Barriers to Rotation of Secondary Amide Peptide Bonds

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Abstract: We present results on the identification and molecular characterization of conformers with secondary *cis* amide peptide bonds for a number of oligopeptides containing tyrosine and phenylalanine in aqueous solution. Employing ^{1}H NMR techniques, peptide bonds adjacent to the aromatic amino acid were found to generate a cis isomer population ranging from 0.1% to 1% in dependence on the chain length and the ionization state of the peptide. The rate constant of the trans → cis interconversion for zwitterionic Ala-Tyr was 2.4×10^{-3} s^{-1} at 298 K and thus in a range typical of imidic prolyl peptide bonds. However, the rate constant $k_{\text{cis}\rightarrow\text{trans}} = 0.6$ s^{-1} of the reverse isomerization revealed a much faster process in Ala-Tyr. Extending the peptide chain in both directions of the Ala-Tyr moiety led to a decrease of both the cis content and the barrier to rotation in the cis → trans direction. The linear Arrhenius plots gave E_a values of 76.7 ± 1.5 and 64.6 ± 1.5 kJ mol^{-1} for the dipeptide Ala-Tyr and the corresponding bond in the pentapeptide Ala-Ala-Tyr-Ala-Ala, respectively. Isomerization rates were affected by both the position and the nature of amino acids flanking the isomerizing bond as could be proved by comparison of peptides containing Gly, Tyr, and Phe residues. These studies provide data that permit the extraction of kinetic events originating from the isomerization of "normal" peptide bonds in protein backbone structuring.

Introduction

Much is known about the kinetics and thermodynamics of the cis/trans isomerization[2] of the imidic peptide bond preceding proline in peptides.[3−10] Moreover, this bond rotation frequently occurs uncoupled from chain collapse and segment rearrangements in protein folding, permitting observation as a kinetically discrete step. Consequently, this conformational interconversion plays an important role as a rate-limiting step in the refolding of many denatured proteins.[11−19] Current knowledge is much less for the cis/trans isomerization of secondary amide peptide bonds, also termed "normal" peptide bonds, which represent, in essence, the overwhelming part of the amide moieties of the protein backbone.

The prototypic secondary amides *N*-methylformamide and *N*-methylacetamide have been characterized with respect to equilibrium constants of cis/trans isomerization by NMR methods in different solvents. Resulting constants are in accord with molecular mechanical calculations and Monte Carlo simulations covering a broad range of cis fractions from 0.014 (1.4% cis; *N*-methylacetamide in D_2O) to 0.117 (10.5% cis; *N*-methylformamide in $CDCl_3$) with the lower numbers occurring in aqueous solution.[20−23]

Conformational energy calculations of the cis/trans isomerization of a peptide backbone composed of secondary amide peptide bonds provide a cis fraction as low as 0.09% for a peptide bond intersecting two alanines in a tetrapeptide.[24] Thus, reliance on the numbers experimentally found for simple amides as a prognostic test for peptides seems doubtful.

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(1) All amino acids are in the L configuration; NOESY, 2D nuclear Overhauser spectroscopy; MT, magnetization transfer.

(2) The term prolyl bond used throughout the paper indicates the peptide bond preceding proline in an amino acid sequence, and prolyl isomerization indicates the cis/trans isomerization of the peptide bond preceding proline. For secondary amide peptide bond isomerizations a similar nomenclature was used referring to tyrosyl isomerization when the cis isomerization of the secondary amide peptide bond preceding tyrosine was considered.

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Secondary amide peptide bonds were found with a frequency of 0.03% in cis conformation in 399 protein structures[25] with a sequence homology of less than 25% from the protein data bank. This is a small number compared to 4.8% resulting from the analysis of the occurrence of cis prolyl bonds in protein structures.[26] The vicinity of the C_α atoms of neighboring amino acids, in close contact to each other in the cis conformation, may be important for the low percentage of secondary cis peptide bonds because the steric strain is released in the trans conformer.

Due to the very small population of amidic peptide bonds in cis conformation, only limited data are available for isomerization kinetics. Barriers to rotation for *N*-methylacetamide of $\Delta G^\ddagger = 79$ kJ mol^{-1} (cis → trans) and $\Delta G^\ddagger = 89$ kJ mol^{-1} (trans → cis) have been measured at 333 K in water.[27] These values are close to those found by MM2 force field[28] and *ab initio* calculations.[29]

Direct comparison of cis → trans isomerization rates became possible by monitoring the 206 nm resonance Raman spectra of *N*-methylacetamide and the dipeptide Gly-Gly at 295 K in aqueous solution.[20] Light absorption by the trans peptide bond causes photoisomerization into an excess population of cis conformer[30] that provides the tool for measuring isomerization kinetics. Given the rate effects found for N-substituted amides,[31−34] extrapolation from the isomerization rates of $k_{cis \rightarrow trans} = 2.3 \pm 0.3$ s^{-1} (*N*-methylacetamide) and $k_{cis \rightarrow trans} = 14 \pm 2$ s^{-1} (Gly-Gly) would lead to faster isomerization rates for any peptide bond but Xaa-Gly linkages.

An alternative way to evaluate isomerization rates of nonproline peptide bonds was provided by the fact that the imidic cis peptide bond at Pro39 of native RNase T_1 retained the exclusive cis state in the Pro39Ala protein variant.[35,36] Rate constants in the range of $k_{cis \rightarrow trans} = 0.9-1.4$ s^{-1} were measured by double-mixing experiments for the cis → trans isomerization of the Tyr38-Ala39 bond in the unfolded protein at 298 K. A quite similar magnitude for $k_{cis \rightarrow trans} = 0.702$ s^{-1} (288 K) of the Tyr92-Ala93 cis peptide bond was monitored for an unfolded state of a RNase A variant.[37] The reverse direction of isomerization ($k_{trans \rightarrow cis} = 2.1 \times 10^{-3}$ s^{-1}) in the RNase T_1 variant, which was determined for an already structured folding intermediate, proved to be rate limiting under refolding conditions.[35]

Secondary amide peptide bond isomerization randomly distributed in unfolded chains has already been discussed as a discrete step during refolding of proteins devoid of native state nonproline cis bonds.[38,39] Because the kinetics of the respective folding phases depend on denaturants, tight coupling between isomerization and structure formation was assumed. Obviously kinetic coupling of cis/trans isomerization with other chain movements would prevent satisfactory identification of individual isomerization processes.

In this paper we identified minor signals for a series of Tyr-(Phe)-containing oligopeptides in the ^{1}H NMR spectra that were attributable to the presence of cis secondary amide peptide bond isomers in aqueous solutions. We were able to quantify cis/trans isomerizations by NMR line shape analysis and magnetization-transfer experiments providing cis fractions and rate constants characteristic of discrete peptide bonds. Experiments were performed to evaluate the influence on isomerization parameters of the peptide bond position, flanking residues, and protonation states at various temperatures.

Experimental Section

Sample Preparation. The peptides Gly-Gly, Ala-Tyr, Ala-Phe, Tyr-Ala, Phe-Ala, Ala-Ala-Tyr, Ala-Ala-Tyr-Ala, and Ala-Ala-Tyr-Ala-Ala were purchased from Bachem (Heidelberg, Germany), recrystallized from water, and lyophilized to remove residual solvents. Samples were prepared by dissolving the peptides in a mixture of 9:1 H_2O/D_2O to a final concentration of 0.5−150 mM. The solution pH was adjusted with dilute HCl or NaOH located on the tip of a glass rod for addition. The pH was measured with a Hamilton special sensor pH electrode in the sample tube before each run and checked afterward at the temperature used for the run.

NMR Measurements. All NMR measurements were performed on a Bruker ARX 500 spectrometer operating at a 500.13 MHz proton frequency. The Bruker B-VT 2000 unit in conjunction with a Keithley thermometer 866 inserted in the sample tube was used for temperature control. The accuracy of the sample temperature was ±0.4 K, and the temperature was maintained within ±0.2 K. The water signal was suppressed by irradiation at the carrier frequency during the relaxation delay of 2.3 s. Proton chemical shifts were referenced to internal TSP (sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$, $\delta = 0$ ppm).

Two-dimensional phase-sensitive NOESY spectra were recorded with a mixing time of 120 ms. The spectra were run with a spectral width of 5050 Hz. Typically a 512 (t_1) × 2048 (t_2) points time domain matrix was collected with 96 scans for each increment. Other parameters were similar to those for the one-dimensional experiments.

Magnetization-Transfer Experiments. One-dimensional magnetization-transfer experiments in the static magnetic field B_0 were used to determine rate constants under slow-exchange conditions.[40−42] Three experimental sets were recorded with typically 16 different mixing times in the range 0−3 s. First, the longitudinal relaxation time T_1 was determined by an inversion recovery experiment. Either the cis or the trans signal of the methyl group of alanine was selectively inverted in two additional experiments, resulting in the antiparallel orientation of the magnetization of the respective signal. The biexponential decays obtained were fitted with a homemade nonlinear least squares fit program based on the Levenberg−Marquart algorithm[43] to extract the T_1 value, the rate constants of cis/trans isomerization k_i, and the populations ρ_i of the isomers.

Line-Shape Analyses. To describe quantitatively kinetics of cis/trans isomerization at high temperatures, line-shape analyses of the one-dimensional ^{1}H NMR spectra were utilized. According to Limbach et

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Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts (ppm) and Selected Coupling Constants (Hz) of Ala-Phe (154 mM) and Ala-Tyr (38 mM) in 9:1 H_2O/D_2O, pH 5.9 at 295 K

		NH	Hα	Hβ	Hδ	Hε	Hζ	$^3J_{HNαH}$	$^3J_{HαβH}$
Ala[1]	trans		3.970	1.491					7.2
	cis		4.043	0.910					7.0
Phe[2]	trans	8.123	4.460	3.203	7.306	7.378	7.290	7.6	5.3
				2.985					8.8
	cis	7.753	*a*	*a*	*a*	*a*	*a*	10.7	
Ala[1]	trans		3.986	1.497					7.2
	cis		4.048	0.926					7.0
Tyr[2]	trans	8.081	4.405	3.121	7.165	6.854		7.6	5.3
				2.909					8.8
	cis	7.733	*a*	3.213	7.212	6.877		10.5	4.0
				2.837					4.5

		CO	Cα	Cβ	Cγ	Cδ	Cε	Cζ
Ala[1]	trans	172.79	51.98	19.35				
	cis	*a*	49.32	18.46				
Phe[2]	trans	180.42	59.68	40.14	140.52	129.68	131.46	132.03
	cis	*a*	62.56	41.20	137.40	129.97	131.15	132.20

[a] These cis signals were not resolved because of overlapping with the trans signals.

al., the quantum mechanical density matrix formalism of a nonsymmetrical two-site exchange problem was applied.[40,44−46] After corrections of phases and baselines, an appropriate part of the spectra was clipped and transferred to an external computer. An in-house Fortran program based on programs of Binsch[46] was used to simulate the line shapes of the ^{1}H NMR signals of the alanine methyl group. The following parameters were included in the calculations: chemical shifts of cis and trans signals, ν_c and ν_t, the coupling constants $^3J_{H\alpha\beta H,c}$ and $^3J_{H\alpha\beta H,t}$, which cause doublet splitting of the alanine methyl signals, the cis contents ρ_c, the natural line width W_i of the signals, and the rate constant $k_{cis\rightarrow trans}$. In high-temperature spectra the coupling constant $^3J_{H\alpha\beta H,c}$ of the cis isomer was not resolved but was extrapolated from the low-temperature values. The natural lines width of the two isomers have proved identical in the low-temperature spectra, where exchange broadening was almost absent. The spectra could be simulated without any further presumption. Best fits between the experimental and calculated line shapes were judged by means of the error square sum, as has been published previously.[47] The similarity of the rate constants extracted from magnetization-transfer experiments and line-shape analyses in an overlapping temperature range constitutes additional proof of the validity of this procedure.

Results

^{1}H NMR of Ala-Tyr. Unexpectedly, the one-dimensional ^{1}H NMR spectrum of the dipeptide Ala-Tyr showed two sets of signals with a ratio of about 270:1 throughout the range of the resonating protons. Impurities as the source of these minor signals were ruled out by using different batches of the recrystallized peptide. Almost the same intensity pattern was found for Ala-Phe. Differences in the chemical shifts of major and minor signals were not uniformly distributed along the proton resonances (Table 1). A similar signal-doubling and intensity pattern was observed in the ^{13}C NMR spectrum under the same conditions. The chemical shifts and the ratio of both signals were independent of the peptide concentration from 0.5 to 120 mM at room temperature in aqueous solution, thereby ruling out the possibility that the minor signals were due to peptide aggregation. However, signal separation tended to disappear gradually by line broadening and signal shift when the sample temperature was gradually raised to 369 K (Figure 1). Repeating the run with this sample at 295 K yielded line

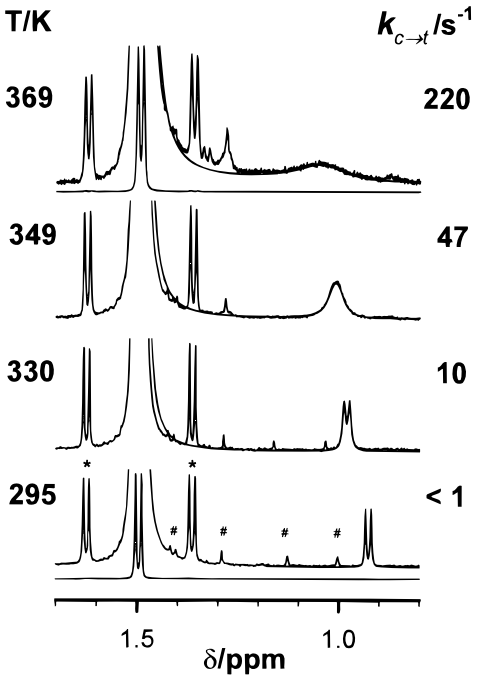

Figure 1. Temperature-dependent experimental and simulated (smooth line) ^{1}H NMR spectra and the corresponding exchange rates $k_{cis\rightarrow trans}$ of Ala-Tyr in 9:1 H_2O/D_2O, pH 5.9. The CH_3-alanine region is shown. Impurities are marked by (#). Asterisks indicate the ^{13}C-satellites of the trans CH_3 signal. The inset at 295 and 369 K shows the full range intensity of the trans signal.

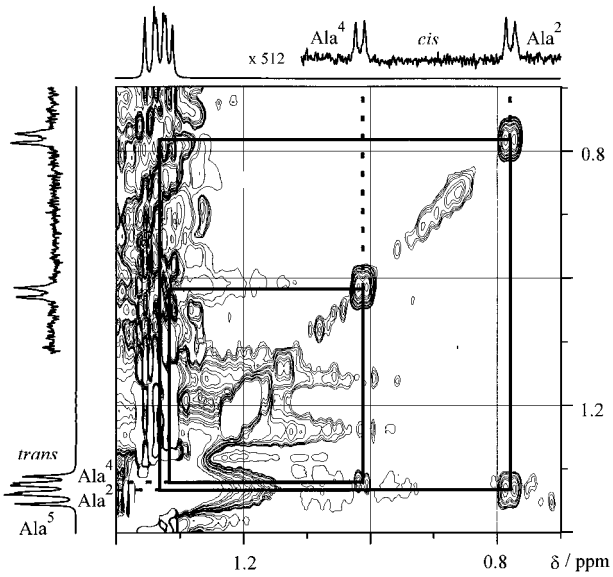

Figure 2. Alanine methyl region of the two-dimensional exchange ^{1}H NMR spectrum given along with the 1-D spectra for Ala-Ala-Tyr-Ala-Ala in 9:1 H_2O/D_2O, pH 4.1 at 295 K. The exchange cross peaks for the cis/trans isomerization of the Ala^2-Tyr^3 and Tyr^3-Ala^4 peptide bond with the corresponding diagonal peaks are marked by straight lines.

shapes and signal ratio for the 1.49 and 0.91 ppm methyl signals identical to those for the original run. New minor signals arose time-dependently at high temperatures (Figure 1) as an indication of partial decomposition of the peptide but showed no reversibility as observed for the signals at 1.49 and 0.91 ppm. Such reversible behavior of proton signals can be interpreted in terms of the conformational isomerism of the peptide rendering the alanine methyl group chemically different in both conformers. Examining exchange cross peaks between the

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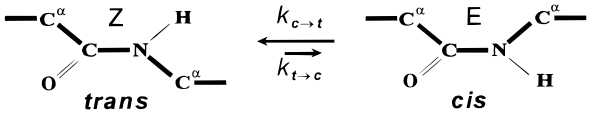

Figure 3. Cis/trans isomerization of a secondary amide peptide bond.

minor and major alanine methyl signals in two-dimensional NOESY spectra at elevated temperature provided direct evidence for the existence of two interconverting Ala-Tyr species stable on the NMR time scale at room temperature (Figure 2).

Now it was supposed that the two conformers found to be present for Ala-Tyr in aqueous solution might be related to a secondary amide peptide bond isomerism.

A coupling constant $^3J_{HN\alpha H}$ of 7.6 Hz was determined for the major conformer whereas the minor isomer was characterized by $^3J_{HN\alpha H} = 10.5$ Hz (Table 1). The latter value corresponds to a backbone torsion angle ϕ of $> -100°$, as was found around the cis secondary amide bonds in cyclic peptides.[48,49]

The magnitude of the Arrhenius activation energy E_a of a conformational interconversion proved to be indicative of its molecular nature because greatly hindered rotation is thought to be the exclusive property of the tyrosyl bond[2] in the Ala-Tyr moiety. The E_a value can be calculated from the temperature dependence of the rate constants that, in turn, becomes available from analyses of the temperature-sensitive line shape exemplified in Figure 1. The equilibrium constant K for the cis/trans isomerization can be defined according to Figure 3 as

$$K = k_{trans \rightarrow cis}/k_{cis \rightarrow trans} = \rho_{cis}/\rho_{trans} \quad (1)$$

where ρ_{cis} and ρ_{trans} represent the populations of the cis and trans isomers, respectively. Considering the dominance of the major conformer (trans) in the spectra, it follows from eq 1 that $k_{trans \rightarrow cis}$ is about 200-fold lower than the reverse rate constant. As a consequence broadening of the trans signals is negligible under the conditions used here. The exact description of the NMR line shape can be performed using a nonsymmetrical two-site model with chemical exchange. Isomerization constants were derived from the best fit of the alanine methyl part of the experimental spectra, as was demonstrated by the drawn line in Figure 1. When the conformers exhibit slow exchange, the rate constants were determined by ^{1}H magnetization-transfer experiments. A progress curve of Figure 4 illustrates the recovery of the magnetization of the cis CH_3-alanine signal with increasing mixing time after the inversion of all signals in the T_1 experiment. The exchange in the magnetization-transfer experiments led to biexponential curves composed of the longitudinal relaxation and the exchange of the cis and trans methyl groups of alanine. At the overlapping temperature 316 K kinetic constants determined by the alternative methods agreed reasonably well. The data were investigated according to Arrhenius (Table 2, Figure 5; empty triangles). The activation enthalpies are in a range typical of imidic peptide bond isomerizations.[5−8] Rate constants and the cis content for the Ala-Tyr dipeptide at 298 K are presented in Table 2. Temperature-dependent spectra were analyzed with the van't Hoff equation, revealing an increased cis population at higher temperature. Collected data of the isomerization of Ala-Tyr and Ala-Phe are presented in the Supporting Information.

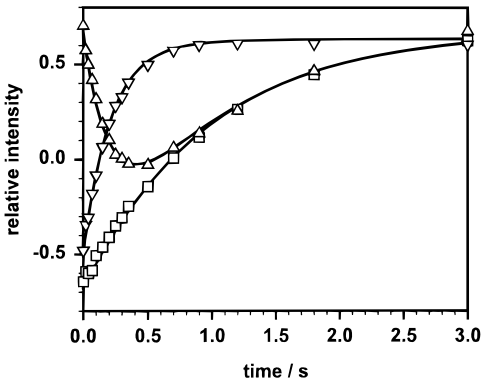

Figure 4. Relative intensities of the time-dependent magnetization of cis CH_3-alanine for Ala-Tyr in 9:1 H_2O/D_2O, pH 5.9 in a T_1 experiment (□) and the magnetization-transfer experiments with the cis signal parallel (△) and antiparallel to the stationary magnetic field (▽) at 316 K. Solid lines represent the correspondingly fitted biexponential decays with $T_1 = 0.984$ s, $k_{cis \rightarrow trans} = 3.7$ s^{-1}, and a cis content of 0.61% as parameters.

The pH dependence of the alanine methyl signals primarily reflects the isomer-specific N-protonation state of Ala-Tyr (Figure 6a). The highest cis fraction of 0.43% occurs in the zwitterionic molecule, decreasing below the carboxyl pK_a (0.14%) and above the amino group pK_a (0.26%) (Figure 6b). At 330 K, the rate constant $k_{cis \rightarrow trans} = 10.8 \pm 0.5$ s^{-1} was independent of pH for Ala-Phe between pH 4.5 and pH 8.6, but it increases to $k_{cis \rightarrow trans} = 17.6 \pm 0.5$ s^{-1} at pH 2.8.

Other Dipeptides. Further studies included the dipeptides Ala-Phe, Tyr-Ala, Phe-Ala, and Gly-Gly. This series of compounds enabled first insight into the general applicability of ^{1}H NMR spectra for the analysis of secondary amide peptide bond isomerism. Dipeptides carrying aromatic side chains showed good signal dispersion for cis and trans isomers (Table 1). The isomer-specific differences depended on the relative position of the side chain being more pronounced for Ala-Tyr-(Phe) (0.58 ppm) than for Tyr(Phe)-Ala (0.33 ppm). However, signal separation was always sufficient to collect data of phenylalanyl, alanyl, and glycyl isomerization for the dipeptides shown here. All isomerization parameters obtained for Ala-Phe were found to be quite similar to those of Ala-Tyr (Table 2) but were different for Tyr(Phe)-Ala bonds. This chemically reasonable result reinforces the assignment of the conformational multiplicity of Ala-Tyr to peptide bond cis/trans isomerization.

The glycyl isomerization of zwitterionic Gly-Gly was clearly indicated by ^{13}C and ^{1}H NMR spectra at pH 5.85. At 295 K the ^{13}C NMR revealed distinct signals of the trans and cis isomers for the peptide bond carbonyl at 170.0 and 172.1 ppm, for the carboxylic carbonyl at 179.3 and 178.6 ppm, for C_α-Gly^1 at 43.6 and 42.1 ppm, and for C_α-Gly^2 at 46.2 and 47.2 ppm, respectively. The cis content at room temperature was 1.0 ± 0.1%, leading to a free energy difference between the cis and trans isomers of $\Delta G° = 11.4$ kJ mol^{-1}. Identical line widths of 2 Hz were measured for both cis and trans signals of the α-protons, excluding chemical exchange at room temperature. At 318 K additional line broadening of the cis signal was still less than 0.5 Hz, which corresponded to a limiting isomerization rate $\ll 1.5$ s^{-1}. At 346 K a rough estimate for $k_{cis \rightarrow trans}$ resulted in 20 ± 10 s^{-1}, giving rise to an activation energy $\gg 65$ kJ mol^{-1}. An accurate determination of the isomerization rates was not possible due to the poor isomer-specific signal dispersion of only 0.02 ppm.

Tyrosine-Containing Oligopeptides. Oligopeptides must be subject to multiple cis/trans isomerizations equilibrating in

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Table 2. Characteristic Constants for the cis/trans Isomerization of Peptide Bonds

compound	bond	log $A_{cis \rightarrow trans}$[a]	$E_{a,cis \rightarrow trans}$[a] (kJ mol^{-1})	$E_{a,trans \rightarrow cis}$[a] (kJ mol^{-1})	$\Delta G°_{trans \rightarrow cis}$[b] (kJ mol^{-1})	% cis	$k_{cis \rightarrow trans}$[b] (s^{-1})	$k_{trans \rightarrow cis}$[b] ($10^{-3}$ s^{-1})	pH
AF	AF	13.1 ± 0.3	75.8 ± 1.6	90.7 ± 2	14.0 ± 1.6	0.36	0.65	2.3	5.9
FA	FA	13.1 ± 0.3	77.4 ± 2.4	90.3 ± 2	13.5 ± 1.2	0.43	0.34	1.5	5.7
YA	YA	13.4 ± 0.4	80.0 ± 2.3	93.6 ± 3	13.2 ± 1.6	0.48	0.24	1.2	5.5
AY	AY	13.2 ± 0.2	76.7 ± 1.5	90.0±2	13.6±1.1	0.41	0.57	2.4	5.9
AAY	AY	13.8 ± 0.4	81.8 ± 3	99.2 ± 5	15.3 ± 2.0	0.21	0.29	0.6	4.4
AAYA	AY	11.6 ± 0.9	65.0 ± 6	81.0 ± 6	16.0 ± 1.5	0.16	1.61	2.6	6.3
	YA	12.7 ± 0.9	74.5 ± 6	92.5 ± 6	15.5 ± 2.0	0.19	0.44	0.8	6.3
AAYAA	AY	11.7 ± 0.2	64.6 ± 1.5	73.7 ± 2	16.3 ± 1.8	0.14	2.38	3.3	4.1
	YA	11.8 ± 0.4	65.9 ± 3	77.3 ± 6	16.8 ± 2.0	0.11	1.77	2.0	4.1

[a] From the Arrhenius equation $\ln k = \ln A - E_a/RT$ for 298 K. [b] From the van't Hoff equation for 298 K.

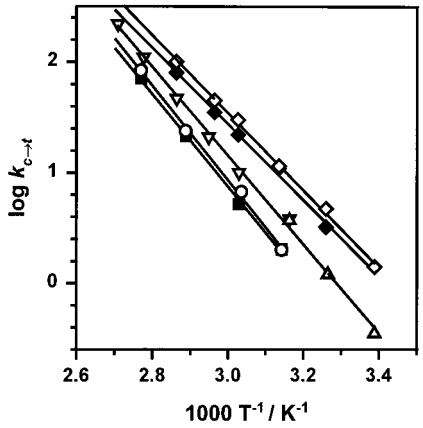

Figure 5. Arrhenius diagram for temperature-dependent $k_{cis \rightarrow trans}$ rate constants for individual peptide bonds in different peptides. Open symbols represent Ala-Tyr bonds, and filled symbols, Tyr-Ala bonds. Rate constants were extracted from line-shape analyses for Ala-Tyr (▽) (△ from magnetization-transfer experiments), Tyr-Ala (■), Ala-Ala-Tyr (○), and Ala-Ala-Tyr-Ala-Ala (◆, ◇) at various temperatures.

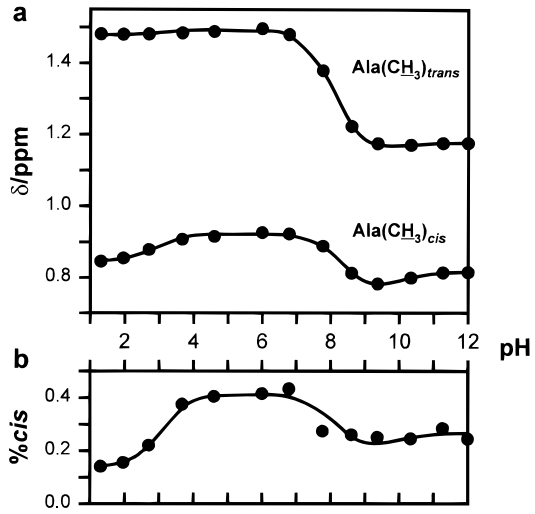

Figure 6. pH dependence of (a) the ^{1}H NMR chemical shifts of the cis and trans CH_3-alanine protons of Ala-Tyr and (b) the corresponding cis content in percent. The solid line represents the calculated curves according to a modified Henderson−Hasselbalch equation using the three pK_a values 3.08 (COOH), 8.05 (NH_3^+), and 10.00 (Tyr-OH) for the trans isomer and 2.89, 8.32, and 9.99 for the cis isomer, respectively.

parallel first-order processes. The isomer-specific intramolecular interaction of aromatic side chains, which considerably affects the chemical shift of flanking alanine methyl protons, proved to be extremely useful in extracting microscopic rate constants for peptides containing Phe or Tyr. Since the tripeptide Ala-Ala-Tyr showed only one cis CH_3-alanine signal (at 0.8 ppm), the isomer-specific influence of tyrosine was identified as a short-range effect. The cis signal broadened with increasing temperature and showed an exchange cross peak with the methyl signal of the trans isomer of the central Ala residue in a two-dimensional NOESY experiment, as it was already indicative of the tyrosyl isomerism in the dipeptide Ala-Tyr. The measured cis contents and activation parameters for the used peptides are presented in Table 2 and Figure 5.

In the pentapeptide Ala-Ala-Tyr-Ala-Ala two peptide bonds flanked tyrosine. Consequently, two cis CH_3-alanine signals were detected at 1.01 ppm (0.12%) and 0.78 ppm (0.14%). They exhibited upfield shifts of 0.31 and 0.55 ppm when compared to the corresponding trans signals. The magnitudes of the upfield shifts were in accordance with those found for the dipeptides Tyr-Ala (0.33 ppm) and Ala-Tyr (0.57 ppm), respectively. Thus we assigned them to isomers having either a cis alanyl bond or a cis tyrosyl bond. Again two-dimensional NOESY spectra confirmed the above assignment due to the appearance of the expected cross peaks (Figure 2). The cis fractions proved to be pH independent within the range 1.5−11. With increasing temperature the two cis signals broadened distinctly. For data analyses, the spectra were thought to be composed of two overlapping subspectra individually described by a two-site exchange model. The microscopic rate constants for the Tyr-Ala and the Ala-Tyr isomerization became available by virtue of this procedure. At 330 K $k_{cis \rightarrow trans} = 22$ s^{-1} for the alanyl isomerization and $k_{cis \rightarrow trans} = 30$ s^{-1} for the tyrosyl isomerization were calculated. The tetrapeptide Ala-Ala-Pro-Ala resembles the pentapeptide in all kinds of analyses (Figure 5, Table 2).

Discussion

The present ^{1}H NMR studies on linear di- and oligopeptides have demonstrated, first, that slowly interconverting conformers exist in aqueous solutions. In fact, ^{13}C NMR spectra confirmed molecular heterogeneity in a quantitative manner (Table 1). In all investigations the ratio of the conformers was ≥100 regardless of whether the peptide structure or the pH value was altered. Conformers were shown to be correlated by chemical exchange. Large conformer-specific differences in proton chemical shifts were recognized for alanine methyl groups flanking aromatic amino acids. Thus, the magnetization-transfer technique and line shape simulation were allowed to quantify conformational dynamics. Remarkably, a similar isomer-specific upfield shift was observed within the cis Tyr-Pro-Phe moiety for the ring protons of proline.[50] An upfield shift occurred in the range between 0.17 and 1.51 ppm for the protons at C^α, C^β, C^γ, and C^δ of proline when two aromatic amino acids flanked proline. Only the protons at C^γ are upfield shifted when a single aromatic amino acid succeeded proline.[50] Conforma-

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tional multiplicity in linear oligopeptides and in proteins with dynamics slow on the NMR time scale has been previously found as an almost exclusive property of proline-containing sequences, because the imidic peptide bond preceding proline causes prolyl isomerization.[3,51−53] Here, however, the peptides were exclusively composed of secondary amide peptide bonds.

Second, the observed activation parameters for the interconversion of the conformers of the dipeptides Ala-Tyr and Ala-Phe are seen to be similar to those reported for the cis/trans isomerization of prolyl bonds.[8] Plotting temperature-dependent rate constants according to the Eyring equation reveals that, typically, the entropies of activation $\Delta S^{\ddagger}$ were found to be in the range 0 ± 5 J mol^{-1} K^{-1} and thus consistent with an unimolecular reaction. The $\Delta H^{\ddagger}$ (E_a) values suggest that the conformational interconversion is enthalpy driven (Table 2). The reaction enthalpies ΔH° (Table 2) also resemble cis/trans isomerizations because increasing the relative population of cis isomer parallels increasing temperature.[7,54] Additionally, diagnostic evidence for a cis peptide bond is found with $^3J_{HN\alpha H} = 10.5$ and 10.8 Hz in the minor conformers of Ala-Tyr and Ala-Phe, respectively.

The obvious similarities to imidic peptide bond isomerizations of the conformational multiplicity of the investigated peptides argue for the secondary amide peptide bond as the origin of heterogeneity in aqueous solution. Moreover, the population found here for cis-Gly-Gly (1.0 ± 0.1%) agrees reasonably with a reported value of 0.5 ± 0.7% resulting from photochemical experiments.[20]

Despite possessing potential protonation sites nearby the isomerizing bond, dipeptides exhibited a flat pH profile of the cis fraction showing a maximum within the physiological pH range (Figure 6b).

Comparing the zwitterionic forms for Ala-Pro and Ala-Tyr, the cis tyrosyl isomer is destabilized by about 12.6 kJ mol^{-1}. The stability difference increased further when the anionic forms were compared. Thus, the increased cis population of anionic Ala-Pro,[3] typical of C-terminal prolines, has no equivalence to that for Ala-Tyr (Figure 6b). However, in both cases the cationic state gave rise to a decreased cis content. Regarding the cis fraction, carboxyl protonation can be mimicked by amidation because we monitored a reduced percentage of cis-Ala-Phe-NH_2 (0.10%) when compared to that of Ala-Phe (0.36%) at pH 5.9. Oppositely charged groups in close contact with the isomerizing bond seem to dominate interactions favorably, contributing to enhanced cis populations, as was optimally realized in dipeptides.

Chain elongation causes destabilized cis isomers for peptide bonds located apart from charged termini. The values of the tetra- and the pentapeptide (Table 2) indicate that the cis fraction of a certain bond decreases when the total number of peptide bonds increases in the molecule. Here a compensating effect might already become visible, keeping the total cis content of an oligopeptide low. Remarkably, it approaches the probability of occurrence of cis-Ala-Ala (0.1%) calculated for the middle position of the tetrapeptide.[24] Specific effects of amino acyl substitution should be detected comparing the prototypic secondary amide isomerization of *N*-methylacetamide with peptides. Since it shows 1.46 ± 0.09% cis isomer in water,[20,22] and the cis-Gly-Gly fraction is rather similar in our experiment, C_α branching needs to be present in the peptides in order for cis isomer destabilization to occur. At the present time it is not appropriate to make a definite conclusion on cis isomer stability regarding individual C_α side chain effects. Local structures may be especially favored by the aromatic side chains used here including isomer-specific peptide bond/aromatic ring and side chain/aromatic ring interactions.[50,55] Reasoning that secondary amide isomerization resembles prolyl isomerization in this respect, the nature of the amino acid preceding proline is thought to have a major effect on the cis/trans ratio.[8,26,50] In contrast, the variability may be much larger for secondary amide peptide bonds because alterations become possible for both flanking positions.

Equilibration of secondary amide peptide bond conformers is a fast process in aqueous solution, since the rate is almost entirely determined by $k_{cis \rightarrow trans}$ (Table 2). This implies typical half-times at close range of 1 s for dipeptides and slightly shorter for oligopeptides. It is not fast enough, however, to exclude rate-limiting isomerization from occurring in protein/peptide and protein/protein interactions, commonly found in biological processes. The rate constant $k_{cis \rightarrow trans}$ increases 4-fold upon positioning the peptide bond in the middle of a longer chain. For Gly-Gly the upper limit of a rough estimation of the rate constant ($k_{cis \rightarrow trans} \ll 1.5$ s^{-1}; 318 K) fits the other dipeptides well but is at variance with the much higher value of 14 ± 2 s^{-1} derived from a photochemical experiment.[20] An Arrhenius activation energy E_a of 49 kJ mol^{-1} was given along with these experiments, which proved to be substantially lower than all values given in Table 2. In our experiments the E_a value had a lower limit of 65 kJ mol^{-1} for zwitterionic Gly-Gly. A rate constant $\ll 1.5$ s^{-1} for Gly-Gly is also in general accord with the steric influence of amide nitrogen substituents on the rotational barrier of *N*,*N*-dialkylacetamide, because C_α atom branching will cause higher isomerization rates.[56] However, a specific pH value was not reported along with isomerization parameters resulting from the photochemical technique. Lowering the pH value to 2.8 caused a 1.6-fold higher rate for Ala-Phe, which may report a critical protonation state of the dipeptide rather than specific acid catalysis. The absence of intermolecular general acid/base catalysis, which was evident for prolyl isomerizations,[8,57] could be confirmed for phenylalanyl isomerizations in buffered solution by measuring unaltered isomerization rates between pH 4.5 and 8.6.

In dipeptides, the rate constant of the reverse trans → cis isomerization was found to be 1.2×10^{-3} and 2.4×10^{-3} s^{-1} for the alanyl and the tyrosyl isomerization, respectively (Table 2). These values are quite similar to that of the trans → cis isomerization in Ala-Pro (1×10^{-3} s^{-1}).[58] Obviously, cis isomers of secondary amide peptide bonds are strongly disfavored due to the ease of crossing of the energy barrier separating the rotational states.

As was already published for simple amides,[59] there was no kinetic solvent deuterium isotope effect detectable for the isomerization rates of Ala-Phe at pH 2.8 and pH 5.9 (data not shown). Therefore, the NH proton is expected to be relatively

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inert toward the distinct rotational states when isomerization proceeds along the reaction coordinate.

Virtually identical rate constants became apparent by the comparison of the Ala-Tyr peptides (Table 2) with both the forward and reverse Tyr38-Ala39 isomerization in a RNase T_1 variant.[35,36] This is not surprising for the cis → trans direction because the rate constants measured under these conditions are characteristic of a randomly ordered peptide backbone for both the protein and the oligopeptides. As for the RNase T_1 variant, the generation of the cis Glu166-Thr167 bond in a cis Pro167Thr mutant of TEM-1 β-lactamase from a premature state is also characterized by a rate constant between 1×10^{-3} and 4×10^{-3} s^{-1}.[32] Significantly enough, these rates have been realized with the oligopeptide too, but at a very low cis stability. It is worth noting that the reisomerization steps have to occur in strongly folded intermediates with the proteins. Ordered structures led to a large decrease of trans → cis isomerization rates for prolyl bonds.[60] Obviously, when attributed to trans → cis isomerizations, the rate constants presented above cannot much contribute to the surprising stability of secondary amide peptide bonds in the native mutant proteins. Instead, considerable deceleration of the cis → trans isomerization in a native-like state has to account for a large part of the thermodynamic cis stability.

Apparently, the suspicion of higher isomerization rates with increasing chain length, which might be inferred from the data of Table 2, did not hold true for polypeptides. Alternatively, coincidence of rate constants of folding and isomerization may accidentally arise under the specific condition of the experiments. Significantly, the unfolded Pro39Ala RNase T_1 variant was responsible for an Arrhenius activation energy as much as 27 kJ mol^{-1} higher for the cis → trans alanyl isomerization when compared to that of Tyr-Ala in the pentapeptide (Table 2).

Quantitative kinetic studies on the folding of small, proline-free proteins, which have *all*-trans peptide bonds in the native state, may serve as a probe to report the influence on refolding of a transient population of cis peptide bonds in the unfolded state. Such proteins having a single activated state between denatured and native state unfold and refold extremely fast with half-times much shorter than 1 ms.[61−63] Apparently "wrong" peptide bonds have not been trapped in a special folding state or intermediate. It may seem puzzling that the native state can be rapidly acquired for the entire population of unfolded molecules irrespective of the participation of the "normal" peptide bond isomerization that has proved to be slow on this time scale.

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Supporting Information Available: Tables with all kinetic and thermodynamic results and the simulation parameters for the line shape analysis of the ^{1}H NMR spectra and the MTRF experiments (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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