Published in final edited form as:

Biopolymers. 2011 November ; 95(11): 755–762. doi:10.1002/bip.21642.

Characterization of Secondary Amide Peptide Bonds Isomerization: Thermodynamics and Kinetics from 2D NMR Spectroscopy

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Abstract

Secondary amide cis peptide bonds are of even lower abundance than the cis tertiary amide bonds of prolines, yet they are of biochemical importance. Using 2D NMR exchange spectroscopy we investigated the formation of cis peptide bonds in several oligopeptides: Ac-G-G-G-NH₂, Ac-I-G-G-NH₂, Ac-I-G-G-N-NH₂ and its cyclic form: I-G-G-N in DMSO. From the NMR studies, using the amide protons as monitors, an occurrence of 0.13% – 0.23% of cis bonds was obtained at 296 K. The rate constants for the trans to cis conversion determined from 2D EXSY spectroscopy were $4-9 \cdot 10^{-3} \text{ s}^{-1}$. Multiple minor conformations were detected for most peptide bonds. From their thermodynamic and kinetic properties the cis isomers are distinguished from minor trans isomers that appear because of an adjacent cis peptide bond. Solvent and sequence effects were investigated utilizing N-methylacetamide and various peptides, which revealed a unique enthalpy profile in DMSO. The cyclization of a tetrapeptide resulted in greatly lowered cis populations and slower isomerization rate compared to its linear counterpart, further highlighting the impact of structural constraints.

Keywords

peptide bond conformation; isomerization; NMR; cis-trans

INTRODUCTION

The planar peptide bond can adopt two conformations, cis and trans, that are separated by a large rotational barrier of approximately 54–84 kJ/mol.^{1–3} Examination of existing protein structures reveals that with exception of proline all other natural amino acids nearly exclusively occur as the trans isomer (99.95–99.97%).^{4–6} In the cis isomer, the C α positions (C α and side chains) of preceding and following amino acid clash while the amide hydrogen offers much less steric repulsion rendering the trans conformation highly preferred. The tertiary amide peptide bond experiences clashes for both the cis and trans isomers which are however energetically similar. Consequently, the fraction of the cis isomer is significantly higher (5%–9%).^{4–6}

Previous studies on the frequencies of cis peptide bonds vary widely.^{4–7} There is a discrepancy between the fraction of cis conformation detected from structural analysis and what would be expected from the free energy difference obtained from model systems.⁴ For a ΔG of 10 kJ/mol the cis population is estimated to be 1.5% at 293K, which is 50 times larger than apparent in PDB data.⁶ This discrepancy may be in part due to the difficulty in detecting cis peptide bonds by lower resolution x-ray crystallography. Indeed, higher resolution structures reveal a higher frequency of cis peptide bonds.^{5–6} Dominant cis peptide bonds in proteins are reliably detected by NMR from characteristic Nuclear-Overhauser (NOE) proton-proton distances.^{8–11} However, if a cis peptide bond occurs only as a minor

species or in unstructured peptides, then the NOE's are not effective and the detection is clearly more difficult. It is also noted that the NMR coupling constants $^3J_{\text{CA-HN}}$ and $^3J_{\text{CA-CA}}$ depend on ω , however the variations are very modest which limits their usefulness. In addition, ^{13}C and ^{15}N -enriched samples are required.¹²

The effect of flanking sequence on the stability of the cis conformation has been explored in a number of studies focusing on the role of local interactions within neighboring residues.^{13–15} Specifically, aromatic residues were observed to be able to stabilize cis conformations by local interactions.^{4,14,16–17} In addition, protein folding constraints and distant residues can also be a source of stabilization. This is supported by mutational studies, where a cis peptide bond was retained even after the proline was replaced by a non-proline residue.^{10,18–22} Structural statistics have shown that cis peptide bonds are located preferably in the region with strained backbones such as coils, bends and VI type turns.^{5,16,23} Interestingly, cis tertiary amide bonds are generally located on the protein surface while cis secondary amide bonds are frequently found in the protein interior.¹⁶

Albeit rare, cis peptide bonds are functionally important. Secondary amide cis peptide bonds often occur near or at the active sites of proteins and are believed to engage in regulating biochemical properties and participate in metal binding.^{19,24–28} For example, the Asp117-Asp118 cis peptide bond²⁸ is involved in Zn^{2+} binding at the active site of *Aeromonas Proteolytica* aminopeptidase. (PDB ID: 1amp). In addition, the strain inherent in a cis peptide bond can potentially serve as an energy reservoir which could be used to facilitate conformation changes or contribute to reactions.⁴ As a consequence of the large rotational barrier, the trans-cis isomerization of both tertiary and secondary amide bonds is a slow process in aqueous solution.⁵ For tertiary amide bonds, the cis to trans rate constant is $1\text{--}5\cdot 10^{-3}\text{ s}^{-1}$ and the reverse process is slightly slower and depends on the flanking sequence.^{29–30} The cis to trans conversion is faster with $0.2\text{--}2.4\text{ s}^{-1}$ for secondary amide bonds while trans to cis reaction rate is $0.6\text{--}3.3\cdot 10^{-3}\text{ s}^{-1}$.^{3,31} As a result, the isomerization of both tertiary^{32–34} and secondary amide^{35–38} bond contribute to rate-determining steps in protein folding, especially when the more rare cis form is present in the folded protein. This is the case for the refolding of RNase T₁ where a rapid initial folding step was followed by an extremely slow trans to cis conversion of the Tyr38-Ala39 bond.³⁵ To facilitate folding, organisms possess both tertiary and secondary amide isomerases.^{32,39–40}

Several experimental^{18,37,41} and theoretical^{42–46} studies have explored the role of tertiary amide bond isomerization in regulating the kinetics and thermodynamics of protein folding and function. Secondary amide cis peptide bonds received less attention in part due to the fact that both low frequency and low percentage of the cis conformer greatly hampers direct experimental studies in peptides and proteins and necessitate indirect approaches. For example fluorescence was used to monitor the cis-trans conversion of Ala39 in the refolding of a RNase T₁ mutant.³⁵ NMR was utilized early on to characterize cis-trans isomers of simple molecules containing N-substituted amide bonds.^{47–48} In addition, UV/visible and UV Raman spectroscopy have also been used to study the cis-trans conversion of a GG dipeptide exploiting the different spectral properties of the two isomers.^{49–50} Small peptides have been investigated by the Fischer and Rabenstein groups using NMR in aqueous solution.^{3,31} In these studies, the target peptide bonds were flanked by aromatic residues which permitted the use of the alanine methyl group as a convenient monitor to study the low abundance cis conformation. In addition, recent theoretical calculations of simple dipeptide model systems have provided information on thermodynamic properties of cis conformers.^{46,51} Encouraged by these studies we investigated peptides without aromatic side chains but with multiple peptide bonds to probe for all possible conformations.

In the present work, quantum mechanical calculations on dipeptide Ac-G-G-NH-Me are followed by experimental studies on tripeptides Ac-G-G-G-NH₂, Ac-I-G-G-NH₂, and an extended tetrapeptide Ac-I-G-G-N-NH₂ using NMR. The formation of cis peptide bond conformers and exchange process between two conformations were monitored with two-dimensional exchange spectroscopy (EXSY).⁵² This permits the identification and study of minor species (<1%) in presence of impurities which would obscure resonances of minor conformers in 1D spectra. Thermodynamic properties were calculated based on temperature dependent population changes. The cis-trans isomerization rates and activation energies were obtained using Perrin & Dwyer's approach to extract exchange data from EXSY spectra.⁵² In addition, a cyclic peptide with the same sequence as the tetrapeptide IGGN was also investigated to explore whether the cyclization constraints would stabilize or inhibit cis conformer formation.

MATERIALS AND METHODS

Materials

All linear peptides are acetylated on the N-terminus and amidated on C-terminus. Both linear and cyclic peptides (purity > 95%) were purchased from GenScript USA Inc. (NJ, USA). DMSO-d₆, Acetone-d₆ and CDCl₃ (isotopic purity > 99.6%) were purchased from Cambridge Isotope Laboratories (Andover, MA). N-methylacetamide (NMA, purity > 99%) was obtained from the Sigma Chemical Co. (St. Louis, MO).

NMR samples

All linear peptides (20mM) and cyclic peptide (30 mM) were prepared in DMSO-d₆. 100mM NMA samples were prepared in DMSO-d₆, CDCl₃ and Aceton-d₆.

Quantum calculations

All *ab initio* Hartree Fock and density functional calculations were carried out using the Gaussian 03 package. Local minima for the Ac-G-G-NHMe peptides were optimized at the density functional B3LYP/6-31G(d) level. NMR calculations were carried out using HF/6-311+g(2d,p).^{53–54} All calculations were carried out in the gas phase and DMSO was used as a reference.

NMR experiments

The NMR experiments were recorded on a Bruker Avance 600 MHz spectrometer with a 5mm QXI probe. Two-dimensional phase sensitive NOESY spectra (6K×1K) were recorded with a series of mixing time from 15 ms to 250 ms, typically 16 scans were acquired. TOCSY (2K×400), COSY (2K×1400) and ROESY (1K×512) spectra were recorded with a sweep width of 10 ppm and 8 scans.

Exchange rate calculation

Chemical exchange rates were determined from 2D Exchange Spectroscopy (EXSY) which provides a map of the exchanging species.⁵⁵ Considering the greatly unequal population between the two exchanging species, as well as similar spin-lattice relaxation time T_1 of the two species, (supplementary material) we found the approach outlined by Perrin & Dwyer⁵² convenient. For a simple two site exchange, the total exchange rate k ($k = k_{c \rightarrow t} + k_{t \rightarrow c}$) is given by the equation:

$$k = \frac{1}{t_m} \ln \frac{r+1}{r-1} \quad (1)$$

The term r accounts for unequal populations and is defined as:

$$r = 4X_c X_t \frac{I_c + I_t}{I_{c \rightarrow t} + I_{t \rightarrow c}} - (X_c - X_t)^2 \quad (2)$$

where I_c and I_t are the diagonal peak intensities of two exchangeable resonances in the EXSY and $I_{c \rightarrow t}$ and $I_{t \rightarrow c}$ are the intensities of the exchange cross peaks, t_m is the mixing time. X_c and X_t are the mole fractions of the cis and trans forms.

RESULTS AND DISCUSSION

Chemical shift calculations

To assess the impact of cis peptide bonds on the chemical shift of the amide protons, a dipeptide model was evaluated (Figure 1A). In this model we focus on the peptide bond between the two glycines. G_7G and GcG describe two models with either a trans or cis peptide bond (ω_1); while cGG , GGc represent models with cis conformations adopted by the N (ω_0) and C (ω_2) terminal peptide-like bonds involving protection groups. The amide proton of G_2 in the GcG model was upfield shifted by 3.31 ppm compared to the G_7G control, indicating that the amide proton in cis conformation is more shielded, which is consistent with previous experimental data.³ (Table 1) These calculations also predict that a cis conformation can cause an upfield shift of an adjacent trans peptide amide proton as predicted for both cGG and GGc .

NMR assignment of 1H resonances

Proton resonances of the peptides were assigned using standard two-dimensional TOCSY, NOESY and ROESY experiments. In addition to regular peptide amide protons which resonate around 8 ppm, we also observed several minor signals (7.3–7.8 ppm) at less than 1% of the main amide proton signals. At this low signal intensity, resonances of minor conformers were identified from EXSY and ROESY experiments which distinguish minor conformers from potential impurities. As shown in Figure 2, exchange peaks clearly correlate the major and minor amide proton (H^N) resonances. Two minor resonances were observed to be associated with Gly1 and Gly2 of $Ac-G-G-G-NH_2$, Gly2 of $Ac-I-G-G-NH_2$ as well as Gly2 and Gly3 of $Ac-I-G-G-N-NH_2$. It is highly unlikely that this indicates the presence of multiple simultaneous cis peptide conformations because this would be energetically strongly disfavored, especially in an unconstrained system. Therefore, only one of the two signals can be from the cis isomer, while the other is suspected to arise from the trans isomer that is affected by a neighboring cis peptide bond, which would be consistent with the chemical shift calculations. Similar findings were also reported previously for peptides containing proline, which had however a much higher percentage of cis isomers.^{56–60} Such an assignment would require that the kinetic and thermodynamic property of a minor trans form is the same as the neighboring cis conformer. As shown in Table 2, the cis conformers (c) are characterized by greater upfield shift which is consistent with reported data.³ In addition, the three bond coupling $^3J_{HN\alpha H}$ of the minor and major conformers are different, signifying a difference in the backbone torsion angle ϕ and allowing us to distinguish two minor conformers. Specifically the cis forms exhibit a larger $^3J_{HN\alpha H}$ coupling compared to the major trans forms, in agreement with previously reported data³. However, the other minor conformers (t) have similar $^3J_{HN\alpha H}$ couplings as the major trans conformers (T) and are thus being assigned as trans conformation that coexist with an adjacent cis peptide bond. For $Ac-I1$ in $Ac-I-G-G-NH_2$ and $Ac-I-G-G-N-NH_2$ only one minor conformer, the cis conformer could be detected (Table 2).

Temperature dependence of the cis-trans isomerization

As shown in Table 3, an average population of 0.18% cis peptide bonds was found for Ac-G-G-G-NH₂ at 296 K. Asn of Ac-I-G-G-N-NH₂ shows a slightly higher percentage of 0.25% at 293 K. Due to signal overlap, no reliable data were obtained for Ac-I-G-G-NH₂ and other residues of Ac-I-G-G-N-NH₂. The equilibrium constants result in an average free energy difference of 16 kJ/mol (Table 3). This value is in line with the previous results of computational and experimental study on N-methylacetamide (NMA).^{2,61} Similar enthalpy changes of isomerization were obtained for the minor trans conformer of G1, (referred to as Ac-*t*G1) and the cis conformer of Gly2 (G1-*c*G2), as well as minor trans conformer G1-*t*G2 and cis conformer G2-*c*G3. This is consistent with the presence of the following conformers for this peptide: Ac-*t*G-*t*G-*t*G-NH₂ (~99.3%), Ac-*t*G-*c*G-*t*G-NH₂ (0.14%), Ac-*T*G-*t*G-*c*G-NH₂ (0.22%) and Ac-*c*G-*T*G-*T*G (~0.3%).

Solvent effects on the cis-trans isomerization

Surprisingly, the percentage of cis conformer detected decreases upon increasing the temperature signifying that the trans to cis conversion in DMSO is exothermic, with the possible exception of G3-*c*N4 in Ac-I-G-G-N-NH₂ for which we obtain an enthalpy of −0.4 kJ/mol. This is in contrast to previous studies which report that the trans to cis conversion is endothermic in aqueous solution.³ To further investigate this, NMA (Figure 1B) was examined in different solvents to explore solvent effects on peptide bond isomerization. We note that earlier studies concluded that the both isomerization equilibria and enthalpy of NMA are insensitive to solvent changes.⁴⁷ However, we observe a higher percentage of cis conformers in solvents with larger dipole moments (Table 4). In addition, a higher amount of cis isomers at higher temperature results in a positive enthalpy of 1.8 kJ/mol, 7.1 kJ/mol and 15.3 kJ/mol in DMSO, acetone and H₂O, respectively. Therefore the enthalpy of the peptide bond isomerization is dependent on the solvent. This also demonstrates that the thermochemistry of peptide bond isomerization depends on the local environment which will change upon protein folding.

Kinetics of the cis-trans isomerization

Temperature-dependent exchange rates are shown in Table 5. At 296 K, the trans to cis rate constant is approximately $4\text{--}9\cdot 10^{-3}\text{ s}^{-1}$, while the cis to trans conversion is about 1000 times faster. Both forward and reverse rates are faster than those reported previously for peptide bonds in an aqueous system.^{3,31} This might be due to solvent effects considering the limited hydrogen bond capability of DMSO compared to H₂O and also to the sequence context which could affect the kinetics of isomerization (discussed in the next section). The activation energy (*E*_a) of both forward and reverse processes were derived based on the Arrhenius equation. Similar kinetics and activation energies were obtained for Ac-*t*G1 and G1-*c*G2, as well as G1-*t*G2 and G2-*c*G3 confirming our conclusion drawn from thermodynamic data.

Effect of flanking sequence on isomerization

To address potential flanking sequence effects Ac-I-G-G-NH₂ was compared to Ac-G-G-G-NH₂ by plotting exchange crosspeaks intensities of each peptide bond against mixing times (Figure 3). The exchange peaks between the trans and cis isomer of I1-G2 are of much lower intensity than for G1-*c*G2 of Ac-G-G-G-NH₂ at all mixing time investigated. This demonstrates that the Ile side chain slows down the trans-cis isomerization compared to Gly at the same position and constitutes a specific sequence effect, which has not been addressed in previous studies. We also note that the cis-trans isomerization rate of NMA is slower than our peptides in DMSO, (Table 5) which may indicate that longer sequences enhance the isomerization rate as was also suggested previously in aqueous solutions.³

Effect of constraint on isomerization

Constraining a peptide could conceptually encourage or inhibit cis peptide bond formation. We have therefore also investigated the cyclic peptide I-G-G-N. Similar to its linear counterpart, we observe several minor conformers in the exchange spectra. However, the intensities of the exchange crosspeaks are much weaker at same mixing time compared to the linear peptides, indicating that the trans-cis isomerization, while still possible, is significantly slowed down. This may also be in part due to the intramolecular hydrogen bond, involving H^N of Ile and Gly2 which further conformationally locks the cyclic peptide (supplementary data). In addition to slowing the kinetics, cyclization also limits the appearance of minor conformers. An approximate occurrence of 0.03% was obtained for the minor conformer of the G2-G3 peptide bond, which is 5–8 times less than that for the linear forms (Ac-G-G-G-NH₂ and Ac-I-G-G-N-NH₂). Interestingly, using two cysteine residues to cyclize a tetrapeptide has previously been reported to have little effect on the population of cis isomers compared to the linear form.³¹

CONCLUSIONS

Peptide bond isomerization is a fundamental property of natural peptides and proteins and is of importance in protein folding/refolding and biochemical activities. We examined peptide bond isomerization of several oligopeptides in both linear and a cyclic form. For linear peptides 0.13% – 0.23% cis conformer was found in DMSO at 296K. In the cyclic form, the occurrence is even lower, demonstrating that backbone constraints affect the cis to trans isomer ratio, in this case negatively. The observation of two minor conformers associated with the same peptide bond signifies the presence of an additional conformer in addition to the expected cis conformer. Thermodynamic and kinetic properties reveal that the minor conformer with smaller upfield shift and similar ³J_{H_NαH} as the major trans conformer is actually also a trans conformer that is impacted by the cis peptide bond on the C terminal side.

The cis-trans equilibrium and enthalpy are solvent dependent. This suggests that peptide bonds located in the interior of folded proteins experience a different thermochemistry from those exposed to an aqueous environment. As a result, the thermodynamics of peptide bond isomerization will change upon protein folding.

Also, higher isomerization rates were obtained in DMSO compared to aqueous solutions demonstrating solvent effects. Small residues also favor isomerization while neighboring bulky side chain may slow the trans-cis isomerization, as seen for I1-cG2 of Ac-I-G-G-NH₂. In addition, the transition was slowed upon backbone cyclization, demonstrating that local structural elements also affect the kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH AI/GM47459 and the Georgia Cancer Coalition. We thank Darrien James for helping with the initial NMR experiments and Alex Spring for suggestions during the preparation of the manuscript. J.Z. is supported by the Brain and Behavior program at GSU.

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Figure 1. Definition of torsion angles of the Ac-G-G-NH-Me dipeptide model (A). N-methyl acetamide (B).

Figure 2. H^N region of 2D EXSY with corresponding 1D spectrum of Ac-G-G-G-NH₂ at 303 K. A data matrix of 6 K $\times$ 1 K was recorded with relaxation delay of 3 s, mixing time of 100 ms. Minor conformers are labeled in the 1D spectrum.

Figure 3.

Build-up of exchange peaks between major and minor conformers. Intensities are scaled based on the highest intensity crosspeak. Ac-G-G-G-NH₂ (left panel): Dashed black line: exchange between major conformer Ac- γ G1 and minor conformers Ac-cG1. Blue line: exchange between Ac- γ G1 and minor conformer Ac-tG1. Dashed blue line: exchange between G1- γ G2 and G1-cG2. Red line: exchange between G1- γ G2 and G1-tG2; dashed red line: exchange between G2- γ G3 and G2-cG3.

Ac-I-G-G-NH₂ (right panel): Dashed black line: exchange between major conformer Ac- γ I1 and minor conformers Ac-cI1. Dashed blue line: exchange between I1- γ G2 and I1-cG2. Red line: exchange between I1- γ G2 and I1-tG2; dashed red line: exchange between G2- γ G3 and G2-cG3. A dashed line indicates a cis conformer and solid line signifies (t). Residue 1 (circle), Residue 2 (open circle), Residue 3 (square).

Table 1
Torsion angles and Gly2 H^N chemical shifts computed for Ac-G-G-NH-Me models.

	ω_0	ϕ_1	ψ_1	ω_1	ϕ_2	ψ_2	ω_2	G2 $\delta_{H(N)}$ (ppm)
G _r G	177.6	81.2	-67.0	-178.1	-80.2	67.5	-178.6	8.2
GeG	-176.9	-120.7	45.0	7.5	-98.7	-11.6	-177.3	4.9
cGG	11.1	-96.9	-2.1	-177.2	-81.2	65.1	-179.4	6.7
GGe	-175.6	-83.1	63.5	176.1	120.7	-65.7	2.0	7.7

Table 2

Amide proton chemical shifts and ³J_{HNαH} couplings for major (trans) and minor peptide bond isomers recorded at 303K.

Pept. Seq.	Gly1(Ile1)			Gly2			Gly3			Asn4		
	<i>T</i>	<i>t</i>	<i>c</i>	<i>T</i>	<i>t</i>	<i>c</i>	<i>T</i>	<i>t</i>	<i>c</i>	<i>T</i>	<i>t</i>	<i>c</i>
Ac-G-G-G-NH ₂	ΔCS	-	0.27	1.0	-	0.35	0.76	-	*	0.69		
	³ J _{NHαH}	5.8	OL	6.7	5.6	OL	6.7	5.9	*	6.2		
Ac-I-G-G-NH ₂	ΔCS	-	*	0.95	-	0.4	1.1	-	*	0.66		
	³ J _{NHαH}	6.5	*	9.5	5.6	OL	6.6	5.6	*	6.4		
Ac-I-G-G-N-NH ₂	ΔCS	-	*	0.72	-	0.37	1.0	-	0.35	0.72	-	0.6
	³ J _{NHαH}	8.3	*	9.9	5.6	OL	OL	5.6	4.9	6.6	8.3	9.8

T indicates major trans, *t* minor trans and *c* minor cis ¹H^N resonances. ΔCS represents the chemical shift difference between major and minor forms. In all cases the minor forms resonate upfield from the major forms. OL: coupling data could not be extracted due to overlap with other resonances that distort the coupling pattern.

* : only one minor form is detected.

Table 3

Thermodynamic parameters for the peptide bonds trans-cis isomerization of Ac-G-G-G-NH₂ peptide at different temperature. Data for cis Ac-cG1 could not be determined due to signal overlap.

	T (K)	% cis	ΔG_{t-c} (kJ/mol)	ΔH_{t-c} (kJ/mol)
Ac-rG1	296	0.13 ± 0.005	16.3 ± 0.1	
	303	0.12 ± 0.020	16.9 ± 0.4	−14.7 ± 2.4
	310	0.10 ± 0.008	17.8 ± 0.2	
G1-rG2	296	0.23 ± 0.015	14.9 ± 0.2	
	303	0.18 ± 0.004	15.9 ± 0.1	−18.6 ± 0.8
	310	0.16 ± 0.012	16.5 ± 0.2	
G1-cG2	296	0.15 ± 0.007	15.9 ± 0.3	
	303	0.16 ± 0.013	16.3 ± 0.3	−14.0 ± 3.9
	310	0.12 ± 0.021	17.3 ± 0.5	
G2-cG3	296	0.20 ± 0.012	15.3 ± 0.2	
	303	0.16 ± 0.013	16.2 ± 0.2	−16.4 ± 6.0
	310	0.15 ± 0.027	16.7 ± 0.5	

Table 4

Thermodynamic parameters for the NMA trans-cis isomerization.

solvent	T (K)	$K_{t-c} \times 10^2$	ΔG_{t-c} (kJ/mol)	ΔH_{t-c} (kJ/mol)
DMSO	293	0.92	11.4	1.8
	303	0.95	11.7	
	313	0.96	12.1	
Acetone	283	0.85	11.2	7.1
	293	0.98	11.3	
	303	1.03	11.5	
H ₂ O	293	1.51	10.2	15.3
	303	2.01	9.8	
	313	2.25	9.9	

Table 5

Isomerization rate constants and activation energies E_{t-c} (trans-to-cis) and E_{c-t} (cis-to-trans) for the Ac-G-G-G-NH₂ peptide. k_{t-c} (trans-to-cis) and k_{c-t} (cis-to-trans) were obtained from: $k_{t-c}/k_{c-t} = K$ and $k_{t-c} + k_{c-t} = k$.

	T (K)	$k_{t-c} \times 10^3$ (s ⁻¹)	k_{c-t} (s ⁻¹)	$E_{a,t-c}$ (kJ/mol)	$E_{a,c-t}$ (kJ/mol)
Ac-tG1	296	7.9 ± 1.0	6.0 ± 0.4		
	303	15.2 ± 2.3	12.3 ± 5.0	86.5 ± 16.2	100.6 ± 23.5
	310	38.4 ± 1.3	38.0 ± 0.7		
G1-tG2	296	4.9 ± 0.7	2.3 ± 0.5		
	303	13.1 ± 1.3	7.3 ± 0.7	98.7 ± 6.1	113.6 ± 10.7
	310	29.9 ± 3.9	18.1 ± 2.1		
G1-tG2	296	8.4 ± 0.8	5.5 ± 1.1		
	303	21.7 ± 4.0	13.8 ± 0.7	81.3 ± 18.8	95.3 ± 3.7
	310	37.5 ± 6.5	31.4 ± 2.1		
G2-tG3	296	4.8 ± 0.8	2.4 ± 0.8		
	303	12.1 ± 2.8	7.4 ± 1.7	97.0 ± 2.1	113.1 ± 8.8
	310	28.3 ± 2.3	18.8 ± 5.8		
HN-CO in NMA	293	1.6 ± 0.3	0.2 ± 0.09		
	303	5.4 ± 1.5	0.6 ± 0.1	98.0 ± 10.9	96.2 ± 12.3
	313	22.3 ± 5.0	2.3 ± 0.3		