Intramolecular Assistance of *cis/trans* Isomerization of the Histidine−Proline Moiety[†]

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ABSTRACT: Peptidyl-prolyl *cis/trans* isomerization is a slow conformational interconversion in the polypeptide backbone that is frequently rate-limiting in refolding of proteins and is thought to play a role in cellular restructuring of proteins. In order to probe the influence of positively charged amino acids located in sequence segments adjacent to proline, the rotational barriers of Arg-Pro- and His-Pro-containing peptides were determined by isomer-specific proteolysis and dynamic NMR spectroscopy for Suc-Ala-His-Pro-Phe-NH-Np, Ac-Ala-Arg-Pro-Ala-Lys-NH_2, Ac-Ala-His-Pro-Ala-Lys-NH_2, angiotensin III, thyrotropin-releasing hormone (TRH), and [His(3-Me)[2]]TRH in aqueous solution. In contrast to the guanidinium group of arginine, the protonated side chain of histidine preceding proline led to an acceleration of the prolyl isomerization up to 10-fold relative to the unprotonated state. Both arginine and histidine residues succeeding proline in an amino acid sequence proved to be ineffective. Under basic and acidic conditions the kinetic solvent deuterium isotope effects $k_{c\rightarrow t}^{H_2O}/k_{c\rightarrow t}^{D_2O}$ for angiotensin III were 1.0 ± 0.1 and 2.0 ± 0.1, respectively. The results are interpreted in terms of intramolecular general acid catalysis of prolyl bond rotation by the imidazolium group that is without precedent in intermolecular catalysis.

Cis/trans isomerization of peptidyl-prolyl bonds[1] is a slow conformational change that is characterized by relaxation times of about 10 s at 37 °C for unstructured peptide chains (*1*, *2*). It interconverts conformers of polypeptide chains being quite different in the spatial organization around the proline residue in a switchlike manner (*3*). In many cellular events the observed ratio of *cis* and *trans* isomers does not transiently correspond to the thermodynamic equilibrium under the actual conditions but reflects the former chemical environment of the peptide chain. Therefore, lag phases in reactions sensitive to the spatial organization of polypeptide chains will occur that may have consequences in biological processes. Isomer-specific proteolysis provides an example in which way prolyl isomerization[1] may affect recognition processes of bioactive compounds *in vitro* as well as *in vivo* (*3*, *4*). Furthermore, the comparable fractions of the prolyl bond isomers often lead to slow kinetic phases in refolding of denatured proteins because of the high rotational barrier (*5*−*8*). Fast-folding molecules have all structurally important prolyl bonds in the native conformation since the functional state of a protein frequently possesses conformational homogeneity for a given prolyl bond.

There is a considerable propensity for *cis* prolyl bonds in the native state of proteins that is documented for 70 out of 205 proline-containing proteins of a nonredundant database of 214 protein structures (Iditis V.3.1, Database 76, Oxford Molecular; *9*). Denatured proteins resemble unstructured oligopeptides with regard to prolyl isomerization. A high fraction of *trans* isomers can be assumed for the unstructured polypeptide chain of native proteins containing *cis* prolyl bonds. This population, in turn, may cause a large fraction of refolding molecules to persist in nonnative folding intermediates. Thus, subsequent to the fast formation of secondary structure and hydrophobic collapse, the particularly slow *trans* to *cis* isomerization must take place.

Even a *trans* state of a prolyl bond in a native protein can cause a rate-limiting prolyl isomerization in refolding since the fraction of *cis* isomer formed in the unfolded state results in a moderately slow *cis* to *trans* isomerization (*6*, *10*).

In cellulo rate-limiting prolyl isomerization may be avoided by catalysis by peptidyl-prolyl *cis/trans* isomerases (PPIases)[2] (*3*, *11*−*13*). These enzymes represent catalysts of prolyl isomerization in oligopeptides and partially folded proteins with acceleration factors of $>10^5$ (*14*).

Numerous examples exist demonstrating that proteins containing proline residues can refold within a few seconds *in vitro* despite the possible slow conformational changes during refolding (*15*, *16*).

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[1] The term prolyl isomerization is used throughout the paper for the *cis/trans* isomerization of the peptide bond preceding proline in an amino acid sequence. Similarly, the term prolyl bond is synonymous with the peptide bond preceding proline.

[2] Abbreviations: Ac, acetyl; δ, chemical shift; BTP, Bis-Tris-propane buffer; DHFR, dihydrofolate reductase; DIEA, *N*,*N*-diisopropylethylamine; DMAP, (*N*,*N*-dimethylamino)pyridine; EXSY, two-dimensional exchange nuclear magnetic resonance spectroscopy; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; ISP, isomer-specific proteolysis; $k_{c\rightarrow t}$, peptidyl-prolyl *cis* to *trans* isomerization rate; KSIE, kinetic solvent isotope effect; MBHA, 4-methylbenzhydrylamine; NH-Np, *p*-nitroanilide; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; PPIase, peptidyl-prolyl *cis/trans* isomerase; Suc, succinyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TRH, thyrotropin-releasing hormone; TSP, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4.

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Table 1: Sequences of the Peptides Used

Oligopeptides	
Suc-Ala-Phe-Pro-His-NH-Np	
Suc-Ala-His-Pro-Phe-NH-Np	
Suc-Ala-Phe-Pro-Lys-NH-Np	
Suc-Ala-Lys-Pro-Phe-NH-Np	
Ac-Ala-His-Pro-Ala-Lys-NH_2	
Peptide Hormones	
H-Arg-Val-Tyr-Ile-His-Pro-Phe-OH	angiotensin III
<Glu-His-Pro-NH_2	TRH
<Glu-His(3-Me)-Pro-NH_2	His(3-Me)^2]TRH

Regardless of the possible insensitivity of the folding probe to prolyl isomerization, a structure-based acceleration of the isomerization also may be responsible for the lack of slow refolding phases. Interactions specific to a certain spatial organization of the polypeptide chain of folding intermediates may either destabilize isomeric ground states or stabilize transition states, thus leading to enhanced prolyl isomerization rates (*17*, *18*). Prediction of structure-based enhancement of prolyl isomerization from the amino acid sequence is not yet possible.

A more general effect on prolyl isomerization could arise from catalysis by functional groups within the polypeptide chain. Specific acid catalysis was found for proline-containing peptides and model compounds but will not affect prolyl isomerization rates under physiological conditions (*19*−*21*).

Recently, intramolecular catalysis of prolyl isomerization by an anilide NH group of *N*-acetylproline anilides was inferred indirectly from comparison with proline esters in nonpolar solvents (*22*).

The aim of this work is to determine whether there is an influence of positively charged amino acid residues histidine and arginine, located at neighboring sequence positions to proline, on prolyl isomerization. Reliable data about the effect of side-chain protonation on isomerization rates were generated by analyzing pH-dependent rate constants for various -His-Pro- moieties. In our experiments, dipeptide units covering potential side-chain protonation sites were located in model peptides as well as in angiotensin III, thyrotropin-releasing hormone (TRH), and a modified TRH ([His(3-Me)^2]TRH) (see Table 1).

In the peptides investigated, the side-chain protonated histidine preceding proline gives the unique possibility of catalyzing prolyl isomerization by intramolecular general acid catalysis.

EXPERIMENTAL PROCEDURES

Materials

Ac-Ala-His-Pro-Ala-Lys-NH_2 was synthesized by a stepwise solid-phase procedure using Fmoc chemistry. For the synthesis a Rink amide MBHA resin (substitution 0.52 mmol/g) from Novabiochem was used. The acetylation was performed using acetic anhydride/DMAP/DIEA (10:5:10) in NMP. The Fmoc-amino acid derivatives (5 equiv) were coupled on the resin with HOBt/TBTU/DIEA (5:5:12.5) and the Fmoc cleavage was performed with 20% pyridine in NMP. The peptide was cleaved from the resin with 95% trifluoroacetic acid, precipitated with diethyl ether, and dried *in vacuo*. The resulting white powder was purified by reversed-phase HPLC using a Nucleosil C_8 column (250 × 25, 7 mm, LiChrosorb RP8) and acetonitrile−water mixtures containing 0.05% trifluoroacetic acid (v/v). The peptide was analyzed by electrospray mass spectroscopy and capillary electrophoresis. Suc-Ala-His-Pro-Phe-NH-Np, Suc-Ala-Phe-Pro-His-NH-Np, Suc-Ala-Gly-Pro-Phe-NH-Np, TRH (<Glu-His-Pro-NH_2), [His(3-Me)^2]TRH (<Glu-His(3-Me)-Pro-NH_2), and angiotensin III (H-Arg-Val-Tyr-Ile-His-Pro-Phe-OH) were all available from Bachem. The protease subtilisin Carlsberg was purchased from Sigma (St. Louis, MO), and bovine α-chymotrypsin was from Boehringer (Mannheim, Germany).

Methods

Protease Coupled Assay. The coupled assay according to Fischer et al. (*11*) was used to determine the rate constants of prolyl isomerization for the peptides Suc-Ala-His-Pro-Phe-NH-Np and Suc-Ala-Phe-Pro-His-NH-Np. A 35 mM potassium citrate buffer was used as solvent for the pH range between pH 3.5 and 5.7. The samples were diluted in 35 mM BTP buffer for measurements from pH 4 to 9.5. An appropriate buffer solution was prepared for each pH. A 50 μM substrate stock solution was prepared by dissolving the peptides in H_2O. Measurements were performed with subtilisin Carlsberg as helper protease at acidic pH below pH 6. α-Chymotrypsin was used above pH 5. A HP 8652 spectrophotometer with thermostated cell holder was used for kinetic measurements. The temperature was probed within the cell and kept at 10.0 ± 0.1 °C. Buffer and protease stock solution (final volume 1.25 mL) were equilibrated for 10 min. The measurements were started by adding 50 μL of substrate stock solution. A total of 1200 data points were collected for each kinetic run. Sampling the data started after 20−30 s, when the initial rapid cleavage of the *trans* isomer was finished. Data were fitted to a first-order rate equation. The same method was used with small modifications for the measurement of prolyl isomerization rates of Suc-Ala-Gly-Pro-Phe-NH-Np in the presence of imidazole. The buffer was 35 mM HEPES. The sample contained different concentrations of imidazole from 0 mM to a maximum concentration of 1.2 M. The ionic strength was kept constantly by adding appropriate amounts of KCl. All measurements were performed at pH 6.1. The helper protease was subtilisin Carlsberg.

NMR Experiments. All NMR experiments were carried out on a Bruker ARX 500 NMR spectrometer with proton resonance frequency at 500.13 MHz. Temperature calibration was performed using an 866 thermometer (Keithley). All samples were referenced to TSP. Samples contained 5−7 mg of peptide dissolved in a H_2O/D_2O mixture (10:1). pH values were measured using a combination pH electrode (Hamilton). The pD values of D_2O solutions were calculated using the relationship pD = (meter reading) + 0.4 (*23*). A set of 1D spectra was recorded in a serial file in pH jump experiments after a fast alteration of pH. The pH jump was initiated by adding small amounts of KOD/KOH and DCl/HCl (all 1:10) to the sample, respectively. The time between the single 1D spectra was 61.6 s. Data were gained by integration and/or deconvolution of *cis* and *trans* signals in each 1D spectrum. Signals of the aromatic protons of the His residue as well as the Ala^1 β-CH proton signal were used for investigation. These data were transferred to a PC and final evaluation was performed with the program

Table 2: Rate Constants $k_{c\rightarrow t}$ of Prolyl Isomerization of Peptides under Basic and Acidic Conditions[a]

compound[b]	pH	T (°C)	$k_{c\rightarrow t}$ 10^{-2} (s^{-1})	acceleration factor[a]
Suc-AHPF-NH-Np[c]	<5.5	10.0	1.4	2.0
	7.5−9.5[d]	10.0	0.7	
Suc-AFPH-NH-Np[c]	5.6−7.8	10.0	0.7	
Suc-AFPK-NH-Np[c]	7.0	10.0	0.6	
Suc-AKPF-NH-Np[c]	7.0	10.0	0.6	
Ac-AHPAK-NH_2[e]	<5.5	4.0	0.5	6.3
	8.0−13.0[d]	4.0	0.08	
Ac-AKPAK-NH_2[e]	4.0	4.0	0.2	
Ac-ARPAK-NH_2[e]	4.0	4.0	0.2	
angiotensin III[e]	3.5	48.1	480	9.6
	11.0	48.1	50	
angiotensin III[f]	3.5	48.1	257	5.1
	11.0	48.1	50	
angiotensin III[c]	3.5	48.1	524	
TRH[e]	4.5	48.1	34	2.1
	8.7	48.1	16	
[His(3-Me)^2]TRH[e]	4.5	48.1	38	3.5
	7.9	48.1	11	

[a] Acceleration factors were calculated by dividing the rate constants measured under acidic and basic conditions. [b] One-letter code is used for amino acids. [c] Measured in H_2O. [d] Several pH values were investigated in the given pH range. [e] Measured in H_2O/D_2O (10:1). [f] Measured in D_2O.

SigmaPlot (Jandel Corp.). The data were fitted to a first-order rate law.

2D NOESY (EXSY) experiments were acquired with the following parameters: spectral width 5050 Hz in both dimensions, 16−32 transients, 512 time increments with 4048 complex points, and mixing times between 200 and 400 ms. Shifted sine bell squared weight functions were applied in both dimensions and data were zero-filled to a final size of 4K × 4K. All resolved exchange peak/diagonal peak sets were used for integration and evaluation of prolyl isomerization rate constants. An error up to 15% resulted from the comparison of the gained rate constants evaluated either from the same spectrum or from different spectra recorded under the same conditions with different mixing times, respectively. Determination of prolyl isomerization rates was done by the method described by Baine (*24*). Additionally, the method by Jeener et al. was used (*25*), depending on the existence of well-resolved and nonoverlapping signals for *cis* and *trans* isomers.

Similar measurements were carried out for angiotensin III in D_2O and H_2O as solvents to estimate the kinetic solvent deuterium isotope effect. In the case of measurements in H_2O we used D_2O as external lock substance in a spherical container immersed in the sample.

RESULTS

Isomerization Rates. The pH dependence of the rate constants for the prolyl isomerization of several peptides (Table 1) containing the -His-Pro- moiety was determined by different methods (Table 2). Isomer-specific proteolysis was used in the case of the chromogenic peptide Suc-Ala-His-Pro-Phe-NH-Np (Figure 1B). We obtained the isomerization rates of angiotensin III, TRH, [His(3-Me)^2]TRH, and Ac-Ala-His-Pro-Ala-Lys-NH_2 at different temperatures by evaluating EXSY spectra. Two different pH values were used to ensure either the fully protonated or deprotonated histidine side chain. All EXSY spectra showed well-resolved exchange peaks for the Pro δ-CH_2 resonance (Figure 2). All

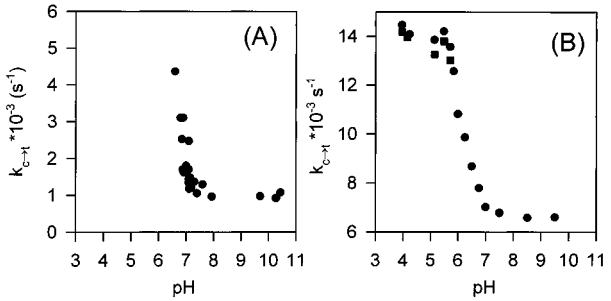

FIGURE 1: pH dependence of prolyl isomerization rates for Ac-Ala-His-Pro-Ala-Lys-NH_2 (A) and Suc-Ala-His-Pro-Phe-NH-Np (B). (A) Prolyl isomerization rates measured for the time course of isomerization by ^{1}H NMR after a pH jump in 20 mM potassium phosphate buffer at 4 °C, initiated by addition of small amounts of KOH and HCl, respectively. The method is limited to rate constants smaller than 5×10^{-3} s^{-1}. Therefore, measurements below pH 6.5 were not performed. (B) Rate constants of prolyl isomerization determined by isomer-specific proteolysis at 10 °C in 35 mM Bis-Tris-propane buffer (●) and in 35 mM potassium citrate buffer (■).

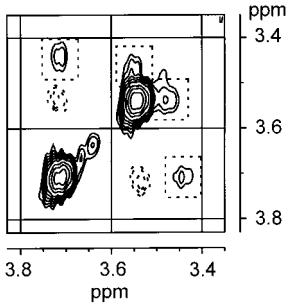

FIGURE 2: Portion of the δ-CH_2 proline region of an ^{1}H EXSY spectrum of angiotensin III in 20 mM potassium phosphate, pH 3.5, at 48.1 °C. The mixing time was 400 ms. The exchange peaks are surrounded by dashed boxes. The cross peaks drawn by dashed lines are antiphase NOE signals.

resolved exchange peak pairs were analyzed in the experiments to verify the kinetic results obtained from the spectra. For comparison of the peptides at different pH values we used the data calculated from the integrals of both Pro δ-CH_2 exchange and diagonal peaks in the appropriate spectrum.

Additionally, we measured the complete pH dependence of isomerization rates by pH jump experiments for the pentapeptide (Figure 1A). This technique utilizes the pH-dependent change in the *cis* content of the peptides. In these experiments a time-dependent set of 1D ^{1}H NMR spectra of the peptide was recorded subsequent to a rapid pH jump. The data recorded contain the complete information with respect to the isomerization kinetics due to slow equilibration of the prolyl isomers. These measurements were limited to a pH range shortly below the pK_a value of the histidine due to a rapid isomerization under more acidic conditions.

All peptides containing His-Pro depicted in Table 2 show an increase in rate constants of prolyl isomerization on going from basic to acidic pH values independent of the peptide concentration in the range from 0.8 to 10 mM. TRH and Suc-Ala-His-Pro-Phe-NH-Np show doubling of the rate constants. A 3.5-fold increase is observed for [His(3-Me)^2]-TRH, whereas the increase in Ac-Ala-His-Pro-Ala-Lys-NH_2 and angiotensin III is even higher (Table 2). We measured the prolyl isomerization of Ac-Ala-His-Pro-Ala-Lys-NH_2 and Suc-Ala-His-Pro-Phe-NH-Np at various pH values using both the NMR-coupled pH jump technique and isomer-specific

Table 3: *Cis* Content and pK_a Values of the Imidazole Ring of the Histidine Preceding Proline of Peptides in H_2O/D_2O (10:1)

	cis content (%)		p$K_a^{[His]}$ [b]	
compound[a]	pH 3	pH 10	trans	cis
Suc-AHPF-NH-Np	8.0	15.3	6.9	6.4
Ac-AHPAK-NH_2	9.5	16.5	6.6	6.3
angiotensin III	5.2	17.5	6.6	6.1
TRH	12.2	17.5	6.5	6.3
[His(3-Me)[2]]TRH	11.5	17.5	6.3	6.2

[a] One-letter code is used for amino acids. [b] p$K_a^{[His]}$ value calculated from the chemical shift (δ) of the aromatic ring protons of His for *cis* and *trans* isomers.

proteolysis to obtain a full titration profile for the rate constants. We obtained curves with inflection points at pH 6.6 and 6.2 that are comparable to the side-chain p$K_a^{[His]}$ values obtained from 1D NMR measurements (Figure 1 and Table 3).

We measured the influence of pH on the prolyl isomerization rate of Suc-Ala-Phe-Pro-His-NH-Np by isomer-specific proteolysis to reveal the positional effect of the histidine. No influence of the pH value on the kinetics of prolyl isomerization was observed. The rate constant was $(6.8 \pm 0.2) \times 10^{-3}$ s^{-1} in a pH range between 5.6 and 7.8 at 10 °C.

In order to compare this behavior with that of peptides containing other charged residues flanking proline we used Ac-Ala-Xaa-Pro-Ala-Lys-NH_2, with protonated Arg and Lys preceding proline. The rate constants $k_{c\rightarrow t}$ are moderately slow for these peptides (Table 2). For an uncharged peptide, as exemplified by Xaa = Ala, the rate constant $k_{c\rightarrow t} = 3.2 \times 10^{-3}$ s^{-1} was even higher (Reimer et al., unpublished experiments). Similarly, prolyl isomerization rates of Suc-Ala-Phe-Pro-Lys-NH-Np ($k_{c\rightarrow t} = 6.1 \times 10^{-3}$ s^{-1}) and Suc-Ala-Lys-Pro-Phe-NH-Np ($k_{c\rightarrow t} = 5.5 \times 10^{-3}$ s^{-1}) give no evidence for an interaction of the charged lysine side chain with the imide bond at pH 7.0 and 10 °C.

An experiment was performed to determine whether externally added imidazole in its protonated state can mimic the catalytic role of the side chain of histidine by an intermolecular attack. Prolyl isomerization rates were determined for Suc-Ala-Gly-Pro-Phe-NH-Np by isomer-specific proteolysis in the presence of imidazole up to a total concentration of 1.2 M. This corresponds to a concentration of 0.6 M protonated imidazole at pH 6.1. All rate constants were independent of the amount of imidazole added and were found to have an average value of $k_{c\rightarrow t} = (5.3 \pm 0.2) \times 10^{-3}$ s^{-1}.

pK_a values of the imidazole side chain of histidine (p$K_a^{[His]}$) were calculated for *cis* and *trans* prolyl isomers from the pH dependence of the ring proton chemical shifts (Table 3). All p$K_a^{[His]}$ values were in the range between 6 and 7 as expected for this side chain. We observed an isomer-specific difference up to 0.55 pH unit in favor of a more basic *trans* isomer.

The *cis* content of the peptides was calculated from the peak area of the *cis* and *trans* signals of the aromatic protons of histidine. An increase of the *cis* content induced by pH changes from acidic to basic pH was observed for all peptides examined (Table 3).

Solvent Deuterium Isotope Effects. The rate constants of prolyl isomerization in D_2O, a mixed solvent of H_2O/D_2O (10:1), and H_2O were measured for angiotensin III. The 1D

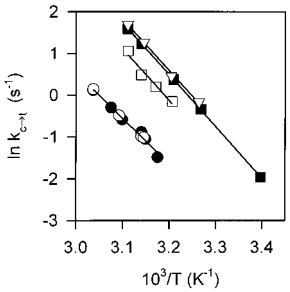

FIGURE 3: Arrhenius plot of the prolyl isomerization of angiotensin III measured in H_2O/D_2O (10:1) at pH 3.5 (■) and pH 11 (●), in D_2O at pH 3.5 (□) and pH 11 (○), and in H_2O at pH 3.5 (▽) using ^{1}H EXSY spectroscopy.

NMR spectra were identical for angiotensin III in H_2O and D_2O for the same protonation state of the peptide. In D_2O a p$K_a^{[His]}$ of 7.1 was determined, whereas the p$K_a^{[His]}$ was 6.6 in 10% deuterated aqueous solution (Table 3). This difference of p$K_a^{[His]}$ values is in good agreement with values calculated from a relationship for weak acids in D_2O and H_2O (*26*). Kinetic measurements were performed at pH/pD 3.5 and 11 where protonation/deuteration of the imidazole side chain of histidine was 100% and 0%, respectively. The measurements were carried out at several temperatures to construct an Arrhenius plot (Figure 3). In the limits of error the slope of the Arrhenius plots is identical for all solvents used but depends on the protonation state. The activation energies are 90 ± 14 kJ·mol^{-1} in basic solution and 103 ± 15 kJ·mol^{-1} for angiotensin III completely protonated at basic sites. The calculation of solvent deuterium isotope effect gives a value of 1.0 ± 0.1 for the unprotonated peptide. We calculated an isotope effect of 2.0 ± 0.1 comparing the prolyl isomerization rates of angiotensin III at acidic pH in D_2O and H_2O. This value was proven by the H_2O/D_2O (10:1) sample leading to a small retardation of the isomerization ($k_{c\rightarrow t}^{H_2O}/k_{c\rightarrow t}^{mix} = 1.1 \pm 0.1$).

The plots in Figure 3 show that the solvent deuterium isotope effect originates from molecular effects expressed in the preexponential factor of the Arrhenius equation.

DISCUSSION

We addressed the question whether a sequence pattern could exist around proline, providing a chemical machinery for an intramolecularly accelerated prolyl isomerization in aqueous solution. The resulting increase in rate for acquiring a native state of a polypeptide would be both sequence-specific and independent of the action of PPIases or a specific environment (membranes). The latter factors are known for enhancing the rate of prolyl isomerization intermolecularly (*11*, *27*). Usually, prolyl isomerization has been found to be independent of pH within a range of pH 2−8 in buffered solution (*1*). This finding clearly indicates the lack of acid/base catalysis functional for this type of reaction under physiological conditions. The observed solvent deuterium isotope effect of $k_{c\rightarrow t}^{H_2O}/k_{c\rightarrow t}^{D_2O} = 1.07$ provides further evidence for the absence of a proton movement in approaching the transition state of prolyl isomerization (*28*).

Despite these facts we observed an increase in the rate of prolyl isomerization for various oligopeptides containing the -His-Pro- moiety (Table 1) when going from basic to acidic pH values (Table 2). Since the rate effects do not depend on peptide concentration, intramolecular assistance becomes obvious.

The rate enhancement varied in dependence on the oligopeptide sequence, from a 2-fold acceleration for TRH and Suc-Ala-His-Pro-Phe-NH-Np up to 10-fold for angiotensin III. The rate effect was always coupled with protonation of the imidazole side chain of the histidine, because the pH profile of the rate constants showed an inflection point between 6.0 and 7.0 (Figure 1). These results coincide with the pK_a values measured for the imidazole group by ^{1}H NMR spectroscopy (Table 3). The hydrogen ion concentration triggers the prolyl isomerization of -His-Pro- moieties due to protonation/deprotonation of the imidazole ring, either by localizing a positive charge adjacent to the rotating bond or by providing an intramolecular hydrogen-bonding facility to stabilize the transition state.

Among the amino acids with protonation sites in the side chain, only protonated histidine seems to fit the spatial requirements for the interaction with the rotating prolyl bond. This was shown for peptides with arginine and lysine flanking proline (Table 2). Also, there is no influence of pH on the isomerization rates when the histidine residue is succeeding proline. Using Suc-Ala-Phe-Pro-His-NH-Np, this result was proven to be indicative of a strictly positional determinant for a catalytic histidine side chain (Table 2).

We evaluated whether there is an intermolecular equivalent of the intramolecular assistance by histidine by measuring the prolyl isomerization in the presence of very high concentrations of imidazolium buffer. We could not obtain any increase in the prolyl isomerization rate for Suc-Ala-Gly-Pro-Phe-NH-Np up to a concentration of 0.6 M protonated imidazole. Therefore, we estimated an effective molarity $\gg 10^2$ mol·l^{-1} for the protonated histidine side chain of angiotensin III at 10 °C.

We determined the kinetic solvent deuterium isotope effect (KSIE) on the prolyl isomerization of angiotensin III to distinguish between general acid catalysis and electrophilic assistance by the suitably arranged imidazolium moiety. Conclusions can be drawn whether there is a proton "in flight" along the reaction coordinate of the assisted prolyl isomerization comparing the KSIEs of the unprotonated and the side-chain protonated peptide (*29*, *30*). Obviously, the lack of a KSIE for the prolyl isomerization in the unprotonated state of histidine in angiotensin III is in accordance with available data relating to other uncharged residues in this position (*28*). However, the KSIE of 2.0 at pH 3.5 has a magnitude typically of primary isotope effects. Such effects are indicative of protons directly involved in the formation of a transition state (*31*).

Thus, we suppose an intramolecular general acid catalysis by an interaction between a proton attached to an imidazole nitrogen of His and the imide nitrogen of the succeeding Pro (Figure 4). Inverse isotope effects would be observed in the case of a specific acid catalysis (*32*). Indeed, the interaction of a proton with the lone pair of the imide nitrogen will be effective in lowering the rotational barrier of the prolyl bond (*33*). Due to imide twisting, the pK_a value of the imide nitrogen increases while progressing along the isomerization path. The pK_a can be estimated as 7.6 ± 1.0 for the transition state by applying the Taft equation for the pK_a values of N-substituted prolines, $pK_a = 10.06 - 1.5\Sigma\sigma^*$ (*34*, *35*). Since the pK_a of the imidazole of histidine is 6.6 for the *trans* and 6.1 for the *cis* isomer, a slightly asymmetric proton position in the transition state is inferred, in accordance with the magnitude of the experimentally determined KSIE. However, this is only applicable to linear transition states. Bent transition states are likely to be important for intramolecular reactions. In such transition states the vibration of the proton (deuteron) largely cancels the contribution of the zero point energy (*36*). For that reason a decrease of the KSIE and a strong influence of activation entropy is observed. In the Arrhenius plot (Figure 3) this effect is indicated by different preexponential factors in isotopic solvents at pH 3.5. Figure 4 shows the possible hydrogen-bond-mediated cyclic transition state of the intramolecular catalysis.

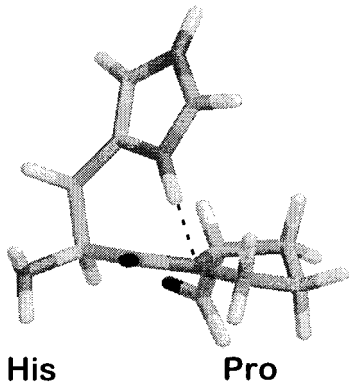

FIGURE 4: Schematic drawing of the His-Pro moiety. The proposed interaction between a nitrogen-linked proton of the imidazole ring of histidine and the imide nitrogen of the proline residue is displayed as a broken line.

Early reports demonstrated the possibility of a specific acid catalysis for the prolyl isomerization in strongly acidic media below pH 1 (*19*, *37*−*39*). General acid catalysis of prolyl isomerization was suggested for a secondary amide, too (*22*, *40*). The formation of an intramolecular hydrogen bond between the anilide NH and either the imide nitrogen or the *N*-acetyl carbonyl oxygen in chloroform was reported for *N*-acetylproline anilides, increasing the isomerization rate up to 188-fold (*22*).

However, both types of catalysis were observed under entirely nonnative conditions. Now we were able to show an intramolecular acceleration of prolyl isomerization by general acid catalysis in aqueous solution at physiological pH values.

There are two special examples where electrophilic assistance by an amino acid side chain has been discussed as a mechanism of acceleration of prolyl isomerization under native conditions. In crystal structures of cyclophilin with bound substrate molecules, interactions of the guanidine group of arginine with the imide nitrogen of the proline in substrates are evident (*41*, *42*). The importance of this interaction has been proven by site-directed mutagenesis, because the R55A mutant of the enzyme seems to have no activity (*43*). However, the essential nature of this arginine has been questioned recently by reinvestigation of the R55A Cyp18 variant (*56*).

Moreover, site-directed mutagenesis implied intramolecular assistance by Arg44 in the isomerization of Pro66 in native dihydrofolate reductase (DHFR) from *Escherichia coli* due to proximal positioning of both residues, as could be inferred from the rate constants of the refolding of urea-denatured enzyme variants. A chemical catalysis was suggested since the crystal structure of ligand-free DHFR revealed hydrogen bonding of the guanidinium group of Arg44 with the imide nitrogen and the carbonyl oxygen of

Pro66. The experiments did not permit calculation of acceleration factors and general acid catalysis could not be established. The sequence distance between the interacting amino acid residues is large in the amino acid sequence. Therefore, catalysis is a consequence of the specific fold of the protein.

These accelerations of prolyl isomerization require a special three-dimensional structure and a spatial proximity of the interacting atoms. In contrast, the acceleration effect due to the -His-Pro- moiety is not coupled to any secondary or tertiary structural requirements but only determined by the primary structure. Under the aqueous conditions used in our experiments the formation of the catalytic hydrogen bond must be compromised by competition with water molecules. Structured polypeptides may overcome this limitation by excluding water from the isomerizing group. Thus, the -His-Pro- moiety in proteins is supposed to isomerize at rates even much higher than could be found in this study.

Long-term persisting folding intermediates may cause misfolding, degradation, misprocessing, and aggregation of proteins. Due to the slow prolyl isomerization, which is uncoupled in rate from most other structural interconversions of polypeptides, native *cis* prolyl bonds of proteins represent a major determinant of late intermediates (*44*−*46*). Evolution provided an arsenal of folding helpers as chaperones and peptidyl-prolyl *cis/trans* isomerases to minimize accumulation of misfolded species (*47*, *10*).

Does -His-Pro- play a general role as a fast conformational switch in proteins? Despite its unique chemical properties, histidine occurs quite rarely in proteins. In the proteins translated from open reading frames in the genomes of six microbial organisms, histidine occurs with a frequency between 1.4% and 2.2% of all amino acids (*48*−*53*). However, histidine covers a position preceding proline with higher propensity than expected from its overall frequency throughout the sequences in all genomes investigated. Only the -Thr-Pro- motif shows the same occurrence. This latter motif is found in repeating sequences typical of proteins that modify DNA structures (*54*) and plays an important role as a target for proline-specific phosphorylation (*55*). The relation of the -His-Pro- moiety to such an important sequence motif might point to a special function that is possibly exemplified by its unique dynamic behavior.

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