Rotational Barriers of *cis*/*trans* Isomerization of Proline Analogues and Their Catalysis by Cyclophilin[§]

Dorothee Kern,*,[†] Mike Schutkowski, and Torbjörn Drakenberg[‡]

Contribution from the Forschungsstelle "Enzymologie der Proteinfaltung", Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V., Kurt-Mothes-Strasse 3, D-06120 Halle/Saale, Germany

Received February 25, 1997[⊗]

Abstract: The rotational barriers for *cis*/*trans* isomerization of different proline analogues have been investigated by dynamic ^{1}H NMR spectroscopy. To this end the analogues (*S*)-azetidine-2-carboxylic acid (Aze), (*S*)-piperidine-2-carboxylic acid (Pip), (*R*)-thiazolidine-4-carboxylic acid (4-Thz), (4*R*)-2-methylthiazolidine-4-carboxylic acid (2Me4-Thz), (*R*)-thiazolidine-2-carboxylic acid (2-Thz), (*S*)-oxazolidine-4-carboxylic acid (4-Oxa), (4*S*,5*R*)-5-methyloxazolidine-4-carboxylic acid (5Me4-Oxa), and (2*S*,4*R*)-4-hydroxypyrrolidine-2-carboxylic acid (Hyp) and several N-alkylated amino acids were incorporated into the sequences Ala-Yaa-(4-)nitroanilide and Ala-Gly-Yaa-Phe-(4-)nitroanilide. NMR line-shape analyses of various *cis* and *trans* proton signals of these peptides were performed at different temperatures, and the rate constants of *cis*/*trans* isomerization were fitted to the Eyring equation. The rotational barriers of all cyclic proline analogues except hydroxyproline were found to be lower than that of proline by about 10 kJ/mol, whereas all noncyclic analogues and hydroxyproline showed rotational barriers similar to that observed for proline. In addition, the ability of cytosolic porcine kidney cyclophilin (Cyp18), a member of the peptidyl prolyl *cis*/*trans* isomerase family, to catalyze *cis*/*trans* isomerization of the peptide bond preceding the proline analogues was investigated. By line-shape analyses we proved efficient catalysis by Cyp18 for the analogues Aze, 4-Thz, and 2-Thz.

Introduction

Proline occupies a special place among the amino acids. On one hand, prolyl residues restrict the conformational space for the peptide chain, but on the other hand, both *cis* and *trans* isomers occur in solution because the energy difference between the two isomers, $\Delta G°$, is small (<8.4 kJ/mol). Since the rotational barrier of *cis*/*trans* isomerization is high ($\Delta G^{\ddagger} = 85 \pm 10$ kJ/mol) this reaction may become rate limiting in protein folding.[1] Furthermore it has been shown that the isomer conformation is important for recognition, stability, and reactivity of proline containing peptides and proteins.[2−11]

Proline residues are frequently found in structural modules regulating protein/protein interactions such as WW[12,13] and SH3 domains.[14,15] Furthermore, collagen, the most common protein in connective tissue, is composed mainly of trimeric repeats of the structure Gly-Xaa-Yaa where proline and 4-hydroxyproline (Hyp) are found in positions Xaa and Yaa.

Proline derivatives are often incorporated into biologically active peptides to study conformational effects[16−23] or to increase the bioavailibility because the unique cyclic structure restricts the attack of proteases.[24,25] While 4-Thz was often incorporated into collagen model compounds[26,27] or bioactive molecules such as thrombin inhibitors,[18] somatostatin[20,28] dipeptidyl peptidase

* To whom correspondence should be addressed.

[†] Present address: Department of Chemistry, University of California at Berkeley, Calvin Lab, Berkeley, CA 94720.

[‡] Department of Physical Chemistry 2, Chemical Center, University of Lund, Box 124, S-22100 Lund, Sweden.

[§] Abbreviations used: CsA, cyclosporin A; Cyp18, 18 kDa cytosolic cyclophilin from porcine kidney; DPIV, dipeptidyl peptidase IV; $k_{c\rightarrow t}$, rate constant of *cis* to *trans* isomerization; NHMe, methylamide; PPIase, peptidyl prolyl *cis*/*trans* isomerase; TSP, sodium 3-(trimethylsilyl)propionate; (4-)-NA, 4-nitroanilide; Yaa, proline analogues and proline.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1997.

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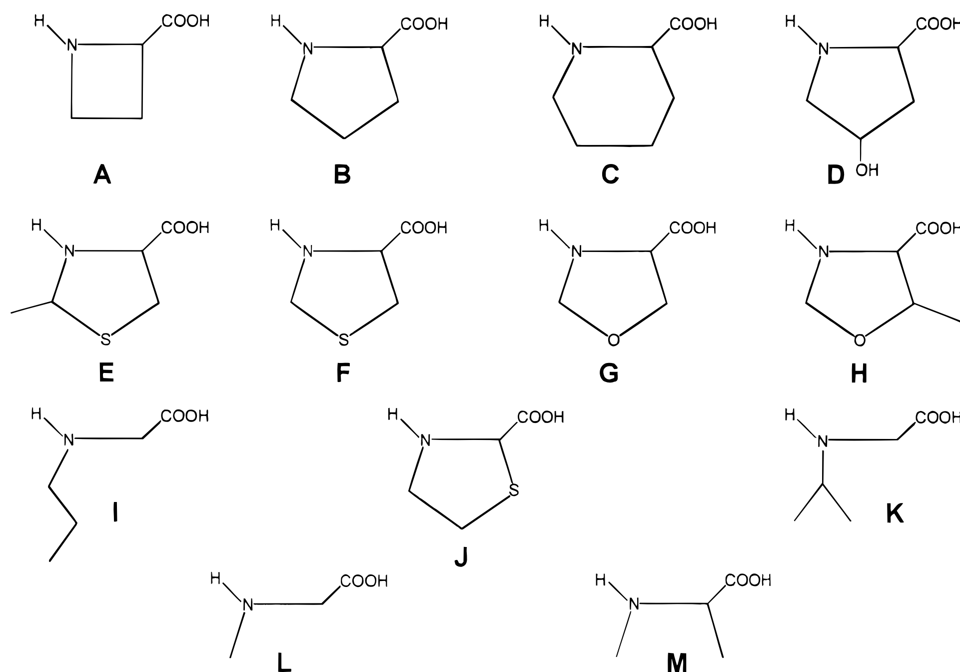

Figure 1. Amino acid analogues which have been used to probe *cis*/*trans* isomerization of the preceding peptide bond and the ability of cyclophilin to catalyze this isomerization: A = (*S*)-azetidine-2-carboxylic acid, Aze; B = proline, Pro; C = (*S*)-piperidine-2-carboxylic acid, Pip; D = (2*S*,4*R*)-4-hydroxypyrrolidine-2-carboxylic acid, Hyp; E = (4*R*)-2-methylthiazolidine-4-carboxylic acid, 2Me4-Thz; F = (*R*)-thiazolidine-4-carboxylic acid, 4-Thz; G = (*S*)-oxazolidine-4-carboxylic acid, 4-Oxa; H = (4*S*,5*R*)-5-methyloxazolidine-4-carboxylic acid, 5Me4-Oxa; I = *N*-(*n*-propyl)glycine, nPrGly; J = (*R*)-thiazolidine-2-carboxylic acid, 2-Thz; K = *N*-isopropylglycine, iPrGly; L = sarcosine, Sar; M = *N*-methylalanine, MeAla.

IV substrates,[29] angiotensin II,[30] angiotensin converting enzyme inhibitors,[17] and oxytocin,[31] 4-Oxa residues are rarely used[17,29] because of the acid lability of the oxazolidine ring and the related synthetic difficulties. On the basis of X-ray studies of *N*-acetylthiazolidine-4-carboxamide[32] and theoretical calculations of polythiazolidine-4-carboxylic acid and polyoxazolidine-4-carboxylic acid, it was suggested that, in contrast to poly-Pro and poly-4-Oxa, poly-4-Thz should not mutarotate from *trans* to the *cis* form in helical structures.[26,27] Piperidine-2-carboxylic acid (Pip) has been incorporated into analogues of bioactive peptides, such as bradykinin, angiotensin II,[19] oxytocin, angiotensin converting enzyme inhibitors, thrombin substrates, and inhibitors.[18] Additionally, the presence of Pip residues is characteristic of the immunosuppressive compound FK506. The contraction of the pyrrolidine ring of proline to the four-membered azetidine ring of Aze has effects on bond angles and internal torsion angle according to calculations of Zagari et al.[33] and ^{1}H NMR measurements of angiotensin II analogues by Matsoukas et al.[19] The effects of Aze residues on model peptides, such as poly-Aze homopolymers, poly(Gly-Pro-Aze), and dipeptides have been summarized.[33−35]

Some comparative ^{1}H-NMR studies have been reported concerning the *cis*/*trans* equilibrium of MeAla, Hyp, Aze, Pip, and Pro in model compounds such as acyl amino acids,[36,37] benzoyl amino acids,[38] and Boc-protected *O*-phenacyl derivatives.[16] However, nothing is known about the rotational barriers of peptide bonds formed by proline analogues. In general the rotational barrier of *cis*/*trans* isomerization could be crucial as a kinetic control of recognition, stability, and reactivity of peptides and proteins containing prolines or proline analogues. To investigate the rotational barriers for *cis*/*trans* interconversion, the proline ring was changed in size, by displacement of ring carbons by oxygen or sulfur, by addition of residues to the ring, or by breaking the ring (Figure 1). Some of the proline analogues have already been incorporated into the sequence Ala-Yaa-(4-)NA, where Yaa represents the different analogues, for studies of the substrate specificity of proline specific peptidases, particularly dipeptidyl peptidase IV (DPIV).[29,39] Therefore we first used these peptides for investigations of the influence of changes in the proline ring on the *cis*/*trans* equilibrium as well as on the rotational barrier.

In the second part of the paper, we investigate the substrate specificity of cyclophilin18, a member of the enzyme class of peptidyl prolyl *cis*/*trans* isomerases (PPIases), directly at the proline position. It has been shown that (PPIases) catalyze the *cis*/*trans* isomerization of peptidyl−prolyl bonds in short peptides[40] and during the refolding of proteins.[41−47] Until now,

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it has been assumed that PPIases are strictly proline specific and only the substrate specificity for the residues N- and C-terminal to proline has been investigated. Using the proline analogues of Figure 1, we investigated whether PPIases are indeed proline specific enzymes or might catalyze the *cis/trans* isomerization of the amide bond preceding proline analogues. For this purpose proline analogues were incorporated into the sequence Ala-Gly-Yaa-Phe-(4-)NA, which has been shown to be a substrate for PPIases of the cyclophilin type if Yaa is proline.

Dynamic NMR spectroscopy was used to determine the isomerization rate and the activation parameters of *cis/trans* isomerization of the appropriate proline analogues. The data show that the *cis/trans* isomerization is accelerated by changing the proline ring in size and structure. In addition, we could demonstrate that the 18 kDa cytosolic porcine kidney cyclophilin (Cyp18) is not proline specific.

Experimental Section

Substrate Synthesis. All tetrapeptide derivatives were synthesized in solution using the Boc moiety for temporary protection of the amino group and isobutyl chloroformate to form the activated mixed anhydride of protected amino acids or peptides.

The purity of all compounds was checked by TLC and HPLC and for the deprotected substances by capillary electrophoresis. The structure was confirmed by electrospray mass spectrometry and NMR spectroscopy.

For the NMR experiments the peptides were dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 10% D_2O for the lock signal. The final stock solutions were 14 mM.

Enzyme Preparation. The preparation of the 18 kDa cytosolic cyclophilin from porcine kidney (Cyp18) and determination of the concentration of active enzyme by active site titration were done as previously described.[48]

NMR Measurements. All experiments were carried out on a GE Omega 500 MHz spectrometer. Conditions of the one-dimensional proton (1D ^{1}H) NMR experiments have already been described.[48] Standard pulse sequences were used for the COSY and ROESY spectra. The TOCSY experiments were modified according to Rance[49] to use the DIPSI-2 mixing sequence (spinlock of 120 ms). The data were acquired in the hypercomplex mode with a spectral width of 6410 Hz, 64 transients, and 256 time increments with 2048 complex data points.

NMR Assignments and Determination of the Activation Parameters. The assignments were made using COSY, TOCSY, and ROESY spectra. For determination of the activation parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$) of all peptides containing the proline analogues, the temperature was varied in steps of 5° from 283 to 338 K. At each temperature the line shapes were analyzed according to a two site exchange model using a locally written program, based on the Bloch equations. The following parameters were necessary for the simulation:

(I) The chemical shifts of the *cis* and *trans* signal in the absence of chemical exchange are directly available from the spectrum of peptide with slow exchange, i.e. at the lowest temperature. However, at higher temperature, both the *cis* and *trans* signals shift not only due to accelerated exchange but also due to changes in temperature. This temperature dependence of chemical shifts was determined in a temperature range where the shifts were not yet influenced by exchange. From these data the chemical shifts in the absence of exchange at higher temperatures could be estimated by extrapolation assuming a linear dependence on temperature.

(II) The vicinal coupling constants of the signals were determined from the spectra taken at low temperature.

(III) The effective transverse relaxation times (T_2) could be determined directly from the line widths of the spectra under slow exchange conditions. At higher temperatures, the signal from the reference sodium 3-(trimethylsilyl)propionate (TSP), which is not affected by exchange, was used as a line shape reference as previously described.[48]

(IV) The populations of the two isomers and finally (V) the rate constants of *cis/trans* isomerization $k_{c\rightarrow t}$ were determined by simulation. The populations did not change in the measured temperature range within the error of measurement (±0.01).

The derived rate constants were fitted to the Eyring equation to obtain the activation parameters and the rate constant of *cis/trans* isomerization at room temperature. The activation parameters for the proline peptides were determined by isomer specific proteolysis described by Schutkowski et al. for the sequence Ala-Pro-(4-)NA[50] and for Ala-Gly-Pro-Phe(4-)NA.[51]

Irreversible changes of the NMR sample caused by heating could be excluded since the NMR spectra of the heated samples, readjusted to 283 K, were identical to the original spectra at 283 K. The peptide concentration in all experiments was 3 mM.

Proof of Cyp18 Catalysis. To a 430 μL peptide sample (300 μM) were added 10 μL aliquots of the Cyp18 stock solution (175 μM) six times. Subsequently 12 μL aliquots of the peptide stock solution (14 mM) were added five times. After each addition a ^{1}H NMR spectrum was acquired at 300 K. The experiments with the inhibitor CsA were done as described by Kern et al.[48]

Table 1. *Cis* Content, Activation Parameters, and Rate Constants of *cis* → *trans* Isomerization of Ala-Yaa-(4-)NA[a]

Yaa	% *cis* (pH 6.0)	$\Delta H^{\ddagger}$ (kJ/mol)	$T\Delta S^{\ddagger\,b}$ (kJ/mol)	$\Delta G^{\ddagger\,b}$ (kJ/mol)	$k_{c\rightarrow t}{}^{b}$
Pro[c]	6 ± 1	78.9 ± 0.2	−1.8 ± 0.6	80.7 ± 0.3	0.055 ± 0.005
4-Thz	7 ± 1	56.0 ± 6	−14.0 ± 7	70.0 ± 0.6	4.0 ± 1
4-Oxa	15 ± 1	55.0 ± 4	−14.0 ± 5	69.0 ± 0.6	6.0 ± 1
Aze	20 ± 1	67.0 ± 3	−7.0 ± 5	74.0 ± 2	0.8 ± 1
Pip	13 ± 1	47.0 ± 6	−23.0 ± 7	71.0 ± 1	2.7 ± 1

[a] In 10 mM sodium phosphate buffer, pH 6.0. [b] At 300 K. [c] At pH 7.8, from ref 50.

Results

Determination of *cis/trans* Ratios and Activation Parameters for *cis/trans* Isomerization of Ala-Yaa-(4-)NA. Figure 1 shows the proline analogues which were used in this study. The analogues 4-Thz, 4-Oxa, Aze, and Pip as well as Pro were used in the sequence Ala-Yaa-(4-)NA for determination of the activation parameters for *cis/trans* isomerization. For all peptides, NMR signals from *cis* and *trans* isomers could be detected at room temperature, showing that both isomers are present and that *cis/trans* isomerization is slow on the NMR time scale under these conditions. The *cis/trans* ratio of the different peptides (Table 1) was determined by integration of separated signals in 1D ^{1}H NMR spectra.

For the proline peptide Ala-Pro-(4-)NA, the rate constant for the uncatalyzed *cis* → *trans* isomerization ($k_{c\rightarrow t}$) and the activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ could be determined by a coupled spectrophotometric assay using the protease DPIV in the assay.[50] Although almost all of the proline analogues incorporated into this sequence are substrates for DPIV,[52] only Ala-Aze-(4-)NA fulfills the requirements for estimation of *cis/trans* isomerization using isomer specific proteolysis because

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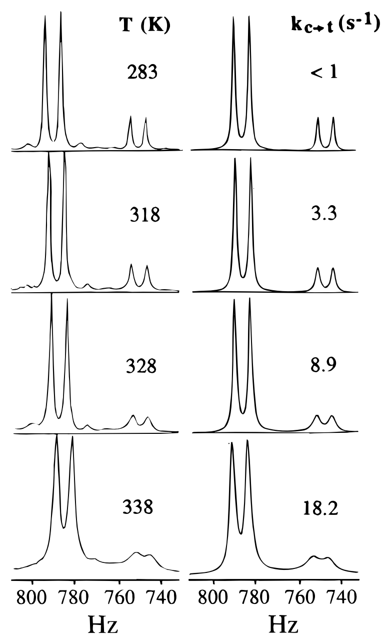

Figure 2. Determination of the rate constants of *cis* → *trans* isomerization ($k_{c\to t}$) in Ala-Aze-(4-)NA at different temperatures. Expansion of ^{1}H NMR spectra of 3 mM Ala-Aze-(4-)NA in H_2O (10 mM sodium phosphate buffer, pH 6.0) at different temperatures showing the *cis* and *trans* signals of the alanine methyl protons: (left) experimental spectra; (right) calculated line shapes according to a two site exchange model with the marked $k_{c\to t}$ values.

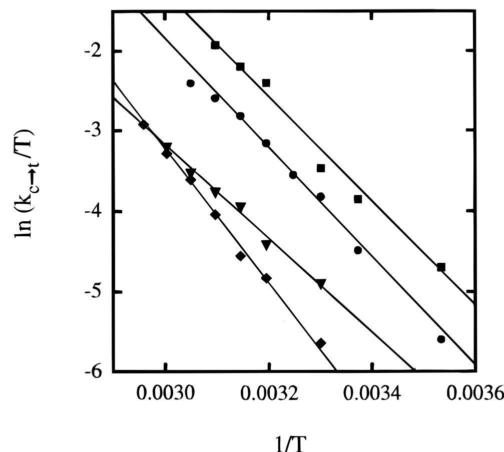

Figure 3. Eyring plot of *cis* → *trans* isomerization of Ala-Yaa-(4-)-NA in 10 mM sodium phosphate buffer (pH 6.0) with Yaa = (●) 4-Thz, (▼) Pip, (■) 4-Oxa, (◆) Aze.

Table 2. Influence of Changes of the Proline Ring on the *cis* Content in Ala-Gly-Yaa-Phe-(4-)NA[a]

Yaa	% *cis*[b] (pH 6.0)	Yaa	% *cis*[b] (pH 6.0)
Pro	23	Aze	40
4-Thz	28	Pip	21
2-Thz	8	Sar	30
2Me4-Thz	33	MeAla	26
4-Oxa	38	nPrGly	33
anti-5Me4-Oxa	33	iPrGly	37
anti-4-Hyp	19		

[a] At 300 K. [b] Error range: ±1%.

either the isomerization is too fast to be detected in this assay or the turnover number k_{cat} for DPIV is too low. Therefore we used dynamic NMR spectroscopy.

NMR spectra at different temperatures were taken between 283 and 338 K. In this range the lines broaden due to accelerated *cis/trans* isomerization as shown in Figure 2 for the alanine methyl protons of Ala-Aze-(4-)NA. For determination of the values of $k_{c\to t}$ at the indicated temperatures, parts of the spectra with well-separated *cis* and *trans* signals and simple spin coupling patterns were selected. Since there is an exchange between the two conformers, *cis* and *trans*, the line shapes were analyzed according to a two-site exchange model. The simulated line shapes with the corresponding rate constants are shown to the right in Figure 2. The activation parameters and the rate constants at room temperature, summarized in Table 1, were determined by fitting the rate constants from the simulations to the Eyring equation (Figure 3).

Cyp18 Catalysis of the *cis/trans* Isomerization of the Amide Bond Preceding the Proline Analogues. To answer the question of whether Cyp18 is specific for peptidyl−prolyl bonds or if this enzyme also accepts variation of the proline ring, all analogues of Figure 1 were incorporated into the sequence Ala-Gly-Yaa-Phe-(4-)NA. The chemical composition and the purity of the peptides were confirmed by COSY, TOCSY, and ROESY spectra.

For the proline peptide, the rate constant and the activation parameters of the uncatalyzed *cis* → *trans* isomerization, as well as the catalysis by Cyp18, were determined by the improved spectrophotometric PPIase-assay based on the isomer specific proteolysis.[40,51] However, this was impossible for the peptides containing the proline analogues due to the fast *cis/trans* isomerization with the exception of Ala-Gly-Aze-Phe-(4-)NA (see below).

We used our previously published dynamic NMR spectroscopy method[48,53] to detect PPIase catalysis for the different peptides. As already shown for the dipeptide 4-nitroanilides, well-separated *cis* and *trans* signals occurred at room temperature. These signals were integrated to determine the influence of the modified proline ring on the *cis/trans* ratio (Table 2). To prove catalysis of *cis/trans* isomerization for the Gly-Yaa peptide bonds by Cyp18, ^{1}H NMR spectra of 300 μM peptide in the absence and presence of increasing amounts of Cyp18 were taken and the line shapes of well-separated *cis/trans* pairs were compared. In the case of the proline peptide, the expected line broadening and shift of the signals due to accelerated *cis/trans* interconversion with increasing amounts of Cyp18 can be observed (Figure 4A). For the peptides containing MeAla, nPrGly, iPrGly, Sar, Pip, anti4-Hyp, 4-Oxa, anti-5Me4-Oxa, and 2Me4-Thz, the line shapes were not altered up to 25 μM Cyp18.

However, for the peptides containing Aze, 4-Thz, and 2-Thz, addition of increasing amounts of Cyp18 results in line broadening similar to that observed for the proline peptide. Figure 4B shows representative results for the Aze derivative. In all cases the original slow exchange spectrum was obtained after incubation of the peptide/Cyp18 solution with excess of the inhibitor CsA (data not shown). These experiments clearly prove that Cyp18 catalyzes *cis/trans* isomerization of the Gly-Aze, Gly-4-Thz, and the Gly-2-Thz peptide bonds.

To calculate the efficiency of catalysis, the uncatalyzed *cis/trans* isomerization rates were determined with the method described above for the dipeptide 4-nitroanilides. As an additional verification of the rate constants determined from the line-shape analyses of the methyl signals, other sets of signals (amide protons of Gly, Phe and (4-)NA) were analyzed by line-shape analyses as well. Within the experimental error given in Table 3, the simulation of different sets of signals gave the same

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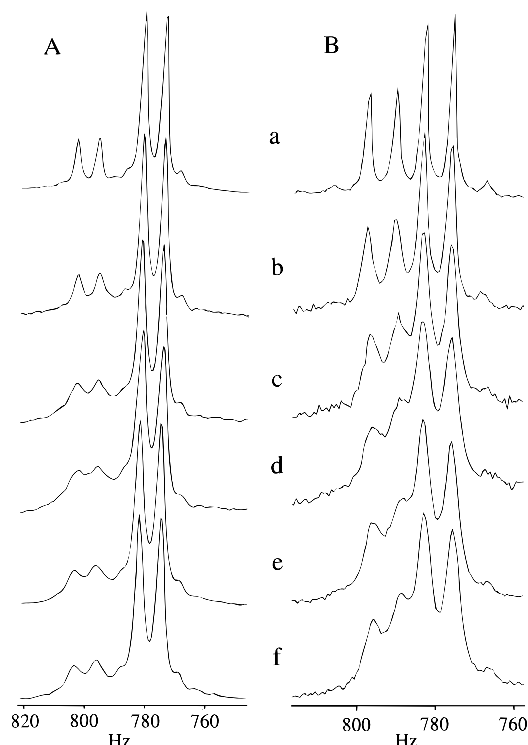

Figure 4. Proof of Cyp18 catalysis in Ala-Gly-Aze-Phe(4-)NA and comparison of catalytic efficiency between Ala-Gly-Pro-Phe(4-)NA (A) and Ala-Gly-Aze-Phe(4-)NA (B) with (a) 300 μM [S_o], 0 μM [E_o], (b) 288 μM [S_o], 7.7 μM [E_o], (c) 278 μM [S_o], 15 μM [E_o], (d) 268 μM [S_o], 22 μM [E_o], (e) 850 μM [S_o], 21 μM [E_o], and (f) 1.41 mM [S_o], 20.5 μM [E_o]. The experiments were done in 10 mM sodium phosphate buffer (pH 6.0) at 300 K.

Table 3. Activation Parameters and Rate Constants of *cis* → *trans* Isomerization of Ala-Gly-Yaa-Phe-(4-)NA and Catalysis by Cyp18[a]

Yaa	$\Delta H^{\ddagger}$ (kJ/mol)	$T\Delta S^{\ddagger\, b}$ (kJ/mol)	$\Delta G^{\ddagger\, b}$ (kJ/mol)	$k_{c\to t}{}^{b}$ (s^{-1})	catalysis by Cyp[c]
Pro[d]	74.7 ± 6	−7.1 ± 7	81.8 ± 0.3	0.036 ± 0.005	+
4-Thz	62.0 ± 5	−8.0 ± 6	70.0 ± 0.7	4.0 ± 1	+
2-Thz	55.0 ± 7	−14.0 ± 8	69.0 ± 0.6	6.0 ± 1	+
4-Oxa	65.0 ± 4	−5.0 ± 5	70.5 ± 0.9	3.30 ± 1	−
Aze	63.0 ± 5	−9.5 ± 8	72.5 ± 2.7	1.5 ± 1	+

[a] In 10 mM sodium phosphate buffer, pH 6.0. [b] At 300 K. [c] At a peptide concentration of 300 μM in the presence of 25 μM Cyp18 no (−) or a significant line broadening (+) in the ^{1}H NMR spectrum at 300 K. [d] Determined by the coupled spectrophotometric assay.[51]

values. Insertion of the obtained rate constants into the Eyring equation allowed calculation of the activation parameters (Table 3).

Comparison between the Two Methods: The Isomer-Specific Proteolysis and the ^{1}H NMR Measurements. To prove that we can directly compare the rotational barriers obtained with two different techniques, we determined the $k_{c\to t}$ values for peptide bonds of proline analogues also by the isomer specific proteolysis method: for Ala-Aze-(4-)NA according to Schutkowsi et al.[50] at 276 K and for Ala-Gly-Aze-Phe-(4-)NA according to ref 51 using subtilisin as the protease at 283 K. The measured rate constants are in accordance with the values extrapolated from the Eyring data obtained by the ^{1}H NMR measurements.

To rule out that the unique slowness of *cis/trans* isomerization of the proline peptide is due to the fact that these experiments were performed at pH 7.8[50,51] whereas the NMR experiments on the proline analogues were done at pH 6.0, we investigated the pH dependence of *cis/trans* isomerization of Ala-Pro-(4-)-NA and Ala-Gly-Pro-Phe-(4-)NA using the methods published.[50,51] The rotational barriers of prolyl *cis/trans* isomerization as well as the *cis/trans* ratio are pH independent in a pH range between pH 5.5 and 8.5 for both peptides.

Discussion

The aim of this study was to elucidate the influence of changes in the proline ring on the *cis/trans* ratio as well as on the activation parameters of interconversion. In addition the ability of Cyp18 to catalyze the *cis/trans* isomerization of peptide bonds formed by proline analogues was investigated.

Cis/trans **Equilibrium of the Proline Analogues.** For the acyclic N-alkylated amino acids, the amount of *cis* isomer increases with the volume of the substituent attached to the amide nitrogen. For the ring analogues, the highest *cis* content was observed for the peptides with the proline analogues Aze and 4-Oxa in both sequences used in our study. The *cis* peptide is further favored thermodynamically by an additional substituent at the δ-position of the ring (2Me4-Thz). This is in accordance with earlier studies showing that, for example, anti-5-methylproline exhibits a relatively high percentage (30−50%) of *cis* isomer in model peptides.[54] In 2-substituted oxazolidine-4-carboxylic acid derivatives, a preference for the *cis* isomer of the amide bond preceding the proline derivative was detected.[55] Incorporation of two methyl groups in δ-position has been shown to give 90% *cis* peptide bond isomer for 5,5-dimethylproline[56] or exclusively *cis* conformer for 2,2-dimethyl-(*R*)-thiazolidine-4-carboxylic acid in model peptides.[57]

In contrast, substituents at the β-position result in an increased population of the *trans* isomer (2-Thz, anti-5Me4-Oxa). However the *cis/trans* ratio is also strongly dependent on the surrounding residues. This is reflected by the different populations of the *cis* isomers for the analogues incorporated into the two different sequences. Whereas in Ala-Pip-(4-)NA the *cis* content is increased by 7% compared to the proline peptide, the *cis* content is decreased by 2% in Ala-Gly-Pip-Phe-(4-)NA compared to the proline peptide. Energy calculations on acetyl-Aze-NHMe and acetyl-Pro-NHMe predicted almost identical *cis/trans* ratios for these two peptides.[38−40] However, for the peptides investigated in the present study, the peptides containing azetidine-2-carboxylic acid show significantly higher populations of the *cis* isomer than in the corresponding proline peptides.

Rotational Barriers of the Analogues. For the analogues Aze, Pip, 4-Oxa, 4-Thz, and 2-Thz, a substantial decrease in the free energy of activation, $\Delta G^{\ddagger}$, and consequently an increased rate constant of *cis/trans* interconversion compared to the proline peptide was detected. This effect seems to be determined mainly by the analogues themselves and not by the neighboring residues, since similar data were obtained for both series of peptide derivatives.

Until now no data have been published about the rotational barriers of *cis/trans* isomerization of peptide bonds preceding proline analogues. Previously, the *cis/trans* isomerization of ring derivatives without the carboxylate group was studied.[58] Investigations of the influence of the ring size on the rotational barrier of the *N*-toluoyl derivatives of azetidine, pyrrolidine, and piperidine[58] show decreased $\Delta G^{\ddagger}$ values of *cis/trans* isomer-

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ization for *N*-toluoylazetidine as well as for *N*-toluoylpiperidine compared to *N*-toluoylpyrrolidine. This is in agreement with the lowered $\Delta G^{\ddagger}$ values obtained in our work for the corresponding carboxylated rings. The X-ray structures reveal a pyramidal nitrogen in *N*-toluoylazetidine whereas it is planar in *N*-toluoylpyrrolidine.[58] The change of hybridization from sp^2 (*p*-toluoylpyrrolidine) toward sp^3 (*p*-toluoylazetidine) has been suggested to be the major factor for the decreased rotational barrier of the four-membered ring compared to the five-membered ring, which is in agreement with *ab initio* calculations on planar and orthogonal formamides.[59] For the six-membered ring an increased steric hindrance might be the reason for a destabilization of the planar conformation of the peptide bond, resulting in a lower rotational barrier.[60,61] In summary, our results agree with the data on the toluoyl derivatives, even though we investigated the *cis*/*trans* isomerization of the corresponding carboxamides.

Systematic investigations of the influence of displacement of the γ-methylene group in pyrrolidine by oxygen or sulfur have not been reported, although rotational barriers for *N*-acetyloxazolidine (73 kJ/mol) and *N*-acetylmorpholine (70.6 kJ/mol) have been determined.[62] The value for the oxazolidine derivative agrees well with our data. The X-ray structures of *N*-acetylproline amide and its isomorphous analogue *N*-acetylthiazolidine-4-carboxamide provide no evidence for the source of the lowered rotational barriers for a thiaprolyl peptide bond since they appear to be very similar.[32] However, in the Oxa and Thz derivatives, the oxygen and sulfur in the ring withdraw electron density from the nitrogen which results in reduced double-bond character of the peptide bond.

In summary, proline seems to be optimized to a high rotational barrier of *cis*/*trans* isomerization of its Xaa−Pro peptide bonds since all alterations of the ring studied in this work except hydroxylation result in lowering of the rotational barrier.

Cyp18 Catalysis of Some of the Analogues. Using the proline analogues in the sequence Ala-Gly-Yaa-Phe-(4-)NA we showed that Cyp18 does not exclusively catalyze *cis*/*trans* isomerization of peptidyl−prolyl amide bonds but also peptide bonds of the analogues 4-Thz, 2-Thz, and Aze. The determination of all rate constants for Cyp18 catalysis of the proline analogues using our recently published method[48] was not possible, since the uncatalyzed *cis*/*trans* isomerization is already too fast. Thus it is impossible to measure the k_{cat}/K_M value using the isomer specific proteolysis assay,[40] which is required to obtain a unique solution of the line-shape analyses.[48] However, a qualitative comparison of the line shapes of the proline peptide and the peptides containing the proline analogues in the presence of identical amounts of Cyp18 allows an estimation of catalytic efficiency for the analogues. The observed line broadening for the peptides in Figure 4 is similar, indicating similar exchange rates in the presence of identical amounts of Cyp18. However, the uncatalyzed rate constants of *cis*/*trans* isomerization are at least 1 order of magnitude higher for the 4-Thz, 2-Thz, and Aze peptide derivatives compared to the proline peptide. This results in a reduced efficiency of Cyp18 catalysis on these proline analogues.

For all other analogues shown in Figure 1, no line broadening was observed for the peptides in the presence of 25 μM Cyp18. The upper limit for the rate constant of interconversion, at which no line broadening is observed, was calculated to about 1 s^{-1} using the modified Bloch equations. This means that for these peptides catalysis by Cyp18 cannot be generally excluded. However, if catalysis exists, the efficiency must be very low.

Acknowledgment. This work was supported by the Studienstiftung des deutschen Volkes, the Deutsche Forschungsgemeinschaft (Fi455/1-3), the Boehringer-Ingelheim Stiftung, and the Swedish National Science Research Council. The authors gratefully acknowledge discussions and advice on this project from Prof. G. Fischer. We thank Prof. D. Wemmer for critical reading the manuscript and B. Hökelmann for technical assistance with the peptide synthesis.

Supporting Information Available: Detailed description of tetrapeptide-4-nitroanilide synthesis including the characterization of all synthetical intermediates and ^{1}H NMR data of the final products (8 pages). See any current masthead page for ordering and Internet access instructions.

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