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Unexpectedly Fast Cis/Trans Isomerization of Xaa-Pro Peptide Bonds in Disulfide-Constrained Cyclic Peptides

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Abstract: Acyclic dithiol and cyclic disulfide forms of the peptides Ac-Cys-Pro-Xaa-Cys-NH_2 (Xaa = Phe, His, Tyr, Gly, and Thr) and Ac-Cys-Gly-Pro-Cys-NH_2 and the peptide Ac-Ala-Gly-Pro-Ala-NH_2 were synthesized and characterized by mass spectrometry and NMR spectroscopy. Rate constants k_{ct} and k_{tc} for cis-to-trans and trans-to-cis isomerization, respectively, across the Cys-Pro or Gly-Pro peptide bonds were determined by magnetization transfer NMR techniques over a range of temperatures, and activation parameters were derived from the temperature dependence of the rate constants. It was found that constraints imposed by the disulfide bond confer an unexpected rate enhancement for cis/trans isomerization, ranging from a factor of 2 to 13. It is proposed that the rate enhancements are a result of an intramolecular catalysis mechanism in which the NH proton of the Pro-Xaa peptide bond hydrogen bonds to the proline nitrogen in the transition state. The peptides Ac-Cys-Pro-Xaa-Cys-NH_2 and Ac-Cys-Gly-Pro-Cys-NH_2 are model compounds for proline-containing active sites of the thioredoxin superfamily of oxidoreductase enzymes; the results suggest that the backbones of the active sites of the oxidized form of these enzymes may have unusual conformational flexibility.

Introduction

Proline is unique among the 20 commonly gene-coded amino acids in that both the cis and trans isomers of the Xaa-Pro (prolyl) peptide bond can be significantly populated.[1] In peptides

and denatured proteins, prolyl peptide bonds exist as an equilibrium mixture of the cis and trans conformations, while in the native, functional state of proteins, most prolyl peptide bonds are exclusively in the trans conformation with a small number in the cis conformation.[2] Cis/trans isomerization of prolyl peptide bonds is a relatively slow process due to the partial double bond character of the C−N bond;[1,3−6] isomerization involves a shift of the nitrogen lone pair of electrons to an sp^3 orbital and rotation around the C−N bond through a twisted transition state.[4] Thus, trans-to-cis isomerization can be a slow step in the folding of proteins that contain one or more cis prolyl peptide bonds in the native state.[7−10] In vivo, cis/trans isomerization of Xaa-Pro peptide bonds is catalyzed by peptidyl prolyl cis/trans isomerases (PPIases), which include the FK506 binding proteins (FKBPs), cyclophilins, and parvulins.[11,12]

In a recent preliminary communication, we reported that the rates of cis/trans isomerization of the Cys-Pro peptide bond in the cyclic disulfide form of the peptides Ac-Cys-Pro-Phe-Cys-NH_2 (**1**) and Ac-Thr-Cys-Pro-Phe-Cys-Arg-NH_2 (**2**) are up to 10 times faster than the rates for the corresponding acyclic dithiol peptides.[13] The rate enhancements were unexpected due to the previously reported effect of steric constraints imposed by intramolecular disulfide bonds on the rates of cis/trans isomerization of prolyl peptide bonds in other peptides.[14−16] For

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Table 1. Amino Acid Sequences of Thiol-Disulfide Oxidoreductase Active Sites that Contain Proline

oxidoreductase enzyme	abbreviated name	active site sequence
thioltransferase	TTase	Cys-Pro-Phe-Cys
disulfide bond forming protein A	DsbA	Cys-Pro-His-Cys
disulfide bond forming protein E	DsbE	Cys-Pro-Thr-Cys
disulfide bond forming protein G	DsbG	Cys-Pro-Tyr-Cys
glutaredoxin	Grx	Cys-Pro-Tyr-Cys
thioredoxin	Trx	Cys-Gly-Pro-Cys

example, cis/trans isomerization of the Leu-Pro peptide bond is significantly slower in the cyclic disulfide form of the peptide Cys-Leu-Pro-Arg-Glu-Pro-Gly-Leu-Cys.[14]

We proposed that the unexpectedly fast rates of cis/trans isomerization of the Cys-Pro peptide bonds of the disulfide forms of **1** and **2** are a result of intramolecular catalysis by a mechanism in which the NH proton of the Pro-Phe peptide bond hydrogen bonds to the sp^3 lone pair of the pyramidalized proline nitrogen in the twisted transition state.[13] Intramolecular catalysis has been proposed previously to account for enhanced rates of cis/trans isomerization for proline peptide bonds in small model peptides and peptides that contain a His-Pro sequence and for catalysis of cis/trans isomerization of Xaa-Pro peptide bonds in proteins by FKBPs.[17−19]

Peptides **1** and **2** are model peptides for the active site of thioltransferase, a member of the thioredoxin superfamily of thiol/disulfide oxidoreductase enzymes. Enzymes in this family all have in common a conserved Cys-Xaa-Xaa-Cys active site motif; the two Cys residues cycle between their acyclic dithiol and cyclic disulfide forms as they catalyze the formation, reduction, and rearrangement of disulfide bonds in folding proteins by thiol/disulfide exchange reactions.[20] Our finding[13] that the rates of cis/trans isomerization of the Cys-Pro peptide bond are faster for the cyclic disulfide forms of **1** and **2** suggests that the peptide backbone at the active site of the oxidized form of thioltransferase has unusual conformational flexibility. Proline is present as one of the two the amino acids between the two Cys residues in several other thiol/disulfide oxidoreductase enzymes (Table 1), which suggests that the active site of the oxidized form of these enzymes might also be conformationally flexible.

To determine if the significantly faster rates of cis/trans isomerization of the Cys-Pro peptide bond are unique to the cyclic disulfide forms of **1** and **2** or if they are general when proline is one of the two amino acids in the Cys-Xaa-Xaa-Cys oxidoreductase motif, we have carried out a systematic investigation of the kinetics of cis/trans isomerization of model peptides for other thiol/disulfide oxidoreductase enzymes that contain proline in the active-site sequence.[20] In this paper, we report the results of studies of the kinetics of cis/trans isomerization of the Cys-Pro peptide bond in the dithiol and disulfide forms of Ac-Cys-Pro-Xaa-Cys-NH_2, where Xaa is His, Tyr, Gly, Leu, and Thr, the Gly-Pro peptide bond in the dithiol and disulfide forms of Ac−Cys-Gly-Pro-Cys-NH_2, and the Gly-Pro peptide bond in Ac−Ala-Gly-Pro-Ala-NH_2 (Table 2).

Experimental Section

Materials. The 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, 1-hydroxybenzotriazole monohydrate (HOBt·H_2O), 1-hydroxy-7-azabenzotriazole (HOAt), and *N*,*N*′-diisopropylcarbodiimide (DIPCDI) were obtained from Millipore Corp. Fmoc-PAL-PEG-PS resin and piperidine were purchased from Millipore Corp. or Novabiochem. Trifluroacetic acid (TFA, Chem-Impex International), *N*,*N*′-dimethylformamide (DMF, Fisher Scientific), triisopropylsilane (Aldrich), and phenol (Aldrich) were reagent grade and were used as received. Sodium 3-(trimethylsilyl)propionate-*2*,*2*,*3*,*3*-d_4 (TMSP) and deuterated DL-1,4-dithiothreitol-d_{10} (deuterated DTT) were obtained from Cambridge Isotope Laboratories. D_2O (99.9%) was supplied by Isotec.

Peptide Synthesis. Linear peptides (peptides **1a**−**8a** and **9** in Table 2) were synthesized on a Millipore model 9050 Plus peptide synthesizer using solid-phase Fmoc peptide synthesis methodology. The amino acid derivatives Fmoc-Cys(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Pro-OH were used for the assembly of the peptide chains, where Trt is trityl, tBu is *tert*-butyl, and Pbf is 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl. Fmoc-PAL-PEG-PS with loading capacities of 0.16−0.22 mmol/g and a 4−5 times excess of each Fmoc-protected amino acid were used for the synthesis. The Fmoc group was removed from the N-terminal amino group of the resin-bound peptide before each coupling step by reaction with 20% piperidine in DMF for 8 min. Entering amino acids were preactivated with HOAt and DIPCDI, and couplings were run for 30−45 min. After each coupling, the peptide on the resin was reacted with capping reagent (3% v/v acetic anhydride and 5% w/w HOBt in DMF) for 10 min to cap unreacted amino groups. Peptide chains were cleaved from the resin and side chain protecting groups were removed by reaction with cleavage reagent (88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) for 3−4 h. The resin was removed by filtration and the filtrate was diluted with water. After extraction 3 times with ether, the water phase was lyophilized and crude product was obtained.

The disulfide forms of the peptides (**1b**−**8b** in Table 2) were prepared by oxidation of the corresponding dithiol peptides with *trans*-$[Pt(CN)_4Cl_2]^{2-}$ or *trans*-$[Pt(en)_2Cl_2]^{2+}$, both of which are reagents for the selective and rapid formation of intramolecular disulfide bonds.[21] The crude product after cleavage[22] was reacted with either *trans*-$[Pt(CN)_4Cl_2]^{2-}$ in dilute HCl or *tran*-$[Pt(en)_2Cl_2]^{2+}$ in phosphate buffer using conditions described previously.[21] In most cases, oxidation was carried out on millimolar concentrations of the peptides.

Peptide Purification. Peptides were isolated by reverse-phase HPLC on a 10 mm × 250 mm C18 column (Vydac, 5 μm particles and 300 Å pore size) using a Bio-Rad model 2800 HPLC system equipped with a Linear model 205 dual wavelength UV detector. Peptides were eluted with an acetonitrile−water gradient mobile phase containing 0.1% TFA. Fast atom bombardment mass spectrometry (FAB-MS) or matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) were used to confirm the peptide identities; the molecular weights obtained by mass spectrometry are reported in Table 2. The purity, as indicated by HPLC and ^1H NMR, was greater than 99% for all the peptides listed in Table 2.

NMR Samples. Most NMR samples were prepared by dissolving peptide in 90% H_2O/10% D_2O at 5−15 mM concentrations; a 10−20 times excess of deuterated DTT was added to sample solutions of the

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(22) In most cases, the crude peptide obtained after cleavage contained more than 80% of the target dithiol peptide.

Table 2. Proline-Containing Peptides Synthesized and Studied in This Research

peptide	amino acid sequence	monoisotopic mass; theoretical (found)	resonances used in magnetization transfer experiments
1a	Ac-Cys-Pro-Phe-Cys-NH_2	509.2 (509)[a]	acetyl-CH_3
1b	Ac-Cys-Pro-Phe-Cys-NH_2	507.2 (507)[a]	acetyl-CH_3
2a	Ac-Thr-Cys-Pro-Phe-Cys-Arg-NH_2	766.3 (766)[a]	Phe NH
2b	Ac-Thr-Cys-Pro-Phe-Cys-Arg-NH_2	764.3 (764)[a]	Thr γ-CH_3
3a	Ac-Cys-Pro-His-Cys-NH_2	499.2 (499.3)[b]	His ring C2-H
3b	Ac-Cys-Pro-His-Cys-NH_2	497.2 (497.3)[b]	His ring C2-H
4a	Ac-Thr-Cys-Pro-Tyr-Cys-Arg-NH_2	782.3 (782.2)[b]	
4b	Ac-Thr-Cys-Pro-Tyr-Cys-Arg-NH_2	780.2 (780)[a]	Thr γ-CH_3
5a	Ac-Cys-Pro-Gly-Cys-NH_2	419.1 (419)[a]	Gly NH
5b	Ac-Cys-Pro-Gly-Cys-NH_2	417.2 (417)[a]	Gly NH
6a	Ac-Cys-Pro-Leu-Cys-NH_2	478.2 (478)	Cys^1 NH
6b	Ac-Cys-Pro-Leu-Cys-NH_2	476.2 (476)	Cys^1 NH
7a	Ac-Cys-Pro-Thr-Cys-NH_2	464.2 (464)	acetyl-CH_3
7b	Ac-Cys-Pro-Thr-Cys-NH_2	462.1 (462)	acetyl-CH_3
8a	Ac-Cys-Gly-Pro-Cys-NH_2	419.1 (419)[a]	Cys^4 NH
8b	Ac-Cys-Gly-Pro-Cys-NH_2	417.1 (417)[a]	Cys^4 NH
9	Ac-Ala-Gly-Pro-Ala-NH_2	355.2 (355)[a]	Ala^1 NH

[a] Measured by Fab-MS. [b] Measured by MALDI-MS.

reduced peptides. In the case of peptide **1b**, a saturated solution (ca. 1−1.5 mM) was used due to its low solubility. TMSP was added for a chemical shift reference and the pH was adjusted to 3.0 by adding HCl and NaOH solutions. For some two-dimensional NMR experiments, the NMR samples were dissolved in 100% D_2O containing TMSP and, in some cases, 0.15 M NH_2OH·HCl, and the pD was adjusted with solutions of DCl and NaOD. Shigemi NMR sample tubes were used for all NMR measurements.

NMR Spectroscopy. One- and two-dimensional ^{1}H NMR spectra were recorded on a 500-MHz Varian Unity-Inova spectrometer. Spectra were referenced to the methyl resonance of TMSP at 0.000 ppm. The water resonance was suppressed in most 1D and 2D experiments with a presaturation pulse. In several experiments where the water resonance overlapped the proline C_αH resonance of the trans isomer, the water resonance was suppressed by the water attenuation by transverse relaxation (WATR) method in 2D spectra measured for the purpose of establishing the presence of both cis and trans isomers.[23] Two-dimensional total correlation spectroscopy (TOCSY) and rotating frame Overhauser effect spectroscopy (ROESY) spectra were measured with standard pulse sequences. Usually, spectra were recorded with 8K points in the directly detected (F2) dimension and 128 or 256 points in the F1 dimension. A mixing time of 120−300 ms was used for TOCSY and ROESY experiments.

Rate constants for cis/trans isomerization were determined by the inversion-magnetization transfer method.[6,24−27] The experimental methodology and analysis of the data to obtain rate constants were similar to that developed by King et al.[6] The trans resonance of a given cis/trans pair was selectively inverted with the pulse sequence: $90°_x-\tau-90°_x-t-90°_{\pm x,\pm y}-$acquisition, where τ is a fixed delay equal to $1/(2|\upsilon_{cis} - \upsilon_{trans}|)$ and t is a variable delay during which transfer of magnetization takes place by interchange between the cis and trans isomers.[6,25,26] For maximum net effect of transfer of inversion on resonance intensity, the more intense trans resonance was selectively inverted and the intensity of the cis resonance was measured as a function of t. Typically, 23−30 t values ranging from 0.01 s to at least 5 times the longest T_1 of the two resonances were used. For example, the cis/trans pair of resonances from C2−H of the imidazole ring of histidine was used in the inversion transfer experiments for peptides **3a** and **3b**; t values were varied from 0.01 to 30 s. For each t value, 128 or 256 transients were collected; in the case of peptide **5b**, 1024 transients were collected due to the low abundance of the cis isomer. The pairs of cis/trans resonances used for the inversion-magnetization transfer experiments are reported in Table 2. The sample temperature (25−70 °C) in the NMR probe was calibrated by using the chemical shifts of neat ethylene glycol.

Results

Assignment of NMR Spectra. The rate of interchange between cis and trans isomers of the prolyl peptide bond is slow on the NMR time scale for the peptides listed in Table 2, with the result that resonances are observed for both isomers. To illustrate, the amide NH region of the ^{1}H NMR spectrum of peptide **3b** is shown in Figure 1. The spectrum consists of two sets of resonances, one much more intense than the other. The following procedure was used to establish that the two sets of resonances are for isomers that have different conformations across the Cys-Pro peptide bond. First, the identities of the amino acid residues giving both the more intense and the less intense backbone amide NH resonances were determined using cross-peaks to the NH resonances in TOCSY spectra.[28] Amide NH resonances for Cys residues were then assigned to Cys^1 using the NOE cross-peak between the Cys^1-NH and acetyl methyl resonances or to Cys^4 using the NOE cross-peak between the His^3-C_αH and Cys^4-NH resonances in ROESY spectra. The proline resonances were assigned using cross-peaks to their C_αH resonances in TOCSY spectra (data not shown). NOE cross-peaks between the Cys^1-C_αH and Pro-$C_\delta H_2$ resonances establish that the more intense set of resonances are for the isomer of **3b** in which the Cys-Pro peptide bond has the trans conformation, while NOE cross-peaks between the Cys^1-C_αH and Pro-C_αH

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(28) TOCSY and ROESY spectra were measured at either 5 or 25 °C, as necessary to obtain resolved amide NH resonances.

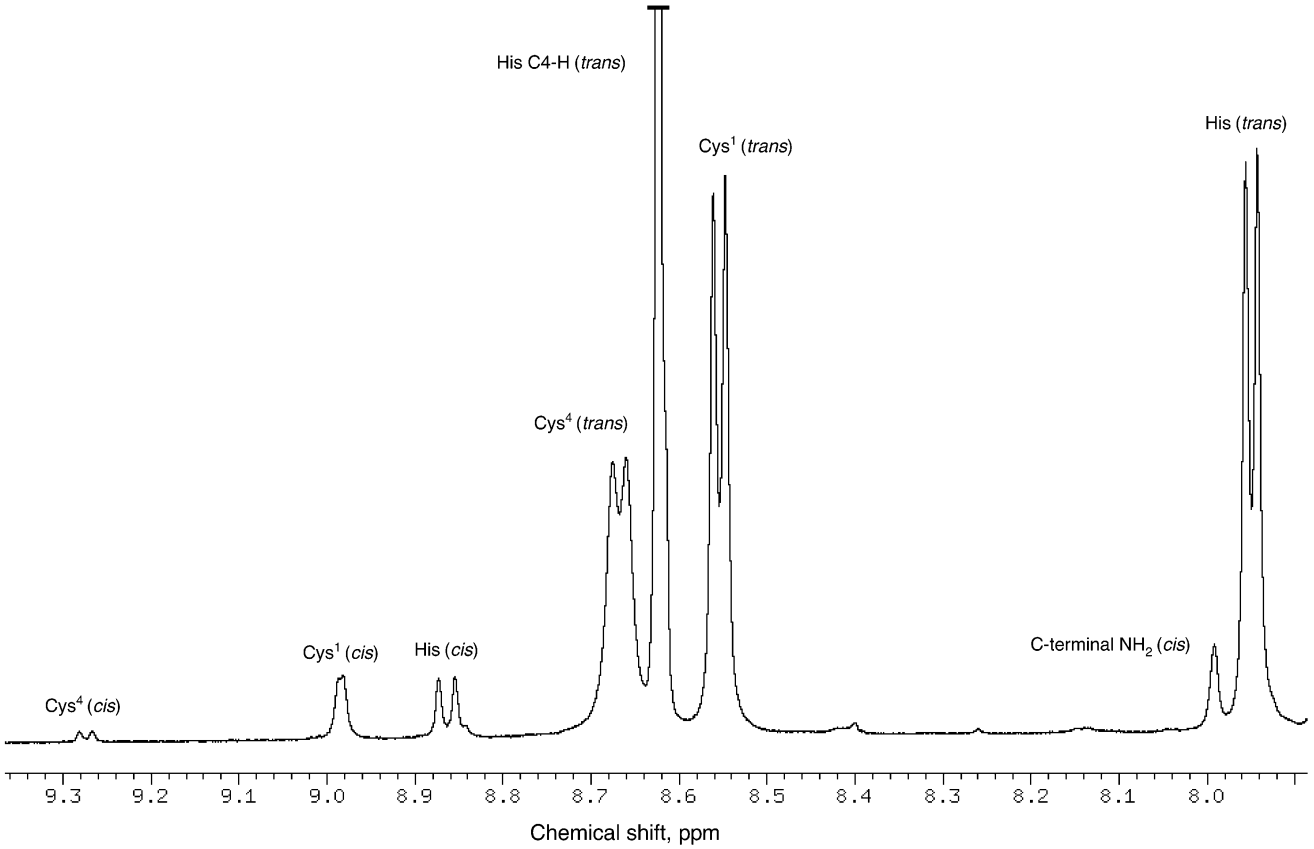

Figure 1. Amide NH region of the 500-MHz ^{1}H NMR spectrum of peptide **3b** in 90% H_2O/10% D_2O at 5 °C. The amide NH resonances are identified by residue and by the conformation of the Cys-Pro peptide bond. The His C-4H resonance of the cis isomer is presumably overlapped by another resonance.

resonances establish that the less intense set is for the cis isomer (Figure 2). Only the dipolar (negative) cross-peaks are plotted, and thus diagonal peaks are absent from Figure 2. Because some resonances of **3b** are obscured by the residual water resonance at 4.88 ppm in 1D and 2D spectra measured with water suppression by presaturation, the ROESY spectrum in Figure 2 was measured with elimination of the water resonance by the WATR method.[23] The WATR-1D spectrum plotted above the ROESY spectrum in Figure 2 shows that the residual water resonance at 4.88 ppm is completely and selectively eliminated, making it possible to observe cross-peaks to the Cys^1-C_αH (trans) resonance in the WATR-ROESY spectrum. The results in Figure 3 from inversion-magnetization transfer experiments for **3b** provide a final proof that the two sets of resonances are for isomers of the same peptide in dynamic equilibrium. In the magnetization transfer experiment, the C2−H resonance of the histidine ring of the trans isomer was selectively inverted, and the intensity of the corresponding resonance assigned to the cis isomer was monitored as a function of mixing time.

For most of the peptides studied, the cis isomer comprises some 5−30% of the total peptide (cf. Table 3), and the NMR signal assignments could be established by using the methods described above. However, peptides **5b** and **7b** are exceptions. Previously, a peptide essentially identical to **5b** (the disulfide form of Ac-Cys-Pro-Gly-Cys-NHMe) was studied by NMR under conditions similar to those used in the present study; however, it was reported that only one conformation was observed.[29] In the present work, both a major and a minor (ca. 2%) set of NMR signals were observed. It was established by the methods described above that the major set of signals is for the isomer having the trans conformation across the Cys-Pro peptide bond. To verify that the minor signals were not from impurities, peptide **5b** was purified twice; in the second purification step, only the central 50% of the HPLC peptide peak was collected. Results from inversion-magnetization transfer experiments (Figure 4) on the highly purified peptide clearly establish that the compound giving the minor peaks is in equilibrium with the isomer that has the trans conformation across the Cys-Pro peptide bond, indicating that ca. 2% of peptide **5b** has a cis prolyl peptide bond.

Equilibrium Constants for Cis/Trans Isomerization. Equilibrium constants for cis/trans isomerization (K = [trans]/[cis]) were determined from the integrated intensities of the same cis−trans pair of resonances used in the inversion-magnetization transfer experiments (Table 2). The resonance intensities were obtained from inversion-magnetization transfer spectra measured with a mixing time $t \geq 5T_1$ or from standard 1D spectra. The equilibrium constants are summarized in Table 3.

Kinetics of Cis/Trans Isomerization. Rate constants for cis-to-trans isomerization, k_{ct}, were derived from the inversion-magnetization transfer data by use of a method described in ref 27. The calculation procedure involves a nonlinear least-squares fit of the inversion-magnetization transfer data[6] to an equation which expresses the resonance intensity as a function of mixing time in terms of known and adjustable parameters, including the rate constant k_{ct}.[27] The rate constant for trans-to-cis isomerization, k_{tc}, was then calculated from k_{ct} and K ($k_{tc} = k_{ct}/K$).

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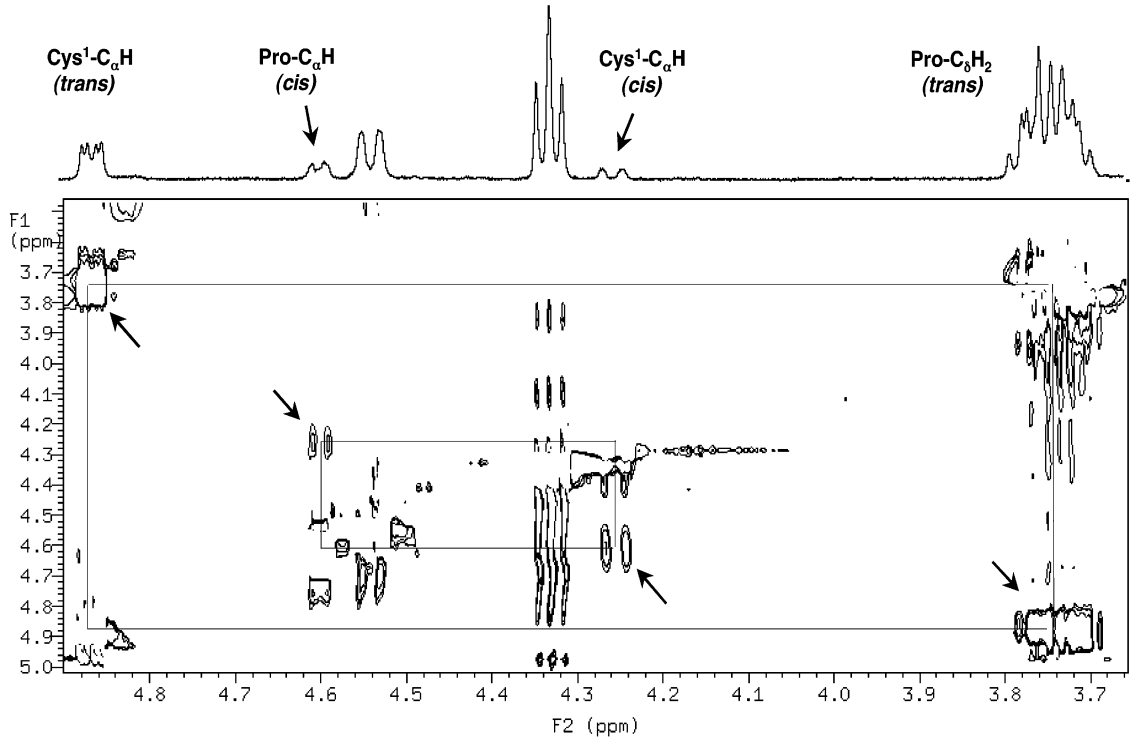

Figure 2. Portion of the ROESY spectrum of peptide **3b** in 90% H_2O/10% D_2O. The one-dimensional spectrum plotted above the ROESY spectrum and the ROESY spectrum were measured with complete and selective elimination of the water resonance by the WATR method.[23] Only the negative cross-peaks (from dipolar interactions) are plotted, and thus diagonal peaks are absent. The cross-peaks that establish the cis and trans isomers across the Cys-Pro peptide bond are identified with arrows; the resonances linked by the cross-peaks can be identified using the labeled resonances in the 1D spectrum.

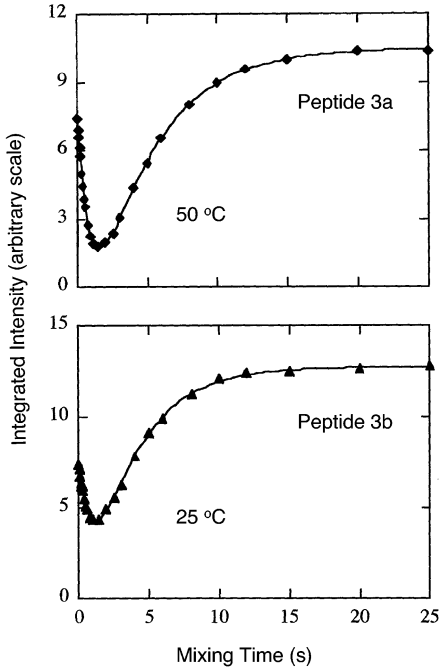

Figure 3. Integrated intensities of the resonances for the histidyl imidazolium C2−H protons of the cis isomers of peptides **3a** and **3b** as a function of the mixing time *t* in the inversion-magnetization transfer pulse sequence. The smooth curves were obtained by nonlinear least-squares analysis of the data.

For the cyclic disulfide peptides **1b**−**6b** and **8b**, cis−trans isomerization is sufficiently fast that it occurs on the magnetization-transfer time scale at 25 °C (cf. Figures 3 and 4). However, the rates of cis/trans isomerization are slower for the acyclic dithiol peptides (**1a**−**8a**) and peptide **9** and thus somewhat higher temperatures were needed to bring their rates onto the magnetization-transfer time scale, as illustrated by the results in Figures 3 and 4 for peptides **3a** and **5a**. Rate constants were measured over a range of temperatures to obtain the activation parameters $\Delta H_{ct}^{\ddagger}$ and $\Delta S_{ct}^{\ddagger}$, which were calculated from the dependence of k_{ct} on temperature with the Eyring equation:

$$\ln(k_{ct}/T) = \ln(k_B/h) + \Delta S_{ct}^{\ddagger}/R - \Delta H_{ct}^{\ddagger}/RT \qquad (1)$$

where k_B is the Boltzmann constant, h is Planck's constant, and R the gas constant. Plots of $\ln(k_{ct}/T)$ vs $1/T$ were linear for all the peptides; the derived activation parameters are summarized in Table 3. Values of k_{ct} and k_{tc} at 25 °C are also reported in Table 3; the values reported for peptides **1b**−**6b** and **8b** were determined directly from inversion-magnetization transfer data measured at 25 °C, while the values reported for the other peptides were calculated from activation parameters determined at higher temperatures using eq 1. The values listed in Table 3 for $\Delta G_{ct}^{\ddagger}$ at 25 °C were calculated from the values for $\Delta H_{ct}^{\ddagger}$ and $\Delta S_{ct}^{\ddagger}$.

Discussion

In the present work, the kinetics of cis/trans isomerization of prolyl peptide bonds have been characterized for an extended series of peptides. The peptides include models for all the enzymes so far found in the thioredoxin superfamily of oxidoreductase enzymes that have active site sequences of -Cys-Pro-Xaa-Cys- or -Cys-Xaa-Pro-Cys-.[20] The kinetic data enables us to thoroughly examine the effect of conformational constraints imposed by the disulfide bond in the oxidized form of the peptides on cis/trans isomerization of the Xaa-Pro peptide bonds.

Table 3. Equilibrium Constants, Rate Constants, and Activation Parameters for Cis−Trans Isomerization of the Xaa-Pro Peptide Bond for the Peptides in Table 2[a,b]

peptide[c]	K[d]	k_{ct}, s^{-1}	k_{tc}, s^{-1}	$\Delta H_{ct}^{\ddagger}$, kcal mol^{-1}	$\Delta S_{ct}^{\ddagger}$, cal K^{-1} mol^{-1}	$\Delta G_{ct}^{\ddagger}$, kcal mol^{-1}
1a	7.3 ± 0.3	0.043 ± 0.004[e]	(6.0 ± 0.8) × 10^{-3} [e]	21.3 ± 1.2	6 ± 4	19.5 ± 1.2
1b	9.8 ± 0.2	0.43 ± 0.03	0.044 ± 0.004	18.6 ± 0.7	2 ± 2	18.0 ± 0.7
2a	10.4 ± 0.2	0.059 ± 0.006[e]	(8.3 ± 0.8) × 10^{-3} [e]	21.0 ± 0.5	6 ± 2	19.2 ± 0.5
2b	17.7 ± 0.3	0.48 ± 0.07	0.027 ± 0.01	18.2 ± 0.5	1 ± 2	17.9 ± 0.5
3a	7.5 ± 0.3	0.044 ± 0.004[e]	(5.8 ± 0.5) × 10^{-3} [e]	19.7 ± 0.2	1 ± 1	19.4 ± 0.2
3b	6.5 ± 0.3	0.46 ± 0.03	0.071 ± 0.006	20.4 ± 0.2	8 ± 1	17.9 ± 0.2
4a[f]						
4b	18.8 ± 0.6	0.42 ± 0.04	0.022 ± 0.003	20.1 ± 0.2	7 ± 1	18.0 ± 0.2
5a	7.5 ± 0.5	0.026 ± 0.003[e]	(3.5 ± 0.4) × 10^{-3} [e]	21.4 ± 0.1	6 ± 1	19.6 ± 0.2
5b[g]	50 ± 5	0.34 ± 0.03	(6.8 ± 0.8) × 10^{-3}			
6a	7.4 ± 0.1	0.062 ± 0.01[e]	(8.3 ± 0.1) × 10^{-3} [e]	24.5 ± 0.9	18 ± 2	19.1 ± 1.1
6b	11.0 ± 0.1	0.26 ± 0.03	0.024 ± 0.003	20.3 ± 1.7	7 ± 5	18.2 ± 1.5
7a	5.7 ± 0.1	0.049 ± 0.002[e]	(8.6 ± 0.4) × 10^{-3} [e]	20.8 ± 1.3	5 ± 4	19.2 ± 1.8
7b[f]	72 ± 0.5					
8a	7.4 ± 0.2	0.061 ± 0.006[e]	(8.2 ± 0.8) × 10^{-3} [e]	19.9 ± 0.3	3 ± 2	19.0 ± 0.6
8b	2.2 ± 0.1	0.041 ± 0.004[e]	0.015 ± 0.002[e]	20.5 ± 0.8	4 ± 2	19.3 ± 0.8
9	10.0 ± 0.2	0.023 ± 0.002[e]	(2.3 ± 0.2) × 10^{-3} [e]	21.8 ± 1.2	7 ± 3	19.7 ± 1.2

[a] In 90% H_2O/10% D_2O at pH 3.0. [b] The rate and equilibrium constants and $\Delta G_{ct}^{\ddagger}$ are for 25 °C. [c] See Table 2 for amino acid sequences. [d] K = [trans]/[cis]. [e] Rate constants were obtained by extrapolation to 25 °C using the activation parameters determined from rate data measured at higher temperatures. [f] No suitable pair of cis/trans resonances for kinetic measuements. [g] Kinetic data measured only at 25 °C due to low abundance of cis isomer.

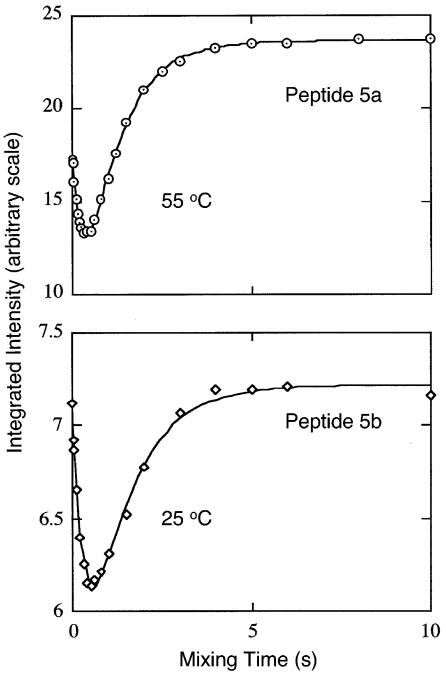

Figure 4. Integrated intensities of the resonances for the Gly N−H protons of the cis isomers of peptides **5a** and **5b** as a function of the mixing time t in the inversion-magnetization transfer pulse sequence. The smooth curves were obtained by nonlinear least-squares analysis of the data.

The results in Table 3 for peptides **1**−**6** clearly indicate that, even though the peptide backbone is constrained by the disulfide bond, the rate of cis/trans isomerization of the Cys-Pro peptide bond is significantly faster for the disulfide form of peptides that have the sequence Cys-Pro-Xaa-Cys. Rate constant k_{ct} is larger by factors of 10.5 for peptide **3b** (Xaa = His), 13.1 for **5b** (Xaa = Gly), and 4.2 for **6b** (Xaa = Leu), while rate constant k_{tc} is larger by factors of 12.2, 1.9, and 2.9, respectively. Due to the lack of a suitable cis/trans pair of resonances for the inversion transfer experiment, it was not possible to determine rate constants for cis/trans isomerization of **4a** (Xaa = Tyr); however, rate constants for **4b** are similar to those for **1b**−**3b** and **6b**, which suggests that the rates of cis/trans isomerization are also significantly enhanced for **4b**. The results of the present study indicate that the enhanced rates we reported[13] previously for cis/trans isomerization of the Cys-Pro peptide bond in the disulfide form of peptides having the sequence -Cys-Pro-Phe-Cys- are not unique to these peptides, but rather are common for peptides having the -Cys-Pro-Xaa-Cys- sequence motif, including those that have the sequence of active sites of thiol/disulfide oxidoreductase enzymes.[20]

The faster rates for the cyclic disulfide peptides could be due to unusually slow rates for the dithiol peptides or unusually fast rates for the disulfide peptides. Rate constants for cis/trans isomerization of the Cys-Pro peptide bonds of the dithiol forms of the peptides are similar to those for the Cys^6-Pro peptide bonds of oxytocin and arginine vasopressin (for oxytocin, k_{ct} = 0.042 s^{-1} and k_{tc} = 0.0035 s^{-1}; for arginine vasopressin, k_{ct} = 0.067 s^{-1} and k_{tc} = 0.0046 s^{-1})[30] and they are similar to those of the Gly-Pro peptide bond in **9**. Taken together, these reference rate constants indicate that the rates of cis/trans isomerization of the Cys-Pro peptide bonds of the acyclic dithiol peptides in Table 3 are normal, while those of the cyclic disulfide peptides are unexpectedly fast.

Cis/trans isomerization of prolyl peptide bonds involves a shift of the lone pair of electrons on the prolyl imide nitrogen from a p_z orbital to an sp^3 orbital and rotation around the C−N bond to give a twisted transition state with the proline ring arranged perpendicular to the carbonyl group (Scheme 1).[1b,4] Cis/trans isomerization is accelerated by factors that destabilize the cis and trans ground states or stabilize the transition state.[1b] Distortion of the ground state conformations can destabilize the ground states,[1b] while solvent effects and intramolecular hydrogen bonding to the lone pair of electrons on the pyramidalized proline nitrogen of the transition state can stabilize the transition state.[4,19,31−33]

Acceleration of the rates of cis/trans isomerization of Xaa-Pro peptide bonds by stabilization of the transition state by intramolecular hydrogen bonding to the pyramidalized proline

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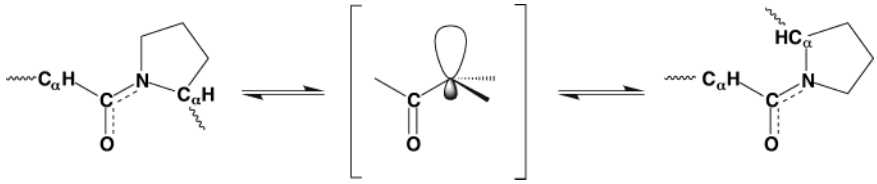
Scheme 1



nitrogen has been reported for model compounds, peptides, and proteins.[1b,18,32,3331−33] Cis-to-trans isomerization of the acyl-prolyl bond in a series of acyl-prolyl-amide model compounds is catalyzed in organic solvents by formation of an intramolecular hydrogen bond between the proline nitrogen and the NH proton of the prolyl amide bond, while cis-to-trans isomerization is accelerated by up to a factor of 10 in peptides that contain a histidyl-prolyl sequence by interaction of an NH proton of the histidyl imidazolium ring with the proline imide nitrogen.[18,32] In dihydrofolate reductase, cis/trans isomerization is accelerated by hydrogen bonding of an NH proton of an arginine side chain to the proline nitrogen; in this case, the arginine is not adjacent to the proline in the amino acid sequence but rather a more distant arginine is positioned proximate to the proline in the native structure of the protein.[33] Of particular significance to the present work, theoretical studies suggest that catalysis of cis/trans isomerization by the FKBPs results in part from stabilization of the transition state by an intramolecular hydrogen bond between the prolyl imide nitrogen and the NH proton of the Pro-Xaa peptide bond of the conformationally constrained enzyme-bound protein substrate.[19]

To account for the increased rates of cis/trans isomerization of peptides **1b** and **2b**, we proposed an intramolecular catalysis mechanism similar to the FKBP mechanism in which a small but kinetically significant fraction of the NH proton of the Pro-Phe peptide bond hydrogen bonds to the proline sp^3-hybridized nitrogen in the transition state.[13] The basis for the proposal is that conformational constraints imposed on the peptide backbone by the intramolecular disulfide bond position the NH proton of the Pro-Phe peptide bond next to the proline nitrogen.[13] Molecular models suggest that cis/trans isomerization of the Cys-Pro peptide bond of peptides **1b**−**8b** proceeds through a syn/exo transition state structure (Scheme 1). A hydrogen bond between the proline nitrogen and the NH proton of the Pro-Xaa peptide bond is predicted to lower the barrier for going from a cis ground-state structure to the syn transition-state structure for a prolyl peptide bond by 1.4 kcal/ mol.[4] $\Delta G_{ct}^{\ddagger}$ for the cyclic disulfide forms of peptides **1**−**3** and **6** is lower than $\Delta G_{ct}^{\ddagger}$ for the acyclic dithiol peptides by from 0.9 to 1.5 kcal/ mol (Table 3), in excellent agreement with the theoretical value, which lends support to the proposed intramolecular catalysis mechanism for the unexpectedly fast rates of cis/trans isomerization of the Cys-Pro peptide bonds in the cyclic disulfide form of peptides having the Cys-Pro-Xaa-Cys oxidoreductase motif.

Finally, the rates of cis/trans isomerization of the Gly-Pro peptide bond in the dithiol and disulfide forms of Ac-Cys-Gly-Pro-Cys-NH_2 (**8**), a model peptide for the active site of thioredoxin, were measured to determine if the rates of cis/trans isomerization are also faster for the Xaa-Pro peptide bond in cyclic disulfide-constrained peptides that have the sequence Cys-Xaa-Pro-Cys. Although rate constant k_{ct} in Table 3 for the Gly-Pro peptide bond of **8b** is less than that for **8a** by 33%, k_{tc} is larger by a factor of 2.3, indicating that rate enhancements are also possible for Xaa-Pro peptide bonds in cyclic disulfide peptides of the Cys-Xaa-Pro-Cys motif.

Conclusion

Although the peptide backbone is constrained by the disulfide bond, the rates of cis/trans isomerization of the prolyl peptide bond are significantly faster for the cyclic disulfide forms of peptides that have the sequences Ac-Cys-Pro-Xaa-Cys-NH_2 and Ac-Cys-Xaa-Pro-Cys-NH_2. The significantly faster rates suggest that the backbones of the active sites of the oxidized forms of thiol/disulfide oxidoreductase enzymes that have a proline in the -Cys-Xaa-Xaa-Cys- active site motif might also have unusual conformational flexibility. Indeed, it has been proposed, on the basis of NMR structures, that functional differences between the oxidized and reduced forms of *E. coli* thioredoxin are related to differences in conformational flexibility in and near the active site loop of the oxidized form.[34] The enhanced rates of cis/trans isomerization also suggest that the disulfide form of peptides having the Cys-Pro-Xaa-Cys sequence might be good model peptides for studying the catalytic mechanism of the FKBPs. The proposed intramolecular mechanism for acceleration of the rates of cis/trans isomerization of the Cys-Pro peptide bond of the disulfide form of the Ac-Cys-Pro-Xaa-Cys-NH_2 peptides is identical to that proposed for catalysis of cis/trans isomerization of protein prolyl peptide bonds by the FKBPs, where constraints from binding in the FKBP-active site force the protein backbone into a type VIa proline turn.[17]

Further studies are in progress to explore the effect of the number of amino acids in the disulfide-constrained ring and the position of the proline on the rates of cis/trans isomerization of prolyl peptide bonds.

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