Direct Measurement Indicates a Slow Cis/Trans Isomerization at the Secondary Amide Peptide Bond of Glycylglycine

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Abstract: Spectral differences between the cis and the trans isomer of a secondary amide peptide bond were used to follow the time course of the cis/trans isomerization of Gly-Gly, Gly-Ala, Ala-Gly, and Ala-Ala dipeptides in the UV/vis region at 220 nm. Isomerization rates and Eyring activation energies were calculated from pH- and LiCl-mediated solvent jump experiments. Rate constants were found to be in a narrow range of 0.29 to 0.64 s^{-1} for the zwitterionic dipeptides at 25 °C. The isomerization rate is about 2-fold higher for the monoionic forms of Gly-Gly. The zwitterionic Gly-Gly has an activation enthalpy $\Delta H^{\ddagger}$ of 71.6 ± 4.9 kJ mol^{-1} that is in the range of the rotational barriers of aromatic side chain dipeptides that have been measured by ^{1}H NMR magnetization transfer experiments. Late stages of protein backbone rearrangements often involve crossing the energy barrier for rotational isomerization of imidic peptide bonds. Our findings are consistent with the idea that a wide range of secondary amide peptide bonds are also able to induce slow rate-limiting steps in protein restructuring.

Introduction

Peptide bonds −C(=O)−NR^1R^2 are special among the covalent bonds forming a polypeptide backbone, in that a considerable barrier to rotation separates the two energetically possible conformers of each peptide unit (Figure 1). Imidic peptide bonds preceding proline (−Xaa-Pro−) provide an example of both a high rotational barrier, $\Delta G^{\ddagger} = 75-90$ kJ mol^{-1}, and a ratio of isomers of about 0.05 to 1.0 (cis/trans) in unstructured polypeptide chains. These conformational features of the imidic peptide bond give rise to isomer-specific bioreactions known to control protein folding,[1,2] enzyme catalysis,[3−5] and certain events in signal transduction.[6−9] Although secondary amide peptide bond isomerization has been proposed to control backbone restructuring in a manner reminiscent of the prolyl peptide bond, much less is known about fundamental characteristics of amidic cis/trans isomerizations. Due to the lack of suitable probes, peptide bonds not involving proline have not been well characterized with the intrinsic Gibbs free-energy difference $\Delta G°$ between the isomers and the Gibbs free-energy barrier to rotation $\Delta G^{\ddagger}$. Therefore, it is unknown whether specific side chain combinations of neighboring amino acids possess the intrinsic potential to promote cis peptide bonds by creating kinetic traps or isomer-specific stabilizing contacts in polypeptide chains, which in turn would affect isomerization rates.

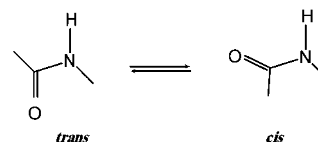

Figure 1. Schematic representation of cis/trans isomers of a secondary amide peptide bond.

Recently, the refolding of a GdmCl-denatured proline-free tendamistate variant was shown to involve secondary amide peptide bond cis/trans isomerization for about 5% of the unfolded molecules.[10] A distinction between certain critical cis peptide bonds and nonessential peptide bonds has not yet been made. Structure-based stabilization of high-energy cis secondary amide peptide bonds was found in native proteins in some cases, leading to a pure cis state for a certain peptide bond.[11,12] There is no clear preference for a particular amino acid to be present at the positions flanking the cis peptide bond but glycine and alanine are frequently involved. Obviously, delineation of amino acid sequences critical to backbone restructuring urgently requires methods applicable to the entire spectrum of secondary amide peptide bonds. Besides the indirect Raman-based method[13−16] fundamental constants for the cis/trans isomeriza-

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tion can be measured by using time-dependent magnetization transfer experiments and line shape analyses of cis specific signals in ^{1}H NMR spectra. Except for the flanking alanine of aromatic side chain peptides, cis isomer-derived signals of most secondary amide peptide bonds suffer from signal overlap, precluding definite signal quantification.[17]

The previous report of a 30-fold higher isomerization rate of Gly-Gly[16] has indicated as-yet-unidentified side chain effects on peptide bond cis/trans isomerizations[18] compared to the ^{1}H NMR-derived rate constants of the few other dipeptides available up to now. Alternatively, peptide association might affect isomerization rates by an unkown mechanism. In the previous report, measurements of the intensity ratio of bands in 206.5 nm excited Raman spectra as a function of the speed of sample translation through a laser beam gave ground-state rate constants ($k_{c/t}$) of 3 s^{-1} for the cationic, 13 s^{-1} for the zwitterionic, and 2 s^{-1} for the anionic form of the dipeptide at 295 K.[15,16]

In this paper we developed a direct UV/vis spectrophotometric method for measuring cis/trans isomerization rates of dipeptides, including Gly-Gly. Experiments were performed to evaluate the influence on isomerization of amino acids neighboring the critical peptide bond, and the ionization state of the dipeptides. Under all conditions, the isomerization rate of Gly-Gly is in the range found for other dipeptides composed of amino acids with uncharged side chains.

Results

Here we report a surprisingly large UV/vis spectral difference between the cis and trans isomers of six representatives of dipeptides including the Gly-Gly originally found to isomerize rapidly. The spectral difference is greatest at 216 nm for zwitterionic Gly-Gly but exhibits a signal/noise ratio more favorable at 220 nm. Using the cis/trans isomer ratio (K) of 0.0101 and 0.003 of Gly-Gly measured by ^{1}H NMR spectroscopy for (Gly-Gly)± and for (Gly-Gly)+, respectively,[17] the difference spectrum (Figure 2) can be converted into the differences of the molar absorption coefficients. This difference was calculated to be 159 M^{-1} cm^{-1} at 220 nm with the higher absorbance for the cis isomer. To examine whether the spectral difference can be utilized to study the kinetics of the cis/trans isomerization of dipeptides, the time course for Gly-Gly was monitored at 220 nm following a pH jump either from pH 2.0 to 7.3 or pH 10.0 to 7.5 on a stopped flow spectrophotometer (Figure 3a). Ionization state alterations, which are known to affect the cis/trans ratio of Gly-Gly and other dipeptides,[17,19] are fast reactions followed by the relatively slow reequilibration of the peptide bond. Reverse jumps demonstrated the reversibility of the spectral changes (Figure 3b). In all cases, single-exponential absorbance changes were recorded, yielding first-order rate constants of about 0. 3 s^{-1} at pH 7.3 and 25 °C. There is no additional kinetic phase present, neither in the dead time of the instrument (1.5 ms) nor on the time scale of minutes. Similar rate constants were obtained when jumping to pH 7.3 or 7.5, respectively, from either side of the pH scale (Figure 3a). As expected from the different ionization states of Gly-Gly in the final reaction mixtures, reverse jumps from pH 7.5 to 2 or from pH 7.5 to 10 reveal slightly different rate constants and absorbance differences (Figure 3b). Next, to exclude possible intermolecular interactions of Gly-Gly that might cause slow kinetic phases, the dipeptide concentration was varied in the range of 1−20 mM. Within the limits of error, rate constants were independent of peptide concentration. Thus, interconverting dipeptide conformations appear to be responsible for the time courses depicted in Figure 3, with the peptide bond chromophore involved in the interconversion.

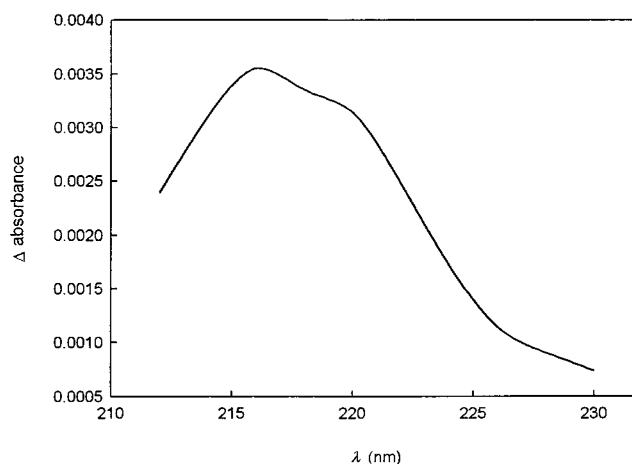

Figure 2. UV/vis difference spectrum for the peptide bond isomers of Gly-Gly. A 113 mM Gly-Gly in 20 mM sodium phosphate buffer (pH 2.0) was diluted in 20 mM sodium phosphate buffer (pH 7.5) 26-fold at 25 °C. The calculated amplitudes of the first-order kinetic phases of the cis/trans isomerization were plotted against the wavelengths in the range 212−230 nm.

Table 1 also shows activation energetics for Gly-Gly and generally similar rate constants and activation energetics for Gly-Ala, Ala-Gly, and Ala-Ala. Examined by a similar approach the dipeptides Ala-Tyr and Phe-Ala show rate constants of $k = 0.60 \pm 0.11$ and 0.35 ± 0.08 s^{-1}, respectively, that are in agreement with values of $k = 0.57$ and 0.34 s^{-1} reported for the cis/trans isomerization of these compounds in magnetization transfer experiments under similar conditions.[17]

A pH profile of the rate constants was determined using a pH jump protocol for different final pH values in the pH range 2.7 to 10.9 (Figure 4). A kinetic model assuming individual interconversion rates of three prototropic Gly-Gly species adequately fits the experimental data (Figure 4; solid line). Moreover, the increase of the population of the putative cis form at increasing temperature ($-\Delta H^0_{c/t} = 9.8 \pm 2.3$ kJ mol^{-1}) and the Eyring activation enthalpy ($\Delta H^{\ddagger}_{c/t} = 71.6 \pm 4.9$ kJ mol^{-1}; $r^2 = 0.971$; $T = 8.8$ to 41 °C) typical of a cis/trans isomerization served to constitute a final confirmation of this model. Moreover, an additional confirmation of the origin of the time-dependent absorbance was established by a solvent-jumping experiment. Ala-Ala dipeptide, dissolved in dry Li$^+$/trifluoroethanol, was injected into aqueous buffer solution at pH 7.5. This experiment with Li$^+$/trifluoroethanol exhibited an initial absorption upon dilution into aqueous buffer that was consistent with a higher fraction of cis isomer than at equilibrium in the buffer. The absorption then decays to its equilibrium value with a first-order rate constant $k_{c/t}$ of 0.85 s^{-1}, and an activation enthalpy $\Delta H^{\ddagger}$ of 62.9 ± 3.7 kJ mol^{-1}, consistent with cis/trans interconversion. Under anhydrous conditions Li$^+$ ions are already known to increase the amount of cis imidic peptide bonds and secondary amide peptide bonds if the peptide bond is preceded

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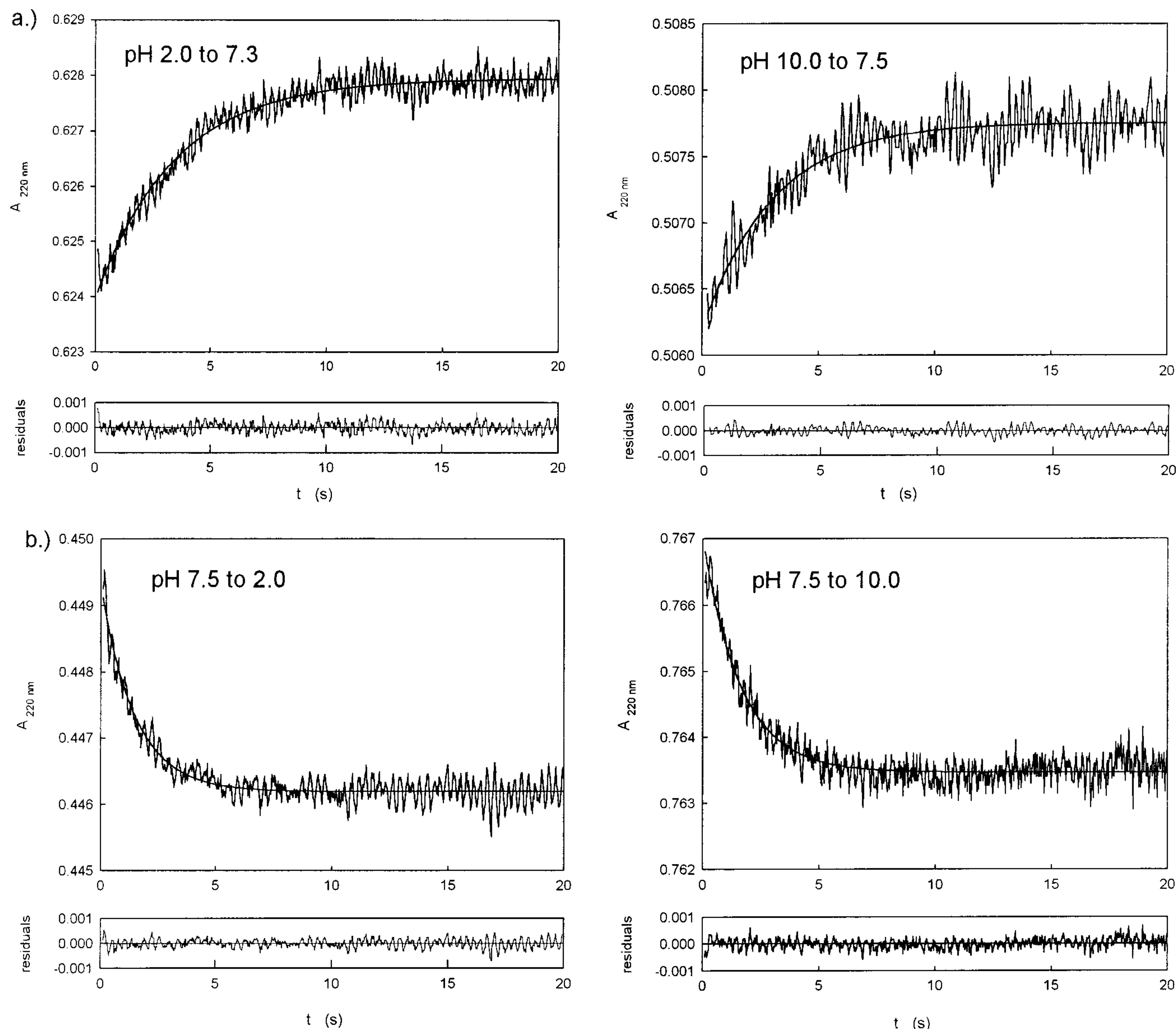

Figure 3. Time course of cis/trans isomerization of Gly-Gly following a pH jump: (a) pH 2.0→7.3 ($k_{c/t}$ = 0.288 s^{-1}) and pH 10.0→7.5 ($k_{c/t}$ = 0.330 s^{-1}) and (b) pH 7.5→2.0 ($k_{c/t}$ = 0.675 s^{-1}) and pH 7.5→10.0 ($k_{c/t}$ = 0.607 s^{-1}). Gly-Gly in 50 mM sodium phosphate buffer with the respective pH (2.0, 7.5, or 10.0) was diluted in 50 mM sodium phosphate buffer with the respective pH (2.0, 7.5, or 10.0) 26-fold at 25 °C. The absorbance at 220 nm was monitored. The kinetic traces were averages of five measurements. Data analyses were performed by single-exponential nonlinear regression resulting in first-order rate constants of the reversible cis/trans isomerization of Gly-Gly ($k_{c/t} = k_{cis\ to\ trans} + k_{trans\ to\ cis}$).

Table 1. Characteristic Constants for the Cis/Trans Isomerization of the Secondary Amide Peptide Bond in Dipeptides at pH 7.3 and for the Anionic (Gly-Gly)− and the Cationic (Gly-Gly)+ States at 25 °C

	$\Delta A_{220\ nm}$ (% of A_{tot}[a])	$k_{c/t}$ (s^{-1})	$\Delta H^{\ddagger}$ [kJ mol^{-1}]	$\Delta S^{\ddagger}$ [J mol^{-1} K^{-1}]
(Gly-Gly)±	0.63 ± 0.06	0.289 ± 0.012	71.6 ± 4.9	−12.4 ± 0.8
(Gly-Gly)+	0.77 ± 0.01	0.675 ± 0.014		
(Gly-Gly)−	0.46 ± 0.01	0.607 ± 0.012		
(Gly-Ala)±	0.56 ± 0.08	0.637 ± 0.042	64.5 ± 8.3	−28.8 ± 3.7
(Ala-Gly)±	0.33 ± 0.06	0.569 ± 0.058	75.7 ± 8.8	−4.1 ± 0.5
(Ala-Ala)±	0.34 ± 0.02	0.437 ± 0.018	69.9 ± 4.5	−10.5 ± 0.7

[a] A_{tot}: Total absorbance of the equilibrated dipeptides.

by alanine.[10,20,21] Consistent with this view, an experiment with the peptide dissolved in trifluoroethanol alone produced no nonequilibrium spectral signal when dilution into buffer took place.

Discussion

Theoretical analyses of the electron populations already suggest isomer-specific differences around the atoms of an amide bond.[22−27] The π component of the ground state resonance structures implies the existence of isomer-specific differences in the electronic transitions of the peptide bond

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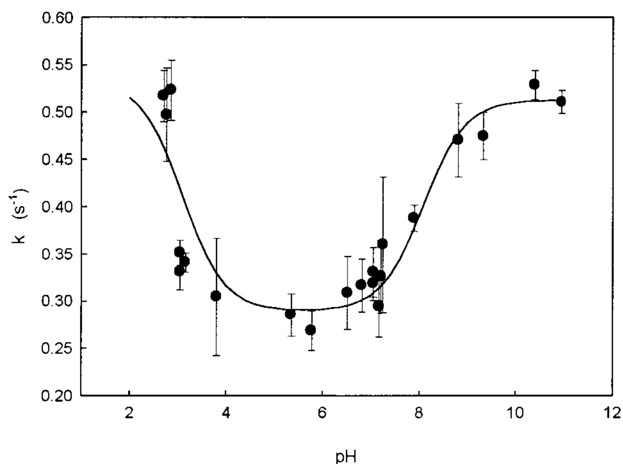

Figure 4. pH dependence of rate constants $k_{c/t}$ for the cis/trans isomerization of Gly-Gly. The solid line represents the calculated curve according to a modified Henderson−Hasselbalch equation using the pK_a values 3.1 (COOH) and 8.08 (NH_3^+).[33]

chromophor. Indeed, a transient difference spectrum was observed for dipeptides as Gly-Gly in the 212−230 nm spectral region following pH or solvent jumps (Figure 2). The difference spectrum ceased within seconds at room temperature in aqueous solution. The time course of the approach to the equilibrium strictly follows first-order kinetics. ^{1}H NMR spectroscopy already established the medium dependence of the cis/trans ratio for secondary amide peptide bonds.[10,17] Thus, reequilibration of the isomers must represent the major cause of the spectral changes depicted in Figures 2 and 3. Taken together, these data indicate that the spectral change in the UV/vis absorption range of the peptide bond chromophore following pH jumps is due to the reversible cis/trans isomerization of Gly-Gly, and that the method should extend to other secondary amidic peptide bonds. Therefore, the new method provides direct access to isomerization kinetics of secondary amide peptide bonds. The high degree of accuracy of the direct assay permits the calculation of the Eyring activation parameters. The extend of molecular reorientation in the transition state for rotation is reflected in the influence that the amino acid side chain on the activation entropy $\Delta S^{\ddagger}$ has in each dipeptide. The highly negative $\Delta S^{\ddagger}$ value of -28.8 ± 3.7 J mol^{-1} K^{-1} for the dipeptide with the carboxyterminal alanine, which is considerably reduced in the dipeptide with an alanine in the N-terminal position, indicates major solvent reorganization in approaching the transition state. On the other hand, both symmetric zwitterionic dipeptides exhibit similar negative activation entropies of -11 ± 2 J mol^{-1} K^{-1} (Table 1). This structural influence emphasizes the importance of attractive orientations, which can be assumed by the charged termini of the zwitterionic ground state. This interaction must be destroyed in the transition state. The ground state interaction between the protonated amino and the carboxy termini is most prominent for Gly-Ala according to the inductive amino acid side chain effects. In comparison, the zwitterionic Ala-Gly has a less basic carboxylate residue and a less acidic protonated amino group, and hence shows a decreased $-\Delta S^{\ddagger}$ value. These results suggest that the higher rotational barriers for prolyl isomerizations in zwitterionic Xaa-Pro dipeptides are mainly enthalpically based.[28]

The data for cis/trans isomerization of amidic peptide bonds of eight zwitterionic dipeptides (Table 1 and ref 17) show a narrow rate constant (k) range of 0.24−0.68 s^{-1} at 25 °C. A formation of dimers and oligomers of the peptides was not found because the rate constants were independent of the peptide concentration. Our isomerization constants closely resemble the rate constants of the secondary amide bond cis/trans isomerization in an unfolded cis (Tyr38-Ala39) RNase T1 variant.[29] Similarly, the proline-free Tendamistat variant P7A/P9A/P50A, which contains 73 peptide bonds, refolds with a first-order rate constant of 2.5 ± 0.2 s^{-1} for a 5% portion of the molecules at 25 °C. This rate constant probably results from the sum of the rate constants of many parallel pathways starting from a manifold of unfolding states distinct in the location of the cis peptide bond.[10]

Taken from the limited database available for secondary amide peptide bonds, neither amino acid side-chain structure nor terminal charges affect isomerization rates to a considerable degree. However, peptide bond isomerizations of all sequences tested proved to be slow on the time scale of biorecognition events that involve polypeptides. It is often believed that secondary amide peptide bond isomerizations play a critical role for differentiating between biologically active and inactive protein states.[30−32] Thus, slow interconversion rates are essential for bioactivity control of proteins by peptide bond rotation because fast interconversion rates would allow peptide bond conformers to be equilibrated in the course of protein/ligand interaction.

Our rate constants and activation energies (Table 1) for Gly-Gly are at variance with the values found with the indirect method of UV resonance Raman spectroscopy[15,16] but agree well with the lower limits estimated from line broadening in ^{1}H NMR spectra.[17] The large difference of isomerization rates determined, on one hand, by ^{1}H NMR spectroscopy and the direct UV/vis method and, on the other hand, by UV resonance Raman spectroscopy must cast some doubt on the reliability of the latter.

Conclusions

We report a direct UV/vis spectrophotometric probe for monitoring the kinetics of secondary amide peptide bond isomerization that is based on the combination of pH and solvent jumps with an isomer-dependent absorption signal at 220 nm in aqueous solution. The first-order rate constants of 0.3−0.7 s^{-1} and the activation energy of $\Delta H^{\ddagger} = 71.6$ kJ mol^{-1} calculated from the Eyring plot indicate a nonexceptional behavior for the cis/trans isomerization of Gly-Gly. Generally, the isomerization rates of secondary amide peptide bonds proved to be insensitive to uncharged amino acid side chains. Because of its technical simplicity the kinetic assay has the potential of broad applicability for studying the dynamics of polypeptide backbone conformation.

Experimental Section

Peptides were purchased from Bachem (Heidelberg, Germany). All measurements were performed on a stopped flow spectrophotometer (Applied Photophysics, Leatherhead, UK). The path length of the observation chamber was 2 mm. The peptides were dissolved in sodium phosphate buffer (20 to 200 mM) of the indicated pH followed by adjusting the required pH with dilute NaOH or phosphoric acid. Small differences in the final concentrations of the peptide result from the pH adjustment. A 26-fold dilution of the peptides into the final buffer was carried out, followed by monitoring the time course of absorbance.

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The time-traces represent averages of five measurements. Data analysis was performed by single-exponential nonlinear regression using a SigmaPlot Scientific graphing system Vers. 4.0 (Jandel Corp, USA) giving first-order rate constants $k_{c/t}$ of the cis/trans isomerizations.

UV/Vis Difference Spectrum. The UV/vis difference spectrum for the peptide bond isomers of Gly-Gly was evaluated by dilution of 113 mM Gly-Gly in 20 mM sodium phosphate buffer, pH 2.0, into 50 mM sodium phosphate buffer, pH 7.5, at 25 °C to give a final pH of 7.3. The amplitude of the time course of the cis/trans isomerization was monitored at various wavelengths in the range 212−230 nm.

Time Course of Cis/Trans Isomerization of Gly-Gly following a pH Jump. Gly-Gly in 50 mM sodium phosphate buffer at pH 2.0 (230 mM), 7.5 (164 and 280 mM), or 10.0 (185 mM) was diluted into 50 mM sodium phosphate buffer at the desired pH 26-fold at 25 °C. The time course at 220 nm was monitored.

For measurement of the concentration dependence of $k_{c/t}$ the final concentration of the peptide was varied from 1 to 20 mM.

pH Dependence of Rate Constants for the Cis/Trans Isomerization of Gly-Gly. Gly-Gly (70 mM in 20 mM sodium phosphate buffer, pH 2.0) was diluted with 200 mM sodium phosphate buffer (pH 7.5) at 25 °C. The time course at 220 nm was monitored. The final pH was measured in a parallel experiment by mixing the solutions manually. Rate constants were plotted against the final pH. Data were fitted to a modified Henderson−Hasselbalch equation using the pK_a values 3.1 (COOH) and 8.08 (NH_3^+).[33]

Temperature Dependence. For monitoring the temperature dependence of the cis/trans isomerization of the peptides Gly-Gly, Gly-Ala, Ala-Gly, and Ala-Ala time courses of pH jumps of the peptides (110 mM in 20 mM sodium phosphate buffer, pH 2.0) into 50 mM sodium phosphate buffer (pH 7.5) were measured at different temperatures (8.8−41 °C). Rate constants were analyzed according to the Eyring equation, yielding the thermodynamic constants of activation $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

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