

UV Resonance Raman Studies of Cis-to-Trans Isomerization of Glycylglycine Derivatives

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Abstract: We report kinetic UV resonance Raman measurements of the pH dependence of the activation barrier for the trans–cis isomerization of glycylglycine (Gly-Gly). We determined the charge-state dependence of the trans–cis ground-state energy difference, ΔG , and the trans–cis isomerization activation barrier, E_a . ΔG for zwitterionic Gly-Gly at pH 5.7 (3.0 ± 0.5 kcal/mol) is below that of pH 10.5 anionic Gly-Gly (4.4 ± 0.7 kcal/mol). The measured value of ΔG for cationic Gly-Gly at pH 3.0 (3.6 ± 0.4 kcal/mol) lies between the zwitterion and anion values. The measured trans-to-cis activation barriers of 13.3 ± 1.0 , 12.6 ± 0.9 , and 13.9 ± 1.1 kcal/mol for the cation, the zwitterion, and the anion, respectively, are identical within experimental error. However, the larger zwitterionic Gly-Gly (13 ± 1.9 s⁻¹) cis-to-trans isomerization rate constant over those of the cation (3 ± 0.4 s⁻¹) and anion (2 ± 0.3 s⁻¹) suggests a ~ 1 kcal/mol decreased activation barrier. The small differences in energy and activation barriers are somewhat surprising since the zwitterionic form can uniquely form ion pairs, which should impact the trans, the cis, and the activated complex energies.

Introduction

Proteins are linear polymer chains which are composed of amino acid residues linked by peptide amide bonds.¹ These linear polymer chains fold into specific three-dimensional structures due to the structural constraints of the amide linkages and the steric and electrostatic constraints associated with side-chain packing. A major structural constraint involves the limited flexibility of the amide bond, due to the planarity of the amide linkage and due to the preference of secondary amides for the trans configuration (Figure 1). The trans form is more stable by ~ 3 kcal/mol for *N*-methylacetamide (NMA) and zwitterionic glycylglycine (Gly-Gly), the smallest fragments containing the amide bond.^{2–7} Larger trans versus cis energy differences occur for amides with side chains, due to the additional steric interactions. Consequently, nearly all peptide linkages in proteins are in the trans configuration.

The presence of a cis amide linkage can significantly affect the protein structure and can dramatically slow the protein

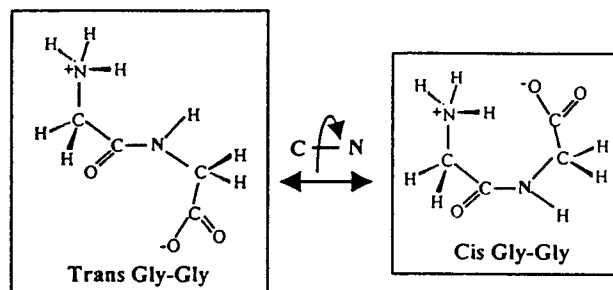


Figure 1. Structures of trans and cis Gly-Gly zwitterion.

folding dynamics.^{8,9} In fact, cis–trans isomerization has been shown to be the slow step in the refolding of both proline- and non-proline-containing proteins.^{8,9} The occurrence of the cis amides is much more common at proline amide linkages, since the trans versus cis energy differences are small for this tertiary amide. Only a few native proteins containing non-proline cis amide linkages have been identified. Examples include carboxypeptidase A¹⁰ and possibly the amyloid protein associated with Alzheimer's disease.^{11,12}

An understanding of the dynamics of protein folding will require a deep understanding of the energetic and dynamics of trans–cis peptide isomerization. Trans–cis isomerization dynamics in proteins has been traditionally studied by nuclear magnetic resonance spectroscopy (NMR).^{2,3,13} NMR has previously been used to study the cis linkages of proline, due to the significant cis concentration compared to the very small cis concentration of the typical secondary amide linkages. NMR

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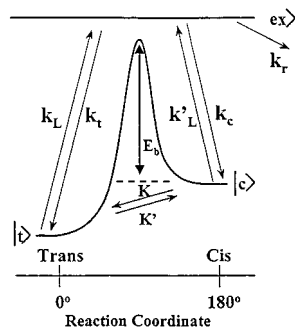


Figure 2. Three-state model, consisting of the cis and trans ground states and the $\pi\pi^*$ excited state. In this model, the amide isomerization reaction coordinate occurs along the CN bond axis. The ground-state cis form is photoisomerized by photoexcitation of the trans species to the $\pi\pi^*$ state, which relaxes back to both the trans and cis ground states. k_L and k_t are the photoexcitation rates from the trans and cis ground states, while k_r and k_c are the relaxation rates from the $\pi\pi^*$ state back to the trans and cis ground states. k_r is the photodegradation rate. K and K' are the ground-state thermal isomerization rates. The energy difference between the cis and trans ground states can be determined by monitoring the cis population as a function of temperature with low CW laser power. The temperature dependence of K is used to determine the cis \rightarrow trans activation barrier (E_b).

measurements have characterized the activation barriers of primary amide linkages for small molecules.¹³ Only recently has the sensitivity of NMR been extended to study the trans-to-cis isomerization of secondary amides.^{11,12,14}

We recently demonstrated a kinetic UV Raman spectroscopic method to probe the energy and activation barriers of trans-to-cis isomerization of secondary amides.⁷ Our earlier study examined the isomerization of NMA and zwitterionic Gly-Gly.⁷ In this study, we examine the dependence of the energy difference between the cis and trans forms and the activation barrier for the different ground states of Gly-Gly.

Experimental Section

Materials. Glycylglycine was purchased from Sigma Chemical Co. and used as received. Sodium perchlorate, used as internal standard, was purchased from Aldrich. Hydrochloric acid and sodium hydroxide were purchased from EM Science and J. T. Baker, respectively.

UV Resonance Raman Spectroscopy. Continuous-wave (CW) 206.5-nm excitation from an intracavity frequency-doubled Kr ion laser was used to determine the ground-state cis-to-trans isomerization rates and to determine the photodegradation quantum yields.^{7,15} The 204.1-nm, 3-ns pulsed excitation, obtained by Raman shifting the third harmonic of a quadrupled Coherent Infinity 100-Hz YAG laser, was used to determine the photoisomerization quantum yields and the ratio of cis excitation to trans excitation.⁷

UVRR spectra were obtained by using a $\sim 135^\circ$ backscattering geometry, and the Raman scattered light was dispersed by a Spex TripleMate spectrometer with an 1800 grooves/mm spectrograph grating.^{16,17a,b} The spectra were detected by using an intensified CCD detector (Princeton Instruments, Inc., model ICCD-1024 MS-E).

We monitored the relaxation rate of photoexcited cis Gly-Gly back to the trans form by using the methodology previously described by Li et al.⁷ This method translates the sample through the laser beam at different rates, and the measured Raman intensity ratio of the cis to

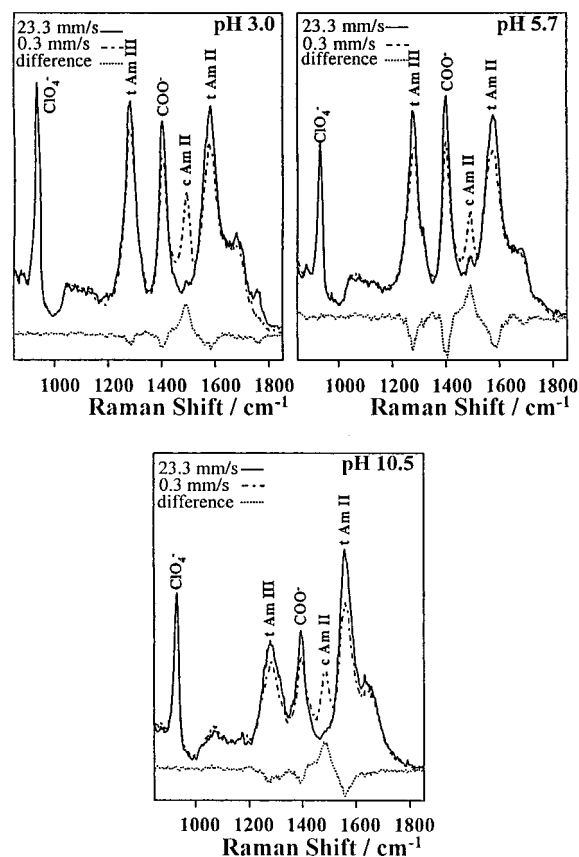


Figure 3. Room-temperature 206.5-nm excited Raman spectra of 8 mM Gly-Gly at various sample cell translation speeds. The cis Am II band intensity increases with decreasing translation speed due to the increase in photon exposure of the sample volume. The band at 932 cm^{-1} derives from the perchlorate (0.32 M), used as an internal standard. The difference spectra between different translation speed spectra are also displayed. The spectra were collected using $\sim 0.2\text{ mW}$ of laser power, using a 5-min accumulation time.

the trans species monitors the relaxation rate of the ground-state cis form back to the ground-state trans form.

We used the three-state model of Li et al. to determine the activation barriers for the trans \rightarrow cis isomerization (Figure 2).⁷ These three states consist of the trans and cis ground states and the $\pi\pi^*$ excited state. We determined the cis \rightarrow trans ground-state isomerization barrier by monitoring the temperature dependence of this isomerization rate. The energy difference between the cis and trans ground states was determined by monitoring the cis population as a function of temperature with low CW laser power.

Results and Discussion

Figure 3 shows the room-temperature 206.5-nm Raman spectra of 0.8 mM Gly-Gly at pH 3.0, 5.7, and 10.5, measured at fast and slow sample translation speeds, and their difference spectra. The prominent spectral features derive from the ClO_4^- (932 cm^{-1}) internal standard band, the trans Am III (1280 cm^{-1}) band, the COO^- ($\sim 1400\text{ cm}^{-1}$) stretching band, the cis Am II ($\sim 1490\text{ cm}^{-1}$) band, the trans Am II (1580 cm^{-1}) band, and the trans Am I (1680 cm^{-1}) band.⁷ The trans Am III band derives from a combination of C-N stretch, N-H bending, and some C=O stretching,¹⁸ while the cis Am II band is composed mainly of the C-N stretching.⁷ The trans Am II vibration is a combination of C-N stretching and some N-H bending motion.¹⁸ The Am I vibration results mainly from a combination

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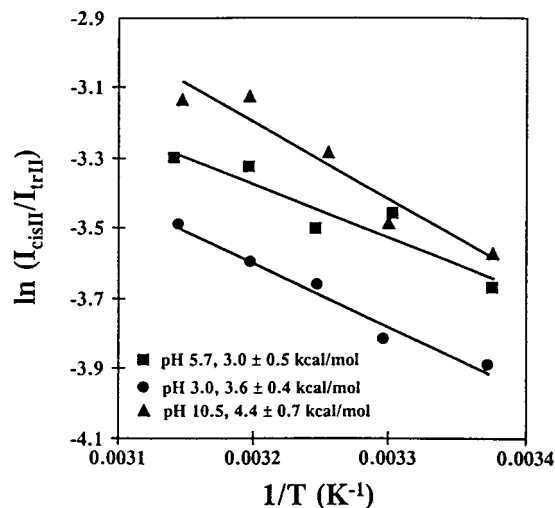


Figure 4. Plot of the log of the cis-to-trans Am II intensity ratio versus $1/T$. The slope is $-\Delta G/R$.

of C=O stretching and N-H bending.¹⁸ The different intensities of the trans amide bands relative to themselves and to the ClO_4^- band signify changes in the $\pi-\pi^*$ absorption band between the different charged species. The decreased pH 3, 1400-cm^{-1} band intensity results from protonation of the carboxylate group and a loss of the carboxylate stretching band contribution, leaving just the CH bending contribution to the 1400-cm^{-1} band.¹⁹

The slow sample translation speed spectra show decreased trans amide band intensities and increased cis Am II band intensities compared to the fast sample translation speed spectra, due to the photochemical isomerization of trans Gly-Gly to cis Gly-Gly. The pH 5.7 and 10.5 difference spectra show similar intensities for the trans bands compared to that of the cis, which indicates similar resonance Raman cross sections for the cis and trans forms. The pH 3 sample shows an increased relative intensity of the cis to the trans forms. The increased relative cis Raman cross section indicates shifts in the absorption spectra between the cis and trans forms in the different pH species. It is much less likely that the intensity differences result from normal mode changes because only small changes occur in the vibrational frequencies. In addition, the trans form shows a weak enhancement of the carboxyl carbonyl band at $\sim 1760\text{ cm}^{-1}$, which appears not to be enhanced in the cis form.

Free Energy Difference between Cis and Trans Gly-Gly.

To avoid photochemical formation of the cis species, we used low CW laser powers to determine the temperature dependence of the trans-to-cis Raman band intensity ratio:⁷

$$\ln\left(\frac{I_{\text{cis}}^{\text{R}}}{I_{\text{trans}}^{\text{R}}}\right) = \ln\left(\frac{\sigma_{\text{cis}}^{\text{R}}}{\sigma_{\text{trans}}^{\text{R}}}\right) - \frac{\Delta G}{R}\left(\frac{1}{T}\right) \quad (1)$$

where $I_{\text{cis}}^{\text{R}}$ and $I_{\text{trans}}^{\text{R}}$ are the cis and trans Am II Raman intensities, respectively, and $\sigma_{\text{cis}}^{\text{R}}$ and $\sigma_{\text{trans}}^{\text{R}}$ are the Am II Raman cross sections of the cis and trans conformers. R is the gas constant, and T is temperature.

Figure 4 plots $\ln(I_{\text{cis}}^{\text{R}}/I_{\text{trans}}^{\text{R}})$ versus $1/T$ for Gly-Gly at pH 10.5, 5.7, and 3.0. The solid lines are the linear least-squares best fits, whose slopes give $\Delta G/R$. Our results show that $\Delta G = 4.4 \pm 0.7$, 3.0 ± 0.5 , and 3.6 ± 0.4 kcal/mol for Gly-Gly at pH 10.5, 5.7, and 3.0, respectively, where the uncertainty given is

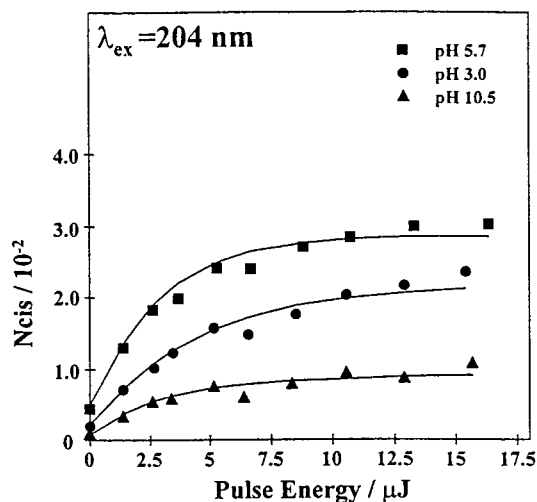


Figure 5. Cis Gly-Gly fractional abundance as a function of the 204.1-nm pulse energy. The solid lines are fits to the temporally and spatially integrated forms of eq 2, from which the cis quantum yield (R_c) and the ratio of cis-to-trans excitation (R_{abs}) are extracted.

Table 1. Cis Isomer Quantum Yield (Φ_c) and Ratio of the Cis-to-Trans Excitation (R_{abs}), Extracted from the Fits to Eq 2^a

pH	Φ_c	R_{abs}	R_t
3.0	0.080	1.90	0.069
5.7	0.082	1.95	0.033
10.5	0.030	2.10	0.0015

^a The photochemical degradation yields (R_t) are determined from difference absorption spectra of Gly-Gly before and after 206.5-nm illumination.

equal to the estimated standard deviation. Although the differences are close to the experimental error limits, the zwitterionic form appears to have the lowest energy difference between the cis and trans forms, while the anionic form has the highest energy difference. Scherer et al.'s study of Ala-Tyr also found that zwitterionic Ala-Tyr has the lowest ΔG (3.2 kcal/mol), while the anionic (pH 11) and cationic (pH 3.0) species both had higher ΔG values (~ 3.6 kcal/mol).¹⁴ These ΔG values would be identical to ΔH in the absence of a temperature dependence of ΔH and ΔS .

Photoisomerization Quantum Yields. Figure 5 plots the fractional abundance of cis Gly-Gly at pH 3.0, 5.7, and 10.5 as a function of the 204.1-nm excitation pulse energy. The solid lines are fits to the model of Figure 2 that utilizes the temporally and spatially integrated forms of the kinetic expressions of eq 2 to relate the changes in the cis and trans fractional abundances (N_c , N_t) to the cis and trans isomer quantum yields (Φ_c , Φ_t). k_L and k_L' are the trans and cis form photoexcitation rates from the ground states to the excited state, and K and K' are the cis \rightarrow trans and trans \rightarrow cis isomerization rates in the ground state.⁷ See Li et al. for a detailed discussion.⁷

$$\begin{aligned} \frac{dN_t}{dt} &= -[(1 - \Phi_t)k_L + K']N_t + (\Phi_t k_L' + K)N_c \\ \frac{dN_c}{dt} &= (K' + \Phi_c k_L)N_t - [(1 - \Phi_c)k_L' + K]N_c \end{aligned} \quad (2)$$

The cis isomer quantum yield (Φ_c) and the ratio of cis-to-trans excitations (R_{abs}) for Gly-Gly at pH 3.0, 5.7, and 10.5 are extracted from the fits in Figure 5 and are listed in Table 1.⁷ Table 1 also includes the photochemical degradation quantum yields (R_t) determined by monitoring the absorption difference spectrum of Gly-Gly before and after 206.5-nm illumination.

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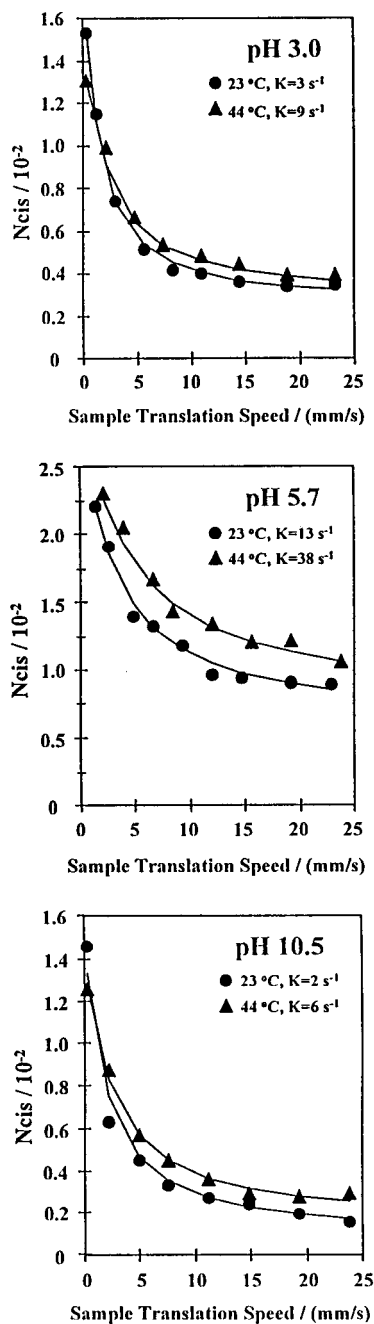


Figure 6. Fractional abundance of cis Gly-Gly at various pH values and sample translation speeds measured at 23 and 44 °C. The solid line is the least-squares fit to the three-state model, from which the cis \rightarrow trans isomerization rates (K) are extracted.

Our R_f value (0.033) for zwitterionic Gly-Gly is higher than that measured by Li et al. (0.022).⁷ We find a systematically increased photodegradation at pH \sim 5.7 for all the temperatures studied over that of Li et al. It is possible that trace impurities can catalyze the degradation process and give rise to the measured differences. The low-pH, cationic form of Gly-Gly shows the highest photochemical degradation quantum yields, which may result from acid-catalyzed hydrolysis. The photodegradation quantum yield at pH 3 is $R_f = 0.069$, just slightly smaller than the quantum yield for cis formation, $\Phi_c = 0.080$.

Ground-State Cis-Trans Isomerization Rate Constants. Figure 6 shows the cis Gly-Gly fractional abundances as a function of the sample translation speed through the laser beam. The solid curves are the least-squares best fits to the three-state model, from which we extract the ground-state cis \rightarrow trans

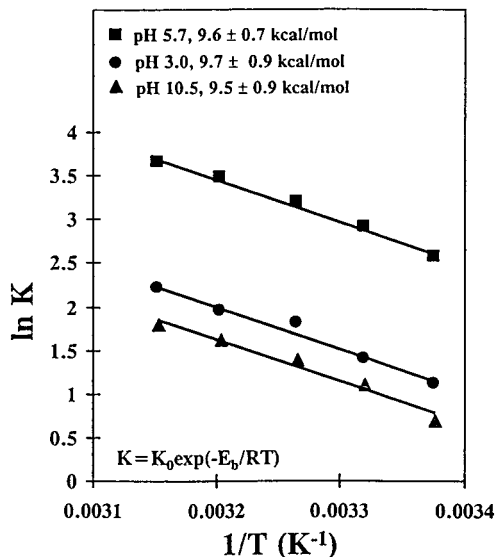


Figure 7. Plot of the log of the cis \rightarrow trans isomerization rate versus $1/T$. The solid lines are the linear least-squares best fits, whose slopes give $-E_b/R$.

Table 2. Energy Differences between the Ground-State Trans and Cis Species (ΔG), the Measured Cis \rightarrow Trans (E_b) and Trans \rightarrow Cis (E_a) Isomerization Barriers, and the Room Temperature Cis \rightarrow Trans Isomerization Rate Constants (K) for Gly-Gly at Different pH Values

pH	ΔG (kcal/mol)	E_b (kcal/mol)	E_a (kcal/mol)	K (s^{-1})
3.0	3.6 ± 0.4	9.7 ± 0.9	13.3 ± 1.0	3 ± 0.4
5.7	3.0 ± 0.5	9.6 ± 0.7	12.6 ± 0.9	13 ± 1.9
10.5	4.4 ± 0.7	9.5 ± 0.9	13.9 ± 1.1	2 ± 0.3

isomerization rates for Gly-Gly at pH 3.0, 5.7, and 10.5. The room-temperature cis \rightarrow trans isomerization rate constant is highest for pH 5.7 ($13 \pm 1.9 s^{-1}$) and decreases for pH 3.0 and 10.5 to values of 3 ± 0.4 and $2 \pm 0.3 s^{-1}$, respectively. Our zwitterionic room-temperature Gly-Gly K value is larger than that obtained by Scherer et al.'s NMR measurements ($\sim 1.5 s^{-1}$).¹⁴ We are unsure of the source of the difference, but it may result from Scherer et al.'s use of a higher Gly-Gly concentration (the concentration they used was not clearly given); formation of dimers and oligomers could slow the isomerization rate.

Ground-State Cis-Trans Isomerization Activation Barriers. To determine the Gly-Gly derivative cis \rightarrow trans isomerization barriers, E_b , we monitored the temperature dependence of their cis \rightarrow trans ground-state relaxation rates, K , following photoisomerization from the trans to the cis configuration (Figure 7).

The trans-to-cis activation energy barrier (E_a) is

$$E_a = E_b + \Delta G \quad (3)$$

Table 2 lists the activation barriers (E_b and E_a), the Gibbs free energy differences between the ground-state cis and trans forms (ΔG), and the room-temperature cis \rightarrow trans isomerization rate constants (K) for Gly-Gly at pH 3.0, 5.7, and 10.5. Our measured zwitterionic E_a value of 12.6 ± 0.9 kcal/mol is close to Li et al.'s E_a value of 11.0 ± 0.7 kcal/mol but is far smaller than Scherer et al.'s quoted zwitterionic Gly-Gly E_a value of 20.2 kcal/mol. Their paper indicates sufficiently large uncertainties in their cis \rightarrow trans isomerization rates ($20 \pm 10 s^{-1}$) to place our measured E_a values easily within their error bars.¹⁴ Scherer et al. also reported higher accuracy activation barriers

for Ala-Phe, Ala-Tyr, Phe-Ala, and Tyr-Ala of 22.1, 22.1, 22.8, and 21.9 kcal/mol, respectively.¹⁴ Much larger E_a values for these derivatives are expected over Gly-Gly due to the bulkier hydrophobic Phe and Tyr side chains.

The ~ 5 -fold increase in the ground-state cis \rightarrow trans isomerization rate constant found for zwitterionic Gly-Gly may derive from a ~ 1 kcal/mol decrease in the E_b value for the zwitterionic species compared to those for the anion and cation. This ~ 1 kcal/mol difference lies within our present experimental error. It is, however, possible that this rate constant difference results from other factors, such as activation entropy and solvent reorganizational and solvent frictional differences for the charged versus zwitterionic species. Better precision data will be necessary to resolve these issues.

Conclusions

We determined the charge-state dependence of the trans-cis ground-state energy difference, ΔG , and trans-cis isomerization activation barrier, E_a , of Gly-Gly in water. ΔG for zwitterionic Gly-Gly at pH 5.7 (3.0 ± 0.5 kcal/mol) is below that of pH 10.5 anionic Gly-Gly (4.4 ± 0.7 kcal/mol). The measured value of ΔG for cationic Gly-Gly at pH 3.0 ($3.6 \pm$

0.4 kcal/mol) lies between the zwitterion and anion values. The measured trans \rightarrow cis activation barriers of 13.3 ± 1.0 , 12.6 ± 0.9 , and 13.9 ± 1.1 kcal/mol for pH 3.0, 5.7, and 10.5, respectively, are identical within experimental errors. However, the larger zwitterionic Gly-Gly cis \rightarrow trans isomerization rate (13 ± 1.9 s⁻¹) over those of the cation (3 ± 0.4 s⁻¹) and anion (2 ± 0.3 s⁻¹) suggests a decrease by ~ 1 kcal/mol in the activation barrier. Whatever the case, these results suggest only a modest impact of the charge state on energy differences and activation barriers for simple amides. This is somewhat surprising in view of the ion pairs that the zwitterion trans and cis forms can have. Furthermore, we might have expected a significant difference in the activation barrier between these different charge states due to entropic differences in water ordering. Possibly, the lack of differences results from numerous fortuitously canceling effects.

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