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Mutational Effects on the Folding Dynamics of a Minimized Hairpin†

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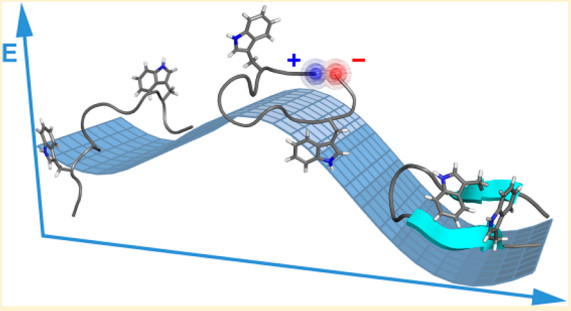
Supporting Information

ABSTRACT: The fold stabilities and folding dynamics of a series of mutants of a model hairpin, KTW-NPATGK-WTE (HP7), are reported. The parent system and the corresponding DPATGK loop species display submicrosecond folding time constants. The mutational studies revealed that ultrafast folding requires both some prestructuring of the loop and a favorable interaction between the chain termini in the transition state. In the case of YY-DPETGT-WY, another submicrosecond folding species [Davis, C. M., Xiao, S., Raleigh, D. P., and Dyer, R. B. (2012) *J. Am. Chem. Soc. 134*, 14476−14482], a hydrophobic cluster provides the latter. In the case of HP7, the Coulombic interaction between the terminal NH_3^+ and CO_2^- units provides this; a C-terminal Glu to amidated Ala mutation results in a 5-fold retardation of the folding rate. The effects of mutations within the reversing loop indicate the balance between loop flexibility (favoring fast conformational searching) and turn formation in the unfolded state is a major factor in determining the folding dynamics. The -NAAAKX- loops examined display no detectable turn formation propensity in other hairpin constructs but do result in stable analogues of HP7. Peptide KTW-NAAAKK-WTE displays the same fold stability as HP7, but both the folding and unfolding time constants are greater by a factor of 20.



The mechanism by which an initially unstructured polypeptide sequence reaches its native structure circumventing the Levinthal paradox[1] is still controversial. It has been suggested that proteins could avoid searching through all possible conformations by taking specific folding pathways. In the diffusion−collision and nucleation models[2−4] of protein folding, stepwise rapid formation of local secondary structures drives the folding. Hydrophobic collapse followed by the acquisition of secondary structure and tertiary packing interactions is an alternative model.[5] The structural elements, or "foldons",[6,7] that collide and coalesce to form a protein's tertiary structure must possess the potential to fold to some extent because of strictly local interactions. Helices, local hydrophobic clusters, and β-hairpins are potential foldons. In Dill's zipping assembly view of protein folding,[8−10] multiple foldon condensation pathways are proposed to accelerate protein folding, but conformational searching is still the ultimate speed limit[10] of protein folding.

The frequent observation, for β-sheet proteins, that one β-hairpin is formed before or at the folding transition state[11−20] is evidence of the involvement of a hairpin in nucleation−condensation pathways.[21−26] As a result, there have been efforts to design β-hairpins for dynamics studies outside of the protein context. Experimental measures of $1/k_F$ for noncyclic β-hairpins at 280−298 K have been in the 0.8−52 μs range;[27−31] contrarily, faster rates ($1/k_F$ = 40−140 ns) have been reported for three- and four-stranded sheet models.[32−34] In the case of the faster folding double and triple hairpins, Gai and coworkers[33,34] have suggested that the rapid folding observed may reflect multiple pathways to the folded state or downhill folding.

A number of reports have revealed a relationship between the stability of a β-hairpin and the turn forming propensity of the loop region.[35−38] Moreover, effects of loop length,[37,39−42] stabilizing cross-strand interactions,[37,42−48] and attractive Coulombic effects between the oppositely charged chain termini[49−52] have also been noted. The influence of these factors on folding rates remains incompletely understood. There have been studies that report an increase in folding rate for reversing loop region mutations that increase the "turn propensity",[29,30,32−34] but other studies[28,41] supported a model for β-hairpin folding in which the rate-limiting step is the loop search process required to establish a near-native cross-strand hydrophobic cluster without prior formation of a well-defined native turn. A recent publication[53] indicates that a choice between these two mechanisms remains elusive for a hairpin with a topology similar to that of the peptides reported herein.

Analogues of the C-terminal hairpin (GB1p) of the B1 domain of protein G,[54,55] including "trpzip" hairpins,[56] have played a prominent role in hairpin dynamics studies.[28,29,57] Indeed, fluorescence-monitored *T*-jump experiments[57] with GB1p were the basis for the original hairpin zipper folding mechanism. Our nuclear magnetic resonance (NMR) relaxation studies[28] revised $1/k_F$ at 298 K for GB1p from 6 to 20 μs, in large part, as a result of a change in the estimated folding

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equilibrium constant. Recalculating the fluorescence-monitored T-jump data using our K_F value brings the two folding time measures ($1/k_F$) within experimental error (± 2 μs). The folding dynamics data for GB1p and related peptides are listed in Table 1.

Table 1. Representative Dynamics Data for GB1 Hairpin Analogues at 297 ± 3 K

		k_F ($\times 10^3$ s^{-1})	k_U ($\times 10^3$ s^{-1}) at 298 K
GB1p	GE WTY-DDATKT-FTV TE	51	179
	D7P	110	90
	K10G	390	780
GB1m2	GE WTY-**NPATGK**-FTV TE	260	156
GB1m3	**KK** WTY-**NPATGK**-FTV **QE**	520	220
trpzip4	GE **WTW**-DDATKT-**WTW** TE-NH_2	67	4.3 (ref 36)
HP7	**KTW-NPATGK-WTE**	1800	210 (this study)
CLN025	**YY-DPETGT-WY**	5200	200 (ref 53)

Loop optimization (GB1m2 vs GB1p) increases the stability of the hairpin fold by 4.5 kJ/mol,[51] this result arising exclusively from an increased folding rate. On the basis of data reported by Du et al.,[29] the even larger fold stability increase (≥7.5 kJ/mol) associated with Trp/Trp interactions in trpzip4 is largely a reflection of retarded unfolding rather than an increase in the folding rate constant. Two loop mutations (D7P and K10G), each of which increases fold stability, have quite different effects on folding dynamics: the D7P mutation increases k_F and decreases k_U,[28] while the K10G mutation increases both rates dramatically.[58] It would appear that loop configurational entropy considerations (e.g., Gly vs Pro content changes), rather than just changes in the net thermodynamic stability, can be a factor in hairpin dynamics. The GB1m3 versus GB1m2 comparison in Table 1 indicates that the hairpin stabilization associated with replacing a repulsive Coulombic interaction near the chain termini (E2/E16) with attractive K/E interactions is largely the result of a 2-fold increase in the folding rate. Given the remoteness from the turn loci, we suggested[28] that this was difficult to rationalize by a "zippering" from the turn mechanism of hairpin folding. Subsequent studies have confirmed hairpin stability enhancement caused by attractive Coulombic interactions between the charges at the extreme termini of this series of peptides.[52] The last entry in Table 1, CLN025, is a peptide with the same turn geometry that has been reported to be an ultrafast folding system.[53] In the CLN025 study, it was concluded that neither the zipper mechanism nor the hydrophobic collapse mechanism could completely rationalize the result and it was suggested that hydrophobic interactions between the terminal aromatic groups lead to a precollapsed structure. In this account, we present hairpin dynamics data from NMR relaxation measurements[28,59−62] for a wide range of strand-terminal and loop mutations of peptide HP7[63] to elucidate the hairpin formation pathways. Mutations at the chain termini and a wide range of mutations in the NPATGK loop, which dramatically altered the loop entropy, are tolerated and do not alter the folded state geometry. These mutations do alter the folding equilibrium and provide, as a result, measures of the effects of both loop and strand mutations on fold stability as well as folding and unfolding rates.

EXPERIMENTAL PROCEDURES

Materials. With the exception of (E12A-NH_2)-HP7, the analogues were synthesized on an Applied Biosystems 433A synthesizer employing standard Fmoc solid-phase peptide synthesis methods. Wang resin preloaded with the C-terminal amino acid in the synthesis provided an unprotected C-terminus upon cleavage. HP7 (KTW-NPATGK-WTE) analogues prepared specifically for this study replaced NPATGK with NGATGK, NPGTGK, NGGTGK, NAAAGK, NAAAKT, NAAAKK, and NAAAKG. For (E12A-NH_2)-HP7, a Rink amide resin provided a C-terminal amide function upon cleavage.

Additional peptides were prepared to ascertain, in other hairpin contexts, the turn propensities of NPATGK, NAAAGK, and NAAAKK with and without the turn-flanking Trp residues. These included KKLWVS-NPATGK-KIWVSA and KKLWVS-NAAAKK-KIWVSA. Fold stability data for these and other constructs appear in the Supporting Information.

Peptides were cleaved using 95% trifluoroacetic acid (TFA), with 2.5% triisopropylsilane (TIS) and 2.5% water. The cleavage product was then purified using reverse-phase high-performance liquid chromatography (HPLC) on a Varian C_{18} preparatory-scale column with a water (0.1% TFA)/acetonitrile (0.085% TFA) gradient. Collected fractions were then lyophilized and their identity and molecular weight confirmed on a Bruker Esquire ion trap mass spectrometer. In some cases, an additional HPLC purification using a C_8 column with a water (0.1% TFA)/acetonitrile (0.085% TFA) gradient or a C_4 column with a water (0.1% TFA)/methanol (0.085% TFA) gradient was required to obtain peptide samples meeting our purity criteria by NMR analysis. All the other HP7 mutants were available from previous studies;[63] these were repurified prior to an additional determination of the circular dichroism (CD) and NMR melts as well as NMR line widths. The structures were fully supported by mass spectrometry, with the NMR assignments confirming the sequence and purity. The thermodynamic stability data from CD and NMR studies of the HP7-related peptides are listed in Table S1 of the Supporting Information.

NMR Spectroscopy. All NMR spectra were recorded on a Bruker DRX-500 or DMX-750 spectrometer. Peptide resonances were assigned through a combination of two-dimensional (2D) TOCSY and NOESY experiments with WATERGATE[64] solvent suppression. The former employed a 60 ms MLEV-17 spin lock[65] and the latter a 150 ms mixing time. The samples for 2D spectra consisted of 1−1.5 mM peptide in buffered water [20 or 50 mM phosphate buffer (pH 6.0)] with 10% D_2O. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal chemical shift reference and set to 0 ppm under all conditions independent of temperature and phosphate buffer concentration.

For NMR line width studies and additional melting data, the peptides were deuterium-exchanged by repeated lyophilization from D_2O and ^1H one-dimensional spectra (512 scans acquired at a resolution of 32K and 64K points for the 500 and 750 MHz spectrometers, respectively) were recorded with 0.6−1 mM peptide concentrations in 20 or 50 mM phosphate-buffered 99.9% D_2O (pH 6.0).

Folding−Unfolding Equilibrium Measurements. Diagnostics of folding for peptide HP7 have already been defined. Expectations based on the pattern of backbone CSD values for hairpins[66] are that the (S ± even) Hα and the (S ± odd) H_N

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should be downfield by ~1 ppm at 100% folding (see Figure 1 for the labeling scheme); in HP7, a number of these are

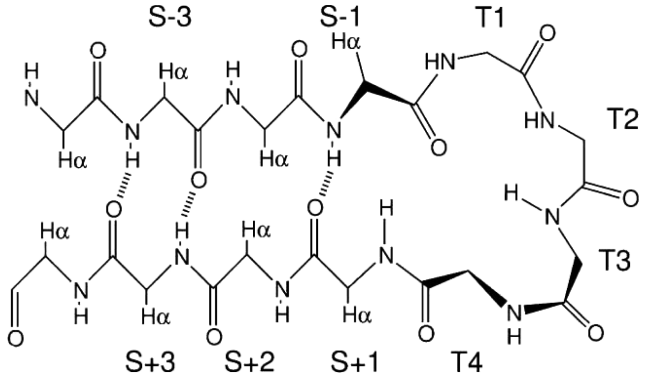

Figure 1. Nomenclature for HP7-related hairpins.

modified by ring current effects. It has also been established that the [4:6]-hairpin loop has diagnostic CSDs, upfield shifts for G8α (T4), T3H_N, and (S + 1) H_N. On the basis of the folding thermodynamics parameters for HP7,[63] the $CSD_{100\%}$ values for these and key sites that experience ring current shifts have been established. These are detailed in the Supporting Information.

Extracting Folding–Unfolding Rate Constants from Exchange Broadening Data. This NMR method relies on the measurement of exchange broadening (Δ^{ex}). In the case of folded–unfolded state exchanges that occur in the <100 μs range, where the probe signal will occur at the population-averaged chemical shift, the relationship among the line width (Δ^{obs}), the exchange rates, and the chemical shift difference ($\Delta\nu$) between the states is given by eq 1

$$\Delta^{ex} = \Delta^{obs} - \Delta^{0} = 4\pi\tau_{ex}\chi_F\chi_U(\Delta\nu)^2 \quad (1)$$

where χ_F is the mole fraction of the folded state ($K_F = \chi_F/\chi_U$), Δ^0 is the "intrinsic" line width expected in the absence of exchange broadening, and $\tau_{ex} = (k_F + k_U)^{-1}$.

For hairpins and other peptide folds that are incompletely populated under accessible conditions, defining fully folded structuring shifts ($CSD_{100\%}$) and whether they may have some residual temperature dependence stands as the greatest potential source of error in dynamic NMR folding rate determinations. Once these have been defined, however, the NMR method has the distinct advantage that the equilibrium constant at any temperature can be derived from the observed chemical shift for the probe (eq 2).

$$\chi_F = (\delta_{obs} - \delta_{coil})/CSD_{100\%} \quad (2)$$

Further details concerning how we extract Δ^{ex} from line shape differences between two first-order doublets with the same coupling constant have been published previously,[28] and their specific implementation in this case appears in the Supporting Information.

Measurements at two field strengths provide a test of an inherent assumption in the method, that Δ^{ex} can be attributed exclusively to exchange broadening caused by the folded/unfolded equilibrium. Exchange broadening due to this mechanism should be proportional to $(\Delta\nu)^2$; thus, the calculated Δ^{ex} should be 2.25 times larger at 750 MHz than at 500 MHz. For Δ^{ex} values of >1.2 Hz at 500 MHz, this condition is met within experimental error (Table S3 of the Supporting Information). When Δ^{ex} is small (<2.5 Hz at 750 MHz), we employ only the value determined at 750 MHz for rate calculations.

Folding rate constants are derived using eq 3. With the folding equilibrium constant established, this also provides the unfolding rate.

$$k_F = 4\pi\chi_U(\chi_F)^2(\Delta\nu)^2(\Delta^{ex})^{-1} \quad (3)$$

Equation 3, which applies near the fast exchange limit, was reported in 1959 by Piette and Anderson[67] and applied to hairpin dynamics[68] in 1999, when it was used to extract upper limits for three-stranded sheet formation time scales. We have previously established the conditions required for the application of eq 3;[28] all of the systems examined herein meet those conditions. To obtain error estimates for k_F, we use χ_U values based on alternative CSD values, one based on a single common value for the chemical shift of the Trp Hε3 probe in the folded state independent of loop mutations, and two others based on the $CSD_{100\%}$ values (and δ_F temperature dependencies) derived from Table S1 of the Supporting Information.

RESULTS

The backbone conformation and the particular edge-to-face (EtF) packing motif of the indole rings in the turn-flanking Trp residues observed in the β-hairpins examined herein are shown in Figure 2. The EtF aromatic interaction results in a dramatic

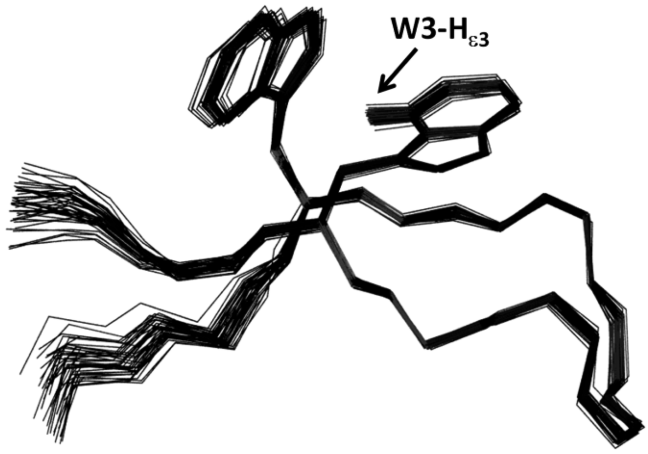

Figure 2. Packing motif for the turn-flanking indole rings (Trp3 and Trp10) and the backbone conformation of HP7.[63] The W3–Hε3 location is highlighted.

(>2.2 ppm) upfield structuring shift for the N-terminal Trp Hε3 resonance (W3-Hε3) due to the ring current effects of the other Trp. The line widths of this shifted resonance provide the dynamics data.

The Hε3 resonance of the Trp at the C-terminus experiences virtually no structuring shift and, thus, is an ideal internal reference for establishing intrinsic broadening effects (Δ^0). We observe significant differential broadening at the upfield-shifted W3-Hε3 resonance of hairpins with this motif (see Figure 3, vide infra).

Verification of two-state folding and accurate $\Delta\nu$ values are the key requirements for extracting meaningful rates by the NMR line width method we employ. The use of several local probes represents a stringent test for two-state folding. Chemical shifts provide a powerful tool for detecting partially

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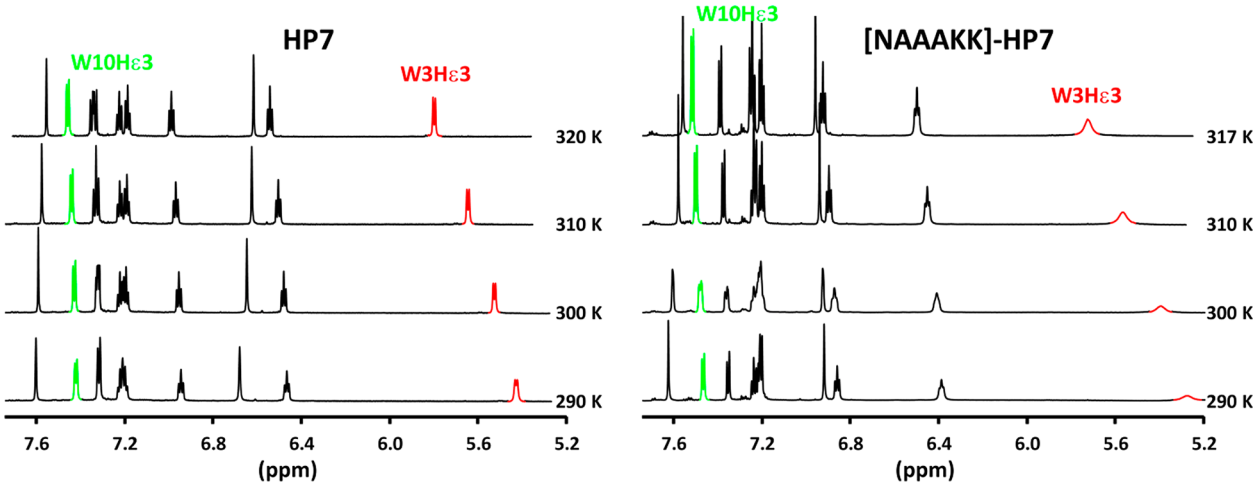

Figure 3. Aromatic region of 750 MHz NMR spectra of HP7 and a loop region mutant in D_2O (pD 6.5). The colored peaks are the Hε3 resonances of the two Trp indole rings: red for W3 and green for W10 (which served as the control for no exchange broadening). Even though the NAAAKK loop mutant displays a somewhat larger upfield shift for W3-Hε3 and a comparable shift melt, the extensive line broadening indicates much slower folding dynamics.

folded states under equilibrium melting conditions. For non-two-state melting behavior, some sequence segments of the peptide would be expected to show structuring shifts (native-like or reflecting an alternative structure), while others would be in a statistical coil conformation, leading to different local melting curves depending on the probe location.[28,41,66,69,70] We employed eight backbone proton sites for measuring the fraction folded values (χ_F) of the hairpins. Five of these monitor β-strand alignment; the other three indicate the extent of turn formation. The agreement between these folding measures for distinct substructural elements supports a two-state unfolding mechanism for all of the species examined (Supporting Information). The melting curves for each of these proton sites revealed that all of them displayed the same fractional degree of unfolding on warming, strong evidence against the formation of partially melted intermediates and a downhill folding scenario.

Because the upfield W3-Hε3 resonance provides both the shift data giving the extent of folding (χ_F) and the line widths that afford the folding dynamics, the accuracy of the extrapolated 100% folded shift value for this site (and its temperature dependence) in each peptide was a major concern. The extrapolated 100% folded CSDs at 280 K for the HP7 series of peptides ranged from −2.39 to −2.64 ppm (Supporting Information). As has been observed in other studies,[71−73] Hε3 ring current shifts in cross-strand Trp/Trp units decrease at higher temperatures. This was incorporated in our dynamics analysis as a temperature-dependent value for the fully folded chemical shift of W3-Hε3. For this set of analogues, two values were required for the analysis: $\delta_F = 5.15 + 0.004(T - 280)$, or $\delta_F = 4.98 + 0.005(T - 280)$. The latter applied to analogues with extensive mutations in the NPATGK loop. Chemical shift melting curves of the W3-Hε3 resonance for all of the peptides in D_2O (the medium employed for the NMR line shape analyses) that support the use of these equations appear in Figures S1 and S2 of the Supporting Information.

One advantage of using the chemical shift as a probe of folding is that this method is insensitive to partial aggregation of the sample at elevated temperatures, a common problem in thermal denaturation studies. Aggregation dynamics are much slower than conformer interconversion with the broad signals for the aggregate not averaged in with those of the monomeric state. However, even under these conditions, the monomer−aggregate interconversion can result in broadening of the monomer signals, but the resulting line width increment due to exchange with an aggregate does not scale with $\Delta\nu$. Rapid exchange between the monomeric folded state and the unfolded ensemble will result in Δ^{ex} values that are proportional to $(\Delta\nu)^2$ and, thus, to (field strength)^2. In every case, the Δ^{ex} ratio values derived for HP7 analogues at 500 and 750 MHz are as expected (2.25): 2.33 ± 0.20 (n = 24) (see Table S5 of the Supporting Information).

The resonances for the two Trp Hε3 sites were resolved in all of the analogues examined, with only the upfield W3 site displaying line broadening. Figure 3 illustrates a large difference in Hε3 line widths observed for two HP7 systems that have essentially the same fold stability and very similar melting behavior.

For dynamics analyses, we always assumed the same coil value (7.58 ppm) for Trp Hε3 but employed the alternative temperature-dependent δ_{folded} values given above. In an alternate analysis, we assumed a common δ_{folded} values of 5.10 ppm, independent of loop residue or mutations in the chain termini. As a rule, the Arrhenius plots derived using the temperature-dependent $CSD_{100\%}$ value provided a more nearly linear plot for ln k_U. The key fold stabilities (as ΔG_U) and folding and unfolding rates appear in Table 2. Table S2 (Supporting Information) provides data at additional temperatures, complementary data measured at 500 MHz, and includes the χ_F value for each entry.

Accurate dynamics data are available over a 6 kJ/mol range ($\Delta\Delta G_U$) of fold stabilities at 300 K. The range of both folding and unfolding rates is larger, 9 kJ/mol in RT(ln k) units; throughout, Arrhenius behavior is observed. In all cases, the plot of ln k_U versus reciprocal temperature is linear within experimental error. The slopes of the Arrhenius plots for k_U are quite similar, reflecting a 2.5 ± 0.2-fold rate increase for a 10 °C temperature increment. Representative Arrhenius plots appear in Figure 4 and the Supporting Information. For examining the correlation between thermodynamic stability and dynamics effects of mutations, we compare $\Delta\Delta G_U$ and Δ(ln k); these are

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Table 2. Effects of HP7 Mutations on Folding and Unfolding Rates in D_2O[a]

mutation	temp (K)	ΔG_U (kJ/mol)	ln k_F	ln k_U
none (KTW-**NPATGK**-WTE)	290	5.8	13.7 ± 0.2	11.3 ± 0.2
	300	5.4	14.60	12.46
	320	3.7	15.71	14.31
strand mutations				
K1A, E12A[b]	300	3.0	14.33	13.11
E12A-NH_2[b]	290	3.1	12.54	11.26
	300	1.85	13.02	12.28
	310	0.46	13.33	13.15
loop mutations of NPATGK				
DPATGK[b]	300	4.2	14.86	13.18
APATGK[c]	300	−0.95	nd[f]	nd[f]
NA**A**TGK	300	4.6	14.28	12.41
NPA**A**GK	300[d]	4.2	13.6 ± 0.3	11.9 ± 0.3
NA**A**AGK	300[d]	6.0	13.1 ± 0.2	10.7 ± 0.2
NAAA**K**G	300	1.9	12.18	11.42
NAAAKK	300	5.3	11.60	9.47
	310	4.4	12.03	10.34
	317	3.5	12.30	10.97
NAAA**K**T	300	4.1	11.69	10.05
	320	1.9	12.52	11.81
at pH 3	300[e]	0.81	10.96	10.63
	315[e]	−1.1	11.69	12.08
NPA**T**AK	300	3.8	12.6	11.07
NP**G**TGK	300	3.3	13.7	12.4
N**G**ATGK	280[d]	4.6	12.13	10.16
	290	4.0	12.51	10.86
N**G**ATGK	300[d]	2.9	12.88	11.73
N**GG**TGK	280[d]	2.6	11.24	10.1
	300[d]	0.17	12.1	12.03
	320[d]	−2.9	12.6	13.7

[a]Data recorded at nominal pH 6 (750 MHz proton observation) unless otherwise specified. The temperature-dependent value used for the fully folded chemical shift of W3-Hε3 (δ_F) was 4.98 + 0.005(T − 280) except as noted. The results using a common temperature invariant 100% folded value (5.10 ppm) are listed in Table S4 of the Supporting Information. [b]A δ_F equal to 5.15 + 0.004(T − 280) was used for HP7 and related species. [c]Stability data from ref 63; dynamics data not available for the same sample. [d]Measurement taken at 500 MHz. [e]Measurement taken at pH 3. [f]Not determined.

listed for single-site loop mutations in Table S3 of the Supporting Information.

DISCUSSION

The NPATGK loop of HP7 was based[51] on location-specific residue statistics for [4:6]-hairpins in proteins. This sequence has subsequently been successfully employed in a number of hairpin and three-stranded constructs.[66,74] A similar analysis led Honda and co-workers to the DPETGT turn sequence that appears in chignolin[75] and further optimized hairpins.[76] This study serves to confirm that the key features of this turn sequence are aryl-Asx-XXXGX-aryl, with the flanking aromatic residues providing significant stability. This is borne out by the effects of single-site residue mutations within this sequence in HP7 analogues (Table S3 of the Supporting Information); an N4A mutation was highly destabilizing (folding rates could not be determined), with the G8A mutation having the largest

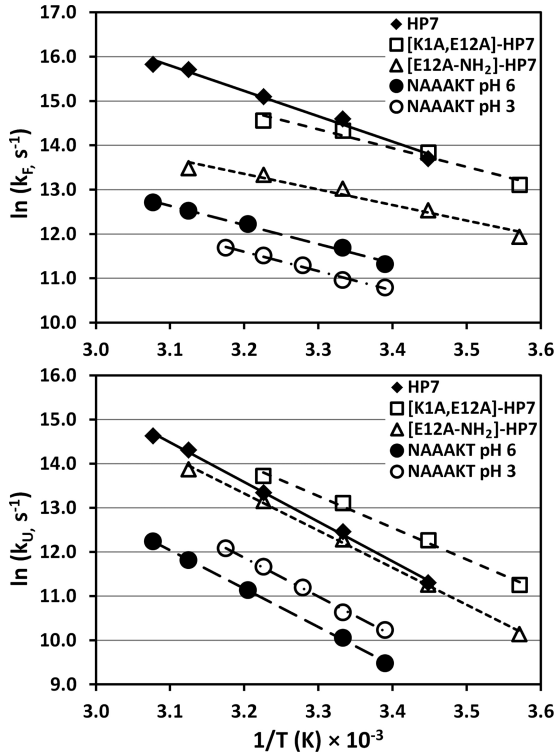

Figure 4. Arrhenius plots for HP7 (◆), [K1A,E12A]-HP7 (□), [E12A-NH_2]-HP7 (△), and the NAAAKT loop analogue at pH 6 (●) and pH 3 (○). The folding rate constants appear in the top panel. The unfolding rate constants appear in the bottom panel. All lines are least-squares fits.

effect on the folding rate (a 7-fold retardation), with a smaller reduction observed for k_U. These results were, to some extent, expected because G8 has ϕ and ψ values of 94° and 47°, respectively, in the HP7 NMR structural ensemble.[63] In the case of the NAAAKK loop mutant, we examined the effect of non-native versus native introduction of glycine: NAAAKK → NAAAKG ($\Delta\Delta G_U$ = −3.4 kJ/mol, Δln k_F = 0.6, Δln k_U = 2.0) versus NAAAKK → NAAAGK ($\Delta\Delta G_U$ = 0.7 kJ/mol, Δln k_F = 1.5, Δln k_U = 1.2). The introduction of glycines at positions other than the "native" one (G8) is destabilizing and results in enhanced melting, but the effects on the dynamics are not uniform (Table S3 of the Supporting Information). In the case of the NAAAKK → NAAAKG mutation, the glycine insertion increases both the folding and unfolding rate (see Figure S6 of the Supporting Information). With a decrease in ΔG_U in this case, the accelerated folding can, in our opinion, be attributed only to a more rapid conformational search for the fold-stabilizing indole/indole geometry at the ends of a more flexible loop upon introduction of a Gly unit.

Turning to the specific questions raised in the introduction concerning the factors that govern hairpin formation rates, we find the HP7 analogue data presented here provide insights into three features: (1) the effects of Coulombic interactions near the chain termini, (2) the correlation between folding rates and thermodynamic stability, and (3) the effects of loop flexibility versus intrinsic conformational preferences. Arrhenius plot comparisons (Figures 4) provide data regarding the Coulombic effect; these probe the effects of the interaction of both oppositely charged side chains near the chain termini and of the N-terminal NH_3^+ and the C-terminal backbone carboxylate on fold stability and dynamics.

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There are also many examples in the literature[45−50,63] in which C-terminal carboxylate protonation reduces hairpin fold stability. In this study, we examined the effects on folding dynamics in the case of the NAAAKT loop mutant. Carboxylate protonation, as for other HP7 analogues,[63] is destabilizing with a 2-fold decrease in the folding rate constant and an acceleration of unfolding.

The chain terminal [K1A,E12A]-HP7 and [E12A-NH_2]-HP7 mutations probe the effects of the interaction of both oppositely charged side chains near the chain termini and of the N-terminal NH_3^+ and the C-terminal backbone carboxylate on fold stability and dynamics. Replacing the N-terminal Lys and the C-terminal Glu with alanine does not alter the folding rate; the slight decrease in thermodynamic stability associated with this change is due to somewhat accelerated unfolding. This analogue retains the attractive interaction between the backbone NH_3^+ and C-terminal CO_2^- units. In contrast, replacing the C-terminal Glu with amidated alanine, which removes this interaction, results in a significant (5-fold) decrease in the folding rate with essentially no change in the unfolding rate constant. This comparison suggests that the fold-favoring Coulombic effect reflects folding acceleration due to the charges at the backbone termini with less contribution from oppositely charged side chain functions. There are cases in the literature[52,63] in which N-terminal acetylation has been demonstrated to decrease hairpin fold stability. These may reflect the same phenomenon.

Substantial folding rate acceleration due to an attractive Coulombic interaction between the extreme termini of the structure would require, with a zippering from the turn mechanism, a late transition state with nearly complete hairpin formation. The alternative is a collapsed structure including Coulombic association between the termini as an early intermediate in folding with cross-strand H-bonding and the formation of the stabilizing turn-flanking Trp/Trp interactions as somewhat later events in the folding pathway.

Turning to the second question, the correlation between folding rates and thermodynamic stability, this has been studied extensively in other systems, particularly proteins. A common feature observed in prior reports of hairpin dynamics comparisons over turn replacements and turn site mutations has been an increased folding rate for turn sequences with an enhanced turn forming propensity.[29,30] With the extensive studies of replacements of the SRSSGR reversing loop in the Pin1 WW domain by Kelly and Gruebele,[77−79] this conclusion has been extended to protein contexts. Among the single-site mutations we examined, NAATGK → NAAAGK ($\Delta\Delta G_U$ = 1.7 kJ/mol, $\Delta \ln k_F = -1.2$, and $\Delta \ln k_U = -1.7$) is a clear exception to this expected correlation. Slow folding, coupled with surprisingly high thermodynamic stability, was observed for all of the NAAAXX loop species examined. For the NP^5AT^7GK reversing loop, the P5A and T7A mutations each decreased fold stability and the folding rate constant; the double mutation resulted in a folding rate retardation that was slighter greater than the sum of the effects of the two individual mutations, but this was coupled with significant fold stabilization. The NAAAKK loop species is the slowest folding species examined, even though it has the same fold stability as the "optimized" NPATGK loop species (HP7). The NMR spectra that provided the folding dynamics for these two species appear in Figure 3. At 300 K, both are 89 ± 1% folded in D_2O; the exchange broadening observed is 1.7 and 34 Hz (at 750 MHz), respectively, corresponding to a 20-fold difference in both the folding and unfolding rates constants.

To explore potential differences between the optimized NPATGK reversing loop and the NAAAXX loops, we examined them as a replacement for β-turn sequences [e.g., XNGK and X-(D-Pro)-GK, where X is S,V, or I] in hairpins and three-stranded sheet models with and without the turn-flanking Trp/Trp pair (see the Supporting Information). A turn-flanking aryl/aryl pair, also a feature of the GB1 peptide,[54−56] is important for high fold stability with both sequences. However, the NAAAKK (and other NAAAXX) sequence cannot replace favorable β-turns in the absence of the flanking aryl/aryl pair, while the NPATGK sequence does appear to have an "intrinsic" chain reversing propensity.[74,80]

Turning to loop search and stiffness considerations, loop contact time studies have established that Pro has the most dramatic rate retarding effect in loop conformational searches while the increased flexibility of Gly can decrease loop-end contact times.[81,82] With the NPATGK loop sequence, however, a significant level of loop prestructuring may occur. Loop mutants with the PATG sequence replaced by AAAK should display comparable flexibility, but turnlike conformations appear to be less significant contributors to the unfolded ensemble for the AAAK species. Thus, with both Pro and Gly at their preferred locations in the loop sequence, the folding direction is favored by loop prestructuring. The observation that unfolding is retarded by the PATG → AAAK change to the same extent as folding suggests that unfolding proceeds through an enthalpically unfavorable loop conformation for the NAAAK species. For this series of hairpins, there is no apparent correlation between fold stability and the rate of fold formation; rather, the data support a role for loop conformational search requirements, including loop flexibility, and the extent to which prestructuring of the loop is favored by the sequence.

How do these new observations regarding hairpin dynamics fit within the context of other literature observations and current questions concerning protein folding mechanisms? Given the close structural analogies between the HP7 hairpins and CLN025 (YY-DPETGT-WY; T_m = 70 °C), this is the first comparison considered here. Although the authors reported[53] the CLN025 dynamics data as relaxation rates rather than specific k_F and k_U values, folding equilibrium constants were also reported; these allow a calculation of $1/k_F$ values of ∼190 ns (298 K) and ∼115 ns (318 K). The fastest folding times we observed for our DPATGK and NPATGK loop species ($1/k_F$) were 530 ns (300 K) and 150 ns (320 K). All of these folding times are faster than the expected[53,83,84] 1 μs speed limit for hairpin formation.

There are some distinctions between the dynamics data for CLN025 and the HP7 analogues. CLN025 displayed a break at 308 K in both the van't Hoff melt analysis and the Arrhenius plot for ln k_R, with probe-dependent kinetics observed in the high-temperature region. In the Arrhenius plot, this corresponds to a radically decreased slope at temperatures above the break point, suggesting either no or a very small (<10 kJ/mol) activation energy. Some of the HP7 analogues display a flattening of the Arrhenius plot for ln k_F; however, we did not extend the studies to sufficiently high temperatures to ascertain whether there are two distinct folding regimes because other evidence suggested that aggregation becomes a competing process at higher temperatures. The unfolding activation energies (E_a) for HP7 analogues are relatively constant (69 ±

6 kJ/mol). The E_a values derived from the linear fits to the Arrhenius plots for folding rates in Figure 4 and Figure S6 of the Supporting Information range from 30 to 50 kJ/mol, values that bracket the ∼45 kJ/mol value observed for the low-temperature portion of the CLN025 Arrhenius plot. The fastest folding system (the NPATGK loop with "wild-type" strands) has the largest activation energy (∼50 kJ/mol) in the folding direction. We view this, in the case of the HP7 systems, as the barrier for an essentially two-state folding process that includes the formation of the Trp/Trp EtF interaction and some cross-strand H-bonds within a short antiparallel β-sheet. CLN025 also has an EtF aryl/aryl pair (Y2/W9), but these side chains, in combination with Y1 and Y10, may represent hydrophobic capping of a turn rather than two-residue β-strands. This may reduce the extent to which CLN025 is an appropriate model for hairpin folding.

Given the observation of probe-dependent dynamics for CLN025,[53] it will be essential to ascertain whether this is also the case for the HP7 hairpins. Efforts to provide this information are in progress. To date, Trp fluorescence-monitored *T*-jump studies (15−26 °C) have confirmed the ultrafast folding of the NPATGK loop species, the folding rate retardation associated with the C-terminal Glu to Ala-NH_2 mutation, the slow folding of the NGGTGK loop species, and the even slower folding of two NAAAKX loop species. Because Trp fluorescence changes may reflect the same structuring transition that determines the ring current shifts used in NMR dynamics, the synthesis of systems with $^{13}C{=}O$ labels at sites that monitor specific H-bond formation within the reversing loops and between the β-strands for *T*-jump experiments is in progress. These should provide the additional probes needed to ascertain whether there is sequential formation of different structural elements (or strict two-state folding) for these hairpin models. However, it is already apparent that this series of HP7 analogues has provided evidence that ultrafast hairpin formation requires both some prestructuring of the loop and favorable Coulombic interactions between the chain termini. The latter is provided by the backbone NH_3^+ and CO_2^- groups. The NPATGK sequence results in loop prestructuring, while a variety of NAAAKX sequences do not, even though stable hairpins result with all of these loop sequences. We anticipate that these sequences in hairpins stabilized with β-capping units at the ends of longer β-strands will provide additional insights into the contribution of loop conformational search times to β-hairpin formation.

■ ASSOCIATED CONTENT

Supporting Information

More detailed descriptions of methods, fold populations (χ_F) derived from NMR shifts (Table S1), folding and unfolding rates obtained using a single temperature invariant δ_{folded} value of W3-Hε3 (Table S4) and a temperature-dependent δ_{folded} (Table S2), exchange broadenings measured at 500 and 750 MHz (Table S5), correlations between mutational thermodynamic and dynamics changes (Table S3 and Figures S4 and S5), and chemical shift melting curves (Figure S1 and S2) for some of the peptides examined. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ADDITIONAL NOTE

†This is the second paper in the series Elucidating Polypeptide Folding Dynamics by ^1H NMR; the first paper of the series is ref 28.

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