Retention of the Cis Proline Conformation in Tripeptide Fragments of Bovine Pancreatic Ribonuclease A Containing a Non-natural Proline Analogue, 5,5-Dimethylproline

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Abstract: Attention is focused on L-5,5-dimethylproline (dmP) as a substitute to lock L-proline (Pro) in a cis conformation in peptides and proteins, to prevent cis/trans isomerization when a protein with cis X-Pro peptide groups unfolds. Procedures have been developed to obtain optically pure L-dmP and to incorporate this sterically hindered residue as the central one in tripeptides that are suitable for fragment coupling to prepare synthetic proteins. Based on the sequences of residues 92−94 (Tyr-Pro-Asn:YPN) and 113−115 (Asn-Pro-Tyr: NPY) in bovine pancreatic ribonuclease A (RNase A), in which the X-Pro peptide groups are in the cis conformation, the tripeptides Ac-Tyr-dmP-Asn (YdmPN) and Ac-Asn-dmP-Tyr (NdmPY) were synthesized, and their structures were determined by 2D ^{1}H nuclear magnetic resonance (NMR) spectroscopy. YdmPN was found to exist solely in the cis conformation between 6 and 60 °C, whereas NdmPY was found to have some trans component that increased from about 10% to about 21% as the temperature increased over the range between 6 and 80 °C. Both YdmPN and cis-NdmPY adopt a type VI reverse turn, as does proline. The NMR structures of YdmPN and cis-NdmPY are comparable with the X-ray structures of the corresponding portions of RNase A, and the NMR structure of trans-NdmPY is compatible with the X-ray structure of the isolated tripeptide, Ac-NPY. These results demonstrate that L-dmP is a promising substitute for proline in a variety of protein problems to constrain the X-Pro peptide group to the cis conformation.

Introduction

Proline is the only naturally occurring cyclic amino acid. Consequently, unlike other peptide groups, which predominantly adopt the trans form, the imidic X-Pro peptide group readily exists in the cis as well as the trans form, even in the absence of structural constraints. In the X-Pro peptide group, the cis and trans states are of similar energies, with about a 20 kcal/mol barrier for their interconversion.[1] In unfolded proteins and short peptides, the X-Pro peptide group exists as a mixture of the two isomers;[2] in native proteins, it almost always adopts only one of these single isomeric states, dictated by other interactions within the protein.

Because of its unique conformational properties, proline plays a critical role in many biologically important processes, including protein folding[3,4] and signal transduction,[5,6] and in biologically active and pharmaceutically relevant peptides.[7] Peptide and protein studies of the role of proline in structure formation

have been hampered by the heterogeneity of the X-Pro peptide group.[8−10] Formation of cis peptide groups at non-proline residues is also biologically significant. A cis Gly−Gly peptide bond has been suggested to play a role in amyloid formation,[11] but investigations have been somewhat limited by the inability to generate peptides containing a high percentage of the cis peptide group. The ability to restrict a peptide group exclusively to the cis conformation, in addition to facilitating progress in the areas discussed above, would also provide an invaluable tool for use in developing peptides for drug use and for the generation of peptide antibodies targeted against the native isomeric state of a protein. The formation of a cis peptide group results in a chain reversal,[3,12] a structural feature that may be of great importance in dictating the formation of early folding intermediates[3,13] and thereby directing productive protein folding pathways.[3,13−18] Indeed, it is the role of proline in influencing

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protein folding pathways that arguably may be its most important role.

In protein folding, numerous examples exist in which the rate-determining step of in vitro folding is the isomerization of an X-Pro peptide group.[15,19-21] The most important purpose of folding studies is not the characterization of the proline isomerization processes but rather the determination of how the sequence directs the folding process. Unfortunately, the heterogeneity of the X-Pro peptide groups in the unfolded state obscures the conformational folding process and prevents meaningful interpretation. In some cases, it has been possible to eliminate the isomerization problem through experiments in which the protein is rapidly unfolded, and refolding is initiated before the X-Pro peptide groups have had time to isomerize from their native state. This procedure has been used successfully to study the disulfide-intact refolding of bovine pancreatic ribonuclease A (RNase A),[22] but it severely limits the experimental conditions and observation methods that can be used and requires specialized equipment. In the case of oxidative protein folding (beginning from the fully denatured and disulfide reduced state), refolding rates are on a time scale similar to that of proline isomerization, and double-jump stopped-flow techniques are ineffective. In that situation, it has been impossible to eliminate complications arising from the cis/trans isomerization of proline peptide groups.

Clearly, the ability to constrain a peptide group to a single unique confirmation is of considerable utility. A number of attempts have been made to constrain the X-Pro peptide group to a single conformation.[23-31] Proline analogues such as methanoproline[24,25] and oxazolidine-4-carboxylic acid[31] are very effective at yielding high percentages of the trans conformer while still retaining the imidic peptide bond. The most common method of retaining a trans peptide bond, however, is simply to replace proline with one of the other naturally occurring amino acids, typically alanine. No such routine methods exist for the incorporation of cis peptide groups. Among the more successful approaches that have been used to constrain peptide groups to the cis conformation is the use of substituted thiazolidines and oxazolidines.[23,30,31] One such analogue, 2,2-dimethylthiazoladine-4-carboxylic acid, has been shown to exist predominantly in the cis conformation in model peptides.[23,30,31] Unfortunately, oxazolidine and thiazolidine compounds are acid labile and, therefore, are not easily incorporated into peptides.[26,32,33] The proline analogues constrain the X-Pro peptide group by the addition of bulky functional groups. In addition, the presence of the heteroatom alters the ring puckering (the dihedral angles, ϕ and χ) and hydrophobicity of the residue as compared to proline, which may result in significant alterations in the conformational properties of peptides containing oxazolidine and thiazolidine proline analogues.[31,34]

The pyrrolidine compound most similar to 2,2-dimethylthiazolidine-4-carboxylic acid is 5,5-dimethylproline (dmP).[26,35] A dmP-containing dipeptide, Boc-Phe-dmP−OMe, has been reported, and nuclear magnetic resonance (NMR) data indicated that the peptide group was predominantly in the cis conformation in methanol.[26] Unfortunately, the results of that experiment were not quantitative because efforts to separate the enantiomers of dmP were unsuccessful. To ascertain the suitability of dmP for use in peptides and proteins, we have investigated the properties of optically pure L-dmP in aqueous solution, and we report here the efficient separation of L-dmP from D-dmP. Since one of our major purposes is to incorporate dmP into synthetic RNase A and peptides derived from that protein, we have synthesized two tripeptides, Ac-Tyr-dmP-Asn (acetylated tyrosine-5,5-dimethylproline-asparagine, YdmPN) and Ac-Asn-dmP-Tyr (acetylated asparagine-5,5-dimethylproline-tyrosine, NdmPY), which correspond to residues 92−94 and 113−115, respectively, of RNase A. In native RNase A, both of these peptides contain cis X-Pro peptide groups.[36] We also report here the solution structure of YdmPN, cis-NdmPY, and trans-NdmpY, determined by ^{1}H NMR spectroscopy.

Experimental Section

Synthesis of 5,5-Dimethylproline (dmP). D,L-dmP was synthesized at the Cornell University Biotechnology Resource Center by the method described by Magaard et al.[26]

Resolution of L-dmP from D-isomer: Determination of Optical Purity. As far as we are aware, optically pure L-dmP has not been prepared previously. A rapid and efficient method for separating the optical isomers of D,L-dmP was developed here, based on a method that proved suitable to resolve the enantiomers of D,L-Pro.[37] This method is based on the differential solubility of the complexes formed between the optical isomers of dmP and D-tartaric acid. The D,L-dmP (1.0 g/7.0 mmol, equivalent to 3.5 mmol of the L form) was dissolved in 2.0 mL of water containing 1.05 g/7.0 mmol of D-tartaric acid at room temperature. Then, 70 mL of absolute ethanol was added slowly with mechanical stirring. After 24 h, a crystalline precipitate was collected by filtration, washed with ethanol, and air-dried; yield of the L-dmP: D-tartaric acid complex, 0.95 g (91% yield, 3.2 mmol). The dmP was separated from the D-tartaric acid by anion-exchange chromatography (Sigma IRA-410, Amberlite) and converted to the methyl ester using $SOCl_2$/MeOH.[38]

The absolute configuration of the isomer of dmP forming the insoluble crystalline complex with d-tartaric acid was identified as L by ^{1}H NMR spectroscopy of the methyl ester of the dmP isomer in (R)-(−)-1-phenyl-2,2,2-trifluoroethanol.[39] The purity of the L-dmP

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(>98%; mp 193.5 °C) was determined by using 2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosyl isothiocyanate (BGIT), which can form diastereomeric thioureas with D,L-dmP without racemization.[40] The diastereomers can be separated on a nonchiral reversed-phase (RP) C-18 column (Vydac C-18, 4.6 × 100) by high-pressure liquid chromatography (HPLC), using as a mobile phase 10 mM phosphate, pH 7, 60−70% MeOH gradient. The specific rotation of the optically pure L-dmP ($[\alpha]_D$ = −51.2°) was determined in water using a Perkin-Elmer model 241 polarimeter. Concentrations were determined by weight using desiccated material.

Synthesis of Tripeptides (Fragments of RNase A). The tripeptides Ac-YdmPN and Ac-NdmPY were synthesized by a combination of solid- and solution-phase methods at the Cornell University BioResource Center by the following methods.

Ac-Y(OBu^t)-dmP-OH and Ac-N(Trt)-dmP-OH (Trt = 1,1,1-Triphenylmethyl). The bulky methyl groups on the δ carbon of dmP hampered formation of the X-dmP peptide bond. In one procedure, Fmoc-Y(OBu^t)-dmP-OMe (Fmoc = (fluorenylmethoxy)carbonyl) was prepared by coupling Fmoc-Y(OBu^t)-OH (138 mg, 0.3 mmol) and dmP-OMe (47 mg, 0.3 mmol) using *N*-dicyclohexylcarbodiimide (DCC, 62 mg, 0.3 mmol) in dry DMF. The Fmoc and the methyl ester were removed by treatment with a minimal excess of 1 M NaOH/methanol and then acetylated with acetic anhydride in a one-pot reaction. The product, Ac-Y(OBu^t)dmP-OH (46 mg, 0.114 mmol, 38% yield), was purified by RP-HPLC on a Nova-Pak C18 (8 × 25 cm) radial compression cartridge employing a Waters model 600E HPLC. Ac-N(Trt)-dmP-OH was prepared in a similar fashion with 32% yield (38 mg, 0.0704 mmol) from Fmoc-N(Trt)-OH (131 mg, 0.22 mmol) and dmP-OMe (35 mg, 0.22 mmol) using DCC (46 mg, 0.22 mmol) in dry DMF. In a second procedure, Boc and Fmoc protecting groups and a variety of coupling reagents were tried, to form the Tyr-dmP peptide bond.

Ac-Y-dmP-N-OH and Ac-N-dmP-Y-OH. Ac-Y(OBu^t)dmP-OH (13.5 mg, 0.033 mmol) was activated with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/*N*-hydroxybenzotriazole (HOBt) and coupled to an equimolar amount of NH$_2$-N(Trt)-Novasyn resin (500 mg, 0.1 mM equivalent NH$_2$, Nova Biochem). The resulting tripeptide resin was cleaved and deprotected with TFA/EDT/thioanisol (95/2.5/2.5, v/v) (EDT = 1,2-ethanedithiol). The Ac-Y-dmP-N-OH was then purified by RP-HPLC as described above with a yield of 55% (8.5 mg, 0.018 mmol). Ac-N-dmP-Y-OH was prepared by coupling Ac-N(Trt)-dmP-OH (12.4 mg, 0.023 mmol) to an NH$_2$-Y(OBu^t)-Novasyn resin in a similar manner. The cleavage of the protecting groups and purification of Ac-N-dmP-Y-OH were accomplished as described above with a yield of 58% (6 mg, 0.013 mmol). The two tripeptides were characterized by analytical HPLC, capillary electrophoresis (CE), and matrix-assisted laser desorption/chemical ionization mass spectrometry (MALDI MS). Each peptide was found to be >98% pure. The (M + H)$^+$ ions of these peptides were 464.8 (±0.1%) and 464.5 (±0.1%) Da, respectively (expected 464.4 Da).

In the second procedure for synthesizing YdmPN, higher yields (75%) were obtained by using a Boc protecting group and the bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) coupling reagent,[41] or an Fmoc protecting group and the tetramethylfluoroformamidinium hexafluorophosphate coupling reagent[42] (TFFH, generously provided by PerSeptive Inc.).

NMR Measurements. For the NMR experiments, 2−3 mg of peptide was added to 240 μL of 90%/10% H$_2$O/D$_2$O solution in Shigemi NMR tubes (BMS-005V). D$_2$O (99.9%) from Cambridge Isotope was used. The pH was adjusted with aliquots of 0.5 M NaOH and checked with a Beckmann Futura Plus electrode without correction for the presence of D$_2$O, which was added for the deuterium lock. The pH for all experiments was 5.3 ± 0.1 at 20 °C.

All ^{1}H NMR experiments were carried out on a Varian Unity 500-MHz spectrometer equipped with a triple-resonance probe and pulsed-field *z*-gradients. The spectral width was 4199.7 Hz. For each 2D NMR experiment, 32 transients of 4096 complex points per transient were

obtained for each of 432 t_1 increments. Chemical shifts were measured and referenced relative to tetramethylsilane (TMS) by assigning the HOD resonance a value of 4.80 ppm. 2D ^{1}H NMR experiments were carried out in the phase-sensitive mode using time-proportional phase incrementation.[43] Suppression of the water signal was accomplished by presaturation during the initial delay between transients. Data were processed with the VNMR data processing program or with the Felix (Molecular Simulations Inc.) program. The data matrix of 432 t_1 experiments was zero-filled to 2K points along both dimensions; prior to Fourier transformation, it was multiplied by a 0°-shifted sine-bell curve along both t_1 and t_2 for the double-quantum-filtered shift-correlated spectroscopy (DQF-COSY)[44] experiment and a 75°-shifted squared sine-bell curve along both t_1 and t_2 for the nuclear Overhauser effect spectroscopy (NOESY),[45] rotating frame nuclear Overhauser effect spectroscopy (ROESY),[46] and total correlated spectroscopy (TOCSY)[47,48] (τ_{mix} = 75 ms) experiments. NOESY spectra were acquired with mixing times of 150, 250, and 450 ms at 25 °C and with a mixing time of 250 ms at 10 and 15 °C. ROESY experiments were carried out with mixing times of 250 ms at 20 and 25 °C.

Determination of Isomerization Rate Constants. As will be shown in the Results and Discussion section, YdmPN is solely in the cis conformation, whereas NdmPY is an equilibrium mixture of cis and trans forms. Assuming that cis/trans interconversion is a two-state process, the rate constants of isomerization were estimated by following the temperature-jump procedures reported by Grathwohl and Wüthrich.[2,49] In these experiments, the kinetics of the isomerization process were followed by measuring the relative intensities of the corresponding ^{1}H resonances of the cis/trans conformers as a function of time after initiating a jump in temperature. For these experiments, the initial temperature was set to 80 °C by heating the NdmPY peptide sample before insertion into the magnet. The final temperatures in the magnet were set to 10, 15, 20, 25, 30, and 35 °C. After an initial delay of 20 s, spectra were recorded at 6.8-s intervals until thermal equilibrium at the final temperature was attained. The cis/trans isomerization rate constants ($k_{c \to t}$ and $k_{t \to c}$; Figure 3A, below) were then calculated from the ratio of the peak areas for the cis and trans species.[2,50,51] For obtaining $k_{c \to t}$, the following equation was used,

$$\frac{[\text{cis}]_t - [\text{cis}]_\infty}{[\text{cis}]_0 - [\text{cis}]_\infty} = \exp\left(\frac{-k_{c \to t}}{[\text{trans}]_\infty}t\right) \tag{1}$$

where [cis]$_0$, [cis]$_t$, [cis]$_\infty$, and [trans]$_\infty$ are the concentrations of cis or trans at times 0, t, and equilibrium, respectively. A corresponding equation was used to obtain $k_{t \to c}$. Cis and trans resonances of the methyl protons on the δ carbon of dmP were selected for the quantitative measurement because they were well separated from each other and from other resonances. The activation energy, $E^\ddagger$, for the cis → trans interconversion was computed from the Arrhenius equation,

$$\ln k_{c \to t} = \ln A - E^\ddagger / RT \tag{2}$$

To obtain the steady-state ratio between the cis and trans populations of the two peptides at a given temperature, the 1D spectra of YdmPN and NdmPY were recorded at 6 °C, in 5−deg steps from 10 to 60 °C, and at 80 °C. Steady-state equilibrium constants were calculated from $K_{c \to t}$ = [trans]/[cis], and a van't Hoff plot was used to obtain the thermodynamic parameters ($\Delta G°$, $\Delta H°$, and $\Delta S°$; Figure 3B, below). At 25 °C, for example, K = 0.143, or the % trans is 13% for NdmPY. Since only one set of resonances was observed for YdmPN throughout the temperature range in this study, it was concluded that no isomerization of the YdmPN group had occurred.

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Table 1. *J* Coupling Constants in Hertz (A) and Cis/Trans Population Ratios at Various Temperatures in H_2O (B)

(A) *J* Coupling Constants

	$J_{HN-H\alpha}$	$J_{H\alpha-H\beta1}$	$J_{H\alpha-H\beta2}$	$J_{H\alpha-H\beta1,2}$	$J_{HN-H\alpha}$	$J_{H\alpha-H\beta1}$	$J_{H\alpha-H\beta2}$
dmP		6.8					8.7
YdmPN		Tyr		dmP		Asn	
	4.3	10.4	5.4	8.4, 2.0	8.2	4.7	8.72
cis-NdmPY		Asn		dmP		Tyr	
	6.4	8.9	4.4	9.2, 1.9	7.25	7.3	7.3
trans-NdmPY		Asn		dmP		Tyr	
	8.3	7.4	7.0	9.3, 2.0	7.36	7.0	7.0

(B) Cis/Trans Population Ratios

temp (°C)	6	10	15	20	25	30	35	40	45	50	55	60	80
% cis-NdmPY	90.3	89.6	88.9	88.5	87.5	86.8	86.2	85.1	84.3	84.1	82.5	80.7	78.9

Dihedral Angle and NOE Constraints. Scalar spin−spin coupling constants (*J*; Table 1) were measured from the $^1H-^1H$ spin−spin splittings in the 1D ^1H spectra. To minimize the overlap of peaks, the amplitude of the free induction decay (FID) was multiplied by a shifted Gaussian window function before the Fourier transformation.

From the analysis of the NOEs from both NOESY and ROESY spectra, similar relative NOE connectivity information was obtained. The intensities of the NOESY cross-peaks from a 250-ms experiment at 10 and 25 °C were measured. Distances were estimated (within upper and lower bounds of ±0.5 Å) by using the isolated spin-pair approximation, with reference to the geminal proton cross-peak of Asn corresponding to an interproton distance of 1.8 Å.[52] This was justified in view of the small size of the two peptides, which should involve negligible spin diffusion.

Stereospecific assignments of the H^β resonances of Asn and Tyr in the cis or trans conformers and their dihedral angles, χ^1, were determined from the $^3J_{H\alpha H\beta}$ coupling constants together with the intraresidue NOE pattern[53] from H^α to $H^{\beta1,2}$ and HN to $H^{\beta1,2}$. For the dihedral angle χ^1 of 180°, the $^3J_{H\alpha H\beta1}$ and $^3J_{H\alpha H\beta2}$ coupling constants are >10 and <5 Hz, respectively. The $H^{\alpha-\beta2}$ and HN−$H^{\beta1}$ NOEs are stronger than their corresponding $H^{\alpha-\beta1}$ and HN−$H^{\beta2}$ NOEs, respectively. For the dihedral angle χ^1 of − 60°, the $^3J_{H\alpha H\beta1}$ and $^3J_{H\alpha H\beta2}$ coupling constants are <5 and >10 Hz, respectively. The $H^{\alpha-\beta1}$ and HN−$H^{\beta2}$ NOEs are stronger than their corresponding $H^{\alpha-\beta2}$ and HN−$H^{\beta1}$ NOEs, respectively.

Calculation of the Structures of YdmPN, cNdmPY, and tNdmPY. The X-PLOR 3.1 program was used, with the IBM SP2 supercomputer of the Cornell Theory Center, to calculate the structures of the peptides.[54] The resulting structures were viewed with the molecular graphics program InsightII (Molecular Simulations Inc.).

Since the dmP residue was a new amino acid, its coordinates, partial charges, and nonbonded parameters were incorporated into the X-PLOR topology and parameter files, respectively, for calculating the structures of YdmPN, cNdmPY, and tNdmPY. The nonbonded parameters, bond lengths, and partial charges for the two methyl groups attached to the δ carbon were taken from the methyl groups of the valine residue in the X-PLOR protein topology and parameter files. The bond angles and dihedral angles of dmP were taken as those of the Pro residue in the down-puckering conformation, the down-puckering having been observed in this NMR investigation.

The default force constants for bond lengths, bond angles, and dihedral angles of all amino acid residues were the same as reported previously.[55] The NOE penalty function was set to 50 kcal mol^{-1} Å^{-2}. Initial sets of 100 random structures each were generated for the YdmPN, cNdmPY, and tNdmPY peptides starting from random values for the dihedral angles ϕ, ψ, and χ, and a value of 180° for the dihedral angle ω, except for dmP in the cis conformation ($\omega = 0°$). The dihedral angle ω was allowed to vary in the calculation, but only within the neighborhood of 0° and 180° for the cis and trans conformations, respectively.

The conformations of the three peptides were then determined by incorporating the NOE distance and dihedral angle constraints into a Powell energy minimization (EM) and simulated annealing (SA) protocol.[56−59] The SA method was based on Verlet restrained molecular dynamics. The EM/SA protocol can be divided into five stages. First, 500 steps of EM were applied with a weight for the van der Waals repulsion force constant set at 0.002 and a slope for the asymptotic constant for the NOEs set at 0.1. Under these conditions, the atoms were allowed to move through each other, providing greater freedom to accommodate the initial NOE constraints. Next, conformational sampling of structures was carried out by applying 10 ps of molecular dynamics at 1000 K, again with the weight for the repulsion force constant set at 0.002 and the slope for the asymptotic constant for the NOEs set at 0.1. Distance penalties were evaluated by using a soft-well square NOE potential. In the third stage, the structures were subjected to 10 ps of molecular dynamics at 1000 K with the weight for the repulsion force constant increased to 0.1 and the slope for the asymptotic constant for the NOE increased to 1.0. Under these conditions, the peptides were given less freedom to sample the conformational space. For the fourth stage, the asymptotic constant for the NOEs remained at 1.0, and the weight for the repulsion force constant was increased to 1.0. The temperature was gradually lowered from 1000 to 100 K in 1000 steps (1 ps/step) of molecular dynamics. In the final stage, the square-well potential was replaced with a soft-well square potential and 1000 steps of EM, and another 1000 steps with inclusion of electrostatic interactions were applied. From the 100 initial random structures of each of the three peptides, 57, 48, and 24 structures of YdmPN, cNdmPY, and tNdmPY, respectively, were selected on the basis of no violations of NOE or dihedral angle constraints greater than 0.1 Å and 5°, respectively. An average structure was then calculated for each peptide, and the 10 structures with the smallest root-mean-square deviations (RMSDs) from the average structure were finally chosen.

Results and Discussion

NMR analyses of dmP, YdmPN, and NdmPY. The chemical shift assignments of L-dmP and the two tripeptides were determined by 1D, DQ-COSY, NOESY, ROESY, and TOCSY experiments using the methods of Wüthrich.[52] The chemical shifts for the free L-dmP residue and the two tripeptides are listed in Table 2. For the free L-dmP, the H^β resonances were resolved and were stereospecifically assigned according to the intensities of their NOEs with the H^α proton. Both $H^{\gamma1}$ and $H^{\gamma2}$ resonances, as well as those of the two methyl groups, were

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Table 2. [1]H Resonance Assignments[a] for L-dmP (A), Ac-YdmPN (B), and cis-Ac-NdmPY[b] (C) in Aqueous Solution

residue	HN[c]	H(Me)[d]	Hα	H$^{\beta 1,2}$	others[e]
			(A) L-dmP		
dmP	8.73, 7.56		4.18	2.46, 2.17	H$^{\gamma 1,2}$, 1.92; H$^{\epsilon 1,2}$, 1.44
			(B) Ac-YdmPN		
Ac		1.94			
Tyr	8.01		4.44	2.84, 2.94	H^{2,6}, 7.17; H^{3,5}, 6.87
dmP	−		3.56	1.66, 1.84	H$^{\gamma 1,2}$, 1.62; H$^{\epsilon 1}$, 1.27; H$^{\epsilon 2}$, 1.47
Asn	8.26		4.36	2.81, 2.66	H$^{\delta}$, 7.53, 6.78
			(C) cis-Ac-NdmPY		
Ac		1.96			
		(1.96)			
Asn	8.29		4.32	2.40, 2.46	H$^{\delta}$, 7.35, 6.72
	(8.17)		(5.14)	(2.71, 2.60)	(H$^{\delta}$, 7.54, 6.81)
dmP	−		4.88	2.28, 1.93	H$^{\gamma 1,2}$, 1.69, 1.50; H$^{\epsilon 1}$, 1.26; H$^{\epsilon 2}$, 1.36
			(4.45)	(2.16, 1.82)	(H$^{\gamma 1,2}$, 1.73; H$^{\epsilon 1}$, 1.31; H$^{\epsilon 2}$, 1.45)
Tyr	7.66		4.33	3.11, 2.96	H^{2,6}, 7.08; H^{3,5}, 6.78
	(7.15)		(4.37)	(2.96)	(H^{2,6}, 7.07; H^{3,5}, 6.80)

[a] Chemical shift (ppm) at pH 5.3. [b] Values in parentheses were observed for the trans-Ac-NdmPY. [c] There are two values for dmP because the free N-terminal group is protonated. [d] Me is the CH$_3$ of the acetyl groups. [e] H$^{\epsilon}$ corresponds to the protons of two methyl groups on the δ-carbon of dmP. H$^{\delta}$ of Asn corresponds to the side-chain amide protons. H^{2,6} and H^{3,5} of Tyr correspond to the phenolic ring protons.
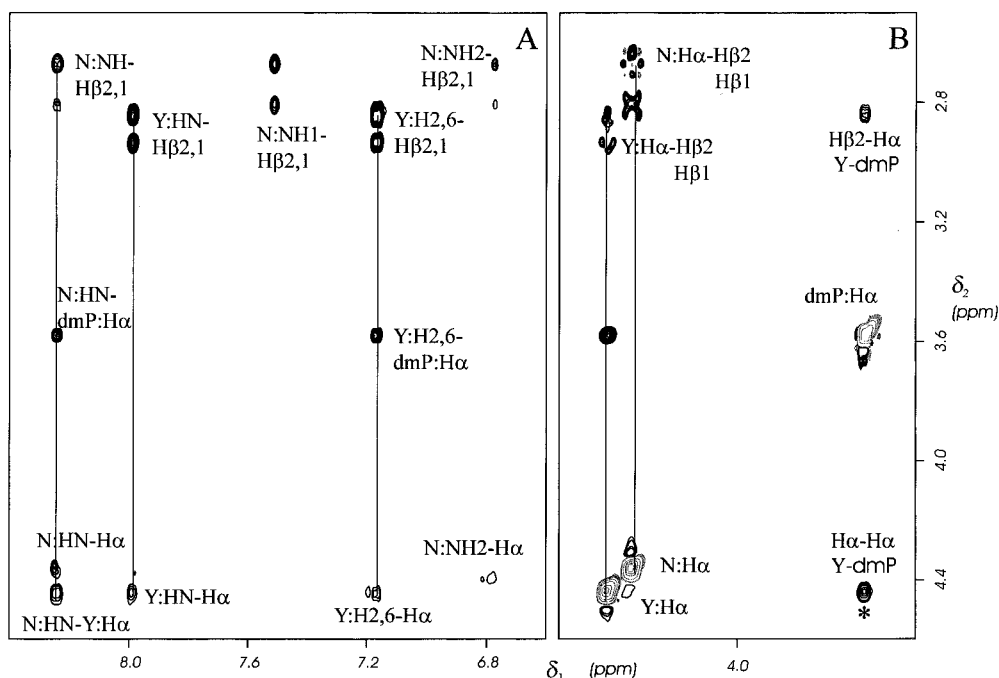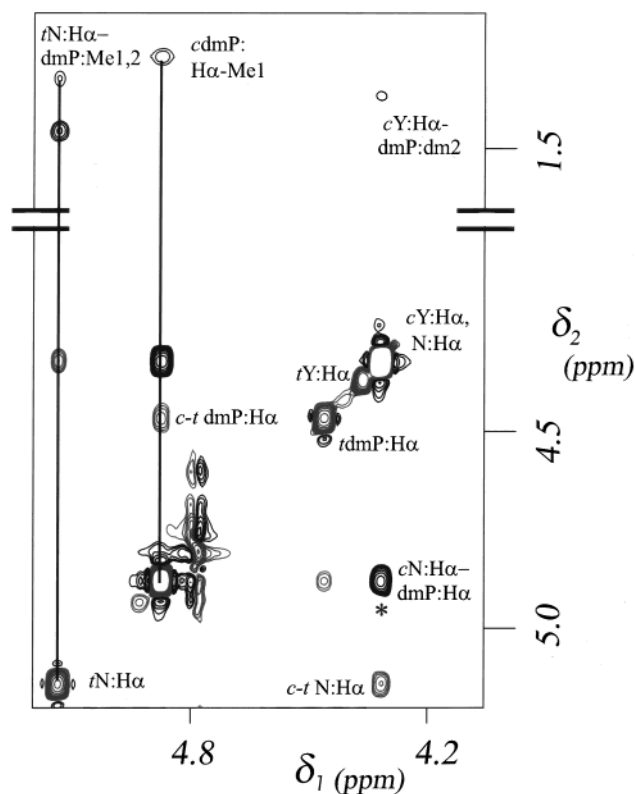

Figure 1. Regions of 2D NOESY spectrum of YdmPN: (A) NH−Hα,H$^{\beta}$ and (B) Hα−Hα,H$^{\beta}$ regions. Experimental conditions for both spectra: 8 mM YdmPN in 90/10% H$_2$O/D$_2$O, pH 5.3, 500 MHz, 32 transients, delay time of 1.0 s, 432 FIDs, τ_{mix} = 250 ms, 25 °C. All spectra were referenced against HOD, 4.80 ppm. The asterisk in (B) indicates the connectivity between the α protons of Tyr and dmP.

degenerate. In the tripeptides, on the other hand, all resonances of dmP were resolved, except the H$^{\gamma}$ resonances of YdmPN and tNdmPY.

The NOESY experiments with the tripeptides were used for determining the conformation of the peptide group preceding the proline residues. For an X-Pro peptide group, the NOE connectivity between the Hα of the X residue and the Hα of the Pro is indicative of a cis conformation, and a strong NOE connectivity between the H$^{\delta}$ of Pro (or the H$^{\epsilon 3}$ of dmP) and the Hα of the X residue is indicative of a trans conformation.[52] For YdmPN, only one set of resonances was observed in the ^{1}H spectrum, demonstrating that a single average conformation exists for this tripeptide. A strong NOE connecting the Tyr Hα to the dmP Hα (Figure 1B) was observed while no significant NOE connectivity between the Tyr Hα and the resonances of the two methyl groups ($^{\epsilon}$CH$_3$) of dmP was observed at 25 °C,

pH 5.3. Therefore, the single conformation adopted by the Tyr-dmP peptide group is cis in aqueous solutions.

Two distinct sets of resonances were observed in the one-dimensional ^{1}H spectrum of NdmPY with relative populations of 90% and 10% at 10 °C, indicating that two average conformations of the Asn-dmP peptide group are present in solution. For the abundant species, a strong NOE was observed between the Asn Hα and the dmP Hα, characterizing the peptide group conformation for this species as cis. For the less abundant species, NOEs between the Asn Hα and the $^{\epsilon}$CH$_3$ protons of dmP were observed (Figure 2), indicating that the Asn-dmP peptide group adopts the trans conformation. The existence of these two species and interconversion between them were also confirmed by the presence of chemical exchange cross-peaks between the cis and trans species for all resonances of NdmPY in the NOESY experiments collected at 25 °C (Figure 2).

Figure 2. Regions of 2D NOESY spectrum of NdmPY: cis-/trans-NdmPY, $H^\alpha-H^{\alpha,\delta}$ region. Experimental conditions for the spectrum were the same as in Figure 1. The asterisk indicates the connectivity between the α protons of Asn and dmP in the cis conformation.

Figure 3. Plot of ln k (A) and ln $K_{c\to t}$ (B) for NdmPY. The kinetic data were obtained from the temperature jump experiments of NdmPY in H_2O, pH 5.3.

Chemical exchange cross-peaks were identified on the basis of their phases, which are in phase with the diagonal peaks but out of phase from the NOE cross-peaks.

The observed coupling constants, together with NOE analyses, indicate that the side-chain rotamers of Tyr in YdmPN and Asn in cNdmPY have a dihedral angle χ^1 of ~180° ± 30°, and the Asn of YdmPN has a dihedral angle χ^1 of −60° ± 30°. These dihedral angles are relatively unusual, since most peptides of this size do not have a specific conformation for the dihedral angle χ^1. $^3J_{H\alpha H\beta}$ coupling constants were not informative for Asn and Tyr of tNdmPY and for Tyr of cYdmPN because their values range between 6 and 8 Hz. In these cases, we assigned H^β stereospecifically on the basis of the relative intensity of the NOESY cross-peak.

Determination of Thermodynamic Parameters. In folded states, X-Pro peptide groups usually adopt a single conformation with some exceptions as in the case of Staphylococcal nuclease, which displays multiple conformations because of cis/trans isomerization of the peptide group.[60] In unfolded proteins or peptides, the population of the cis form varies from 0% to 70%[2,61] and depends primarily on the preceding residue type, as well as on the solvent, pH, temperature, and neighboring residues. For YdmPN, only one set of resonances was observed throughout the entire temperature range, indicating that, within the detection limit of 1%, the Y-dmP peptide group adopts only the cis conformation.

The relative populations of the cis and trans conformers of NdmPY peptide are listed in Table 1 for the range of temperatures from 6 to 80 °C. From the two distinct sets of resonances,

(60) Evans, P. A.; Dobson, C. M.; Kautz, R. A.; Hatfull, G.; Fox, R. O. *Nature* **1987**, *329*, 266.

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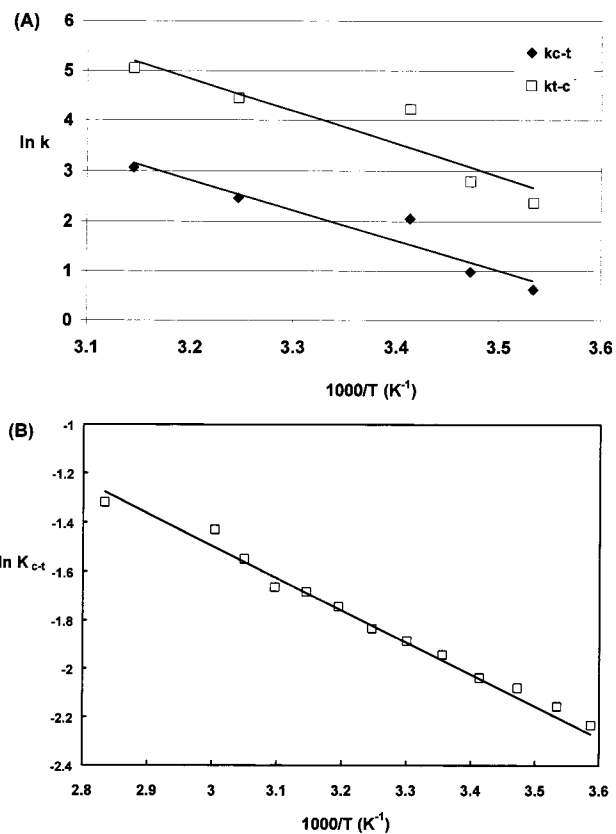
the relative populations of cis and trans species were determined from the integrated areas of the peaks corresponding to the resonances of the methyl groups on the δ carbon of dmP. The percentage of trans conformer increased by ~11% over the temperature range of 6–80 °C. Assuming that cis/trans isomerization is a two-state process, the equilibrium constant was expressed as [cis]/[trans] at each temperature, from which the thermodynamic parameters (ΔG°, ΔH°, and ΔS°) and the time constant for isomerization were determined (Figure 3).[2] The values of ΔG°, ΔH°, and ΔS° are ~1.15 ± 0.01 kcal/mol at 25 °C, ~2.40 ± 0.01 kcal/mol, and ~4 ± 0.1 cal mol^{-1} deg^{-1}, respectively. The transition from the cis to the trans conformation is unfavored for NdmPY. The equilibrium constants increased with increasing temperature. The free energy difference between cis and trans isomers of dmP (cis → trans) in this peptide was different from the corresponding value for the proline-containing peptide Asn-Pro-Tyr (~ −0.93 kcal/mol at 25 °C, unpublished results).

From the temperature dependence of the rate constant $k_{c\to t}$ of eq 1, we obtained a barrier, $\Delta E^\ddagger$, of about 13 ± 1.0 kcal/mol between the cis and trans forms of Asn-dmP in NdmPY, which was smaller than that which is usually found for the X-Pro group (~20 kcal/mol).[2]

NOE Connectivity for a Turn. In small peptides, even those with a tendency to adopt a unique structure, NOEs are usually very small or not seen, and their dihedral angles (i.e., *J* couplings) do not provide any useful information, because of the presence of an ensemble of conformations. Even though the tripeptides investigated here are small, they give rise to NOEs and coupling constants that indicate the existence of populations with folded conformations. More long-range NOEs were found for YdmPN than for NdmPY.

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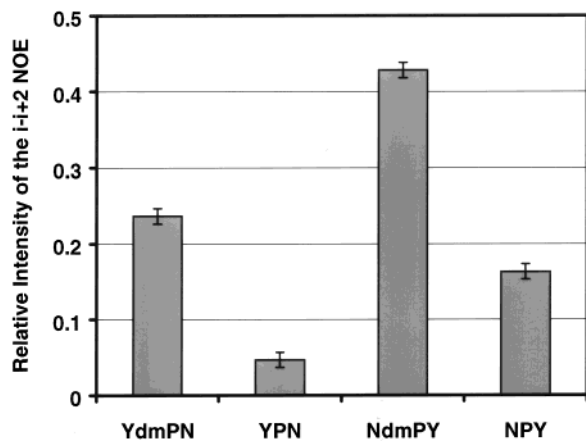
Figure 4. Comparison of the $i-i+2$ NOEs of YdmPN, YPN, NdmPY, and NPY (10 °C). NOEs were normalized with respect to the corresponding NOEs between H$^\alpha$ of the preceding amino acid and H$^\alpha$ of Pro/dmP in cis conformations.

The most convincing evidence for a turn comes from an NMR criterion suggested by Yao et al.[61] for indicating the presence of a type VI turn (with a cis X–Pro peptide group) in a sequence which contains aromatic residues preceding and/or following proline, viz., a cross-turn NOE between H$^\alpha$ of the residue preceding and HN of the residue following Pro ($i-i+2$ NOE). Observation of an $i-i+2$ NOE for the peptides in both the trans and the cis conformations indicates the presence of type I and VI turn conformations,[52,62] respectively. NOEs between H$^\alpha$ of Tyr and HN of Asn, and between H$^\alpha$ of Asn and HN of Tyr, were observed in the spectra of YdmPN and cNdmPY, respectively, indicating that they have a turn conformation. The relative intensities of the $i-i+2$ NOEs of YdmPN and cNdmPY were normalized with respect to the NOE between H$^\alpha$ of the residue preceding dmP and H$^\alpha$ of dmP. The corresponding $i-i+2$ NOEs from cis-YPN and cis-NPY were also normalized with respect to their NOEs between H$^\alpha$ of the residue preceding Pro and H$^\alpha$ of Pro, respectively. The intensities of the NOEs from YdmPN and NdmPY were larger than those from YPN and NPY, respectively (Figure 4). These results indicate that the tripeptides with dmP have a higher percentage of a type VI turn conformation than tripeptides with Pro. It seems that dmP increases not only the cis propensity but also the turn propensity in the cis population of the peptides and perhaps in proteins as well.

Temperature Coefficient of HN Resonances. The temperature dependence of the amide proton chemical shifts can be used to determine whether these protons are protected from the solvent. The temperature coefficients with an absolute value lower than 5 ppb/deg have been used for identifying amide protons which might be involved in hydrogen bonding in a close family of peptides.[61] We examined whether hydrogen bonding contributes to the stabilization of the folded population of these peptides. The 1D spectra of YdmPN and NdmPY, recorded from 6 to 60 °C in 5-deg steps, were analyzed. The temperature coefficients of the YdmPN amide protons indicate that the backbone HNs of Tyr and Asn may not be involved in hydrogen bonds ($\Delta\delta/\Delta T \approx -9$ ppb/K). However, the NOE between H$^\alpha$ of Tyr and HN of Asn in YdmpN suggests that HN of Asn is positioned close to the carbonyl oxygen of Ac. For cNdmPY, on the other hand, the temperature coefficients of Asn HN and Tyr HN were ~ -7.9 and -6.4 ppb/K, respectively, suggesting some protection for Tyr HN from the solvent. Interestingly, in
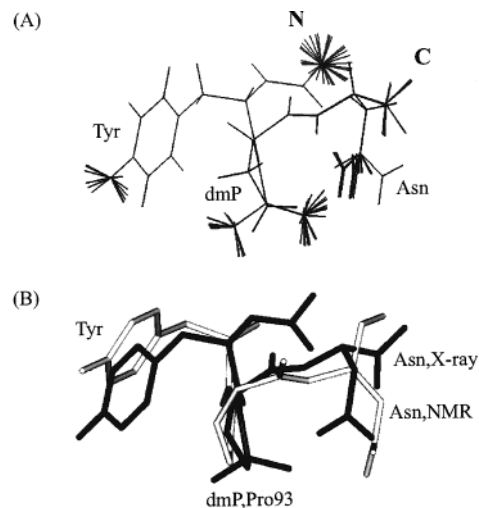
Figure 5. Ensemble of 10 structures of YdmPN (A) and superposition of that NMR structure which was closest to the average on the 92–94 fragment of the X-ray structure[36] of RNase A (B). The N-terminus of YdmPN is CH$_3$CO, and the C-terminus is COO$^-$. White and black models in (B) represent the 92–94 YPN stretch of the X-ray structure[36] and the NMR structure, respectively. N and C refer to the N- and C-termini of the fragments.

tNdmPY, the temperature coefficients of HN of Asn and Tyr were ~ -5.5 and -5.7 ppb/K, respectively, suggesting the existence of some interaction that involves these amide protons. These results are similar to previous results from Pro-containing peptides that also did not show a strong hydrogen bond even though their structures contained a type VI turn without a hydrogen bond.[62,63]

These data suggest that there is a turn without a strong intramolecular hydrogen bond involving the amide protons; i.e., there is no substantial stabilization of the turn conformation by hydrogen bonding.

Solution Structures of YdmPN, cNdmPY, and tNdmPY. For calculating the structures of YdmPN, cNdmPY, and tNdmPY, 75, 42, and 18 NOEs, respectively, were used with the X-plor program. Since the percentage of NdmPY molecules containing a trans-NdmP group is low, the number of NOEs is small and their intensities are weak. Three and two dihedral angle constraints were used for the structures of YdmPN and cNdmPY, respectively.

All structures are consistent with the constraints, with the distance or dihedral angle violations being less than 0.1 Å or 5°, respectively. Superpositions of the 10 best structures, which converged close to one conformation, are shown in Figures 5A, 6A, and 7A. The backbone RMSDs of each of the 10 structures of YdmPN, cNdmPY, and tNdmPY, with respect to their own average structure, are 0.022 ± 0.001, 0.131 ± 0.014, and 0.361 ± 0.012 Å, respectively, suggesting that good convergence was obtained in the calculation of these structures. The corresponding backbone and side-chain heavy-atom RMSDs are 0.460 ± 0.020, 0.848 ± 0.072, and 0.954 ± 0.141 Å for YdmPN, cNdmPY, and tNdmPY, respectively. The corresponding all-atom RMSDs are 0.633 ± 0.017, 0.907 ± 0.187, and 1.033 ± 0.133 Å for YdmPN, cNdmPY, and tNdmPY, respectively. The tNdmPY structures are not as well characterized as are the other two cis forms, because fewer NOEs were observed and used in the calculation.

From the best structures in Figures 5A, 6A, and 7A, a close interaction between the dmP side chain and the Tyr rings is

(62) Dyson, H. J.; Rance, M.; Houghton, R. A.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1988**, *201*, 161.

(63) Oka, M.; Montelione, G. T.; Scheraga, H. A. *J. Am. Chem. Soc.* **1984**, *106*, 7959.
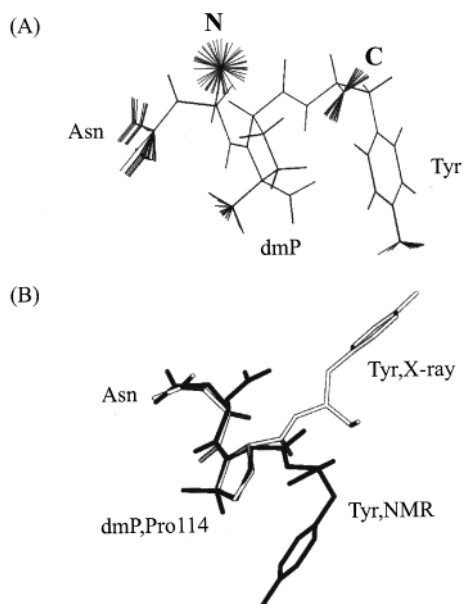
Figure 6. Ensemble of 10 structures of cNdmPY (A) and superposition of that NMR structure which was closest to the average on the 113–115 NPY fragment of the X-ray structure[36] of RNase A (B). The N-terminus of cNdmPY is CH_3CO, and the C-terminus is COO^-. White and black models in (B) represent the 113–115 stretch of the X-ray structure[36] and the NMR structure, respectively. N and C refer to the N- and C-termini of the fragments.
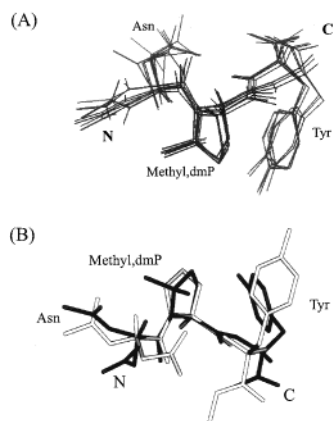
Figure 7. Ensemble of 10 structures of tNdmPY (A) and superposition of that NMR structure which was closest to the average on the X-ray structure[25] of the isolated NPY peptide (B). The N-terminus of tNdmPY is CH_3CO, and the C-terminus is COO^-. White and black models in (B) represent the X-ray fragment and the NMR structure, respectively. N and C refer to the N- and C-termini of the fragments.

evident in all three peptides with the same degree of ring stacking. The structures of YdmPN are consistent with the widely different $J_{H\alpha H\beta}$ coupling constants (10.4 and 5.4 Hz), which indicate that there is not much flexibility of the aromatic rings and the side chain is quite rigid, since most of the $J_{H\alpha H\beta}$ coupling constants from similar size peptides fall between 6 and 8 Hz. The $H^{\epsilon 1}$ of dmP is close to the Tyr ring preceding dmP in YdmPN, while the $H^{\epsilon 2}$ of dmP is close to Tyr following dmP in both cNdmPY and tNdmPY, which are consistent with the NOEs and with the stereospecific assignments of the dmP ring protons.

Type VI turns are common in proteins with a cis proline residue. There are two sets of type VI turns in proteins, type VIa (hydrogen-bonded) and VIb (non-hydrogen-bonded) with dihedral angles ψ close to ~0 and 150°, respectively.[44] The values of ψ of dmP in YdmPN and cNdmPY are ~ 24.9° and

−23.3°, respectively, in the structures shown in Figures 5A and 6A. Even though the temperature coefficients were not significantly low, suggesting no hydrogen bonding, these values suggest that there is some type VIa turn character in these peptides. Yao at al. found a similar kind of turn in a longer peptide with a proline in the cis form.[61]

Finally, to examine the dependence of the structures on the NMR constraints, 500 steps of EM without any NOE and dihedral angle constraints were applied to the 10 best structures. The minimized structures without any experimental constraints, especially the backbone heavy atoms, did not deviate much from the best starting structures, indicating that the best structures with NOE constraints were, indeed, close to their low-energy state. The backbone RMSDs of each of the newly minimized 10 structures of YdmPN, cNdmPY, and tNdmPY, with respect to the previous average structure, are 0.159 ± 0.011, 0.196 ± 0.100, and 0.385 ± 0.048 Å, respectively. The ring stacking between Tyr and dmP was maintained, but the ring of Tyr moved out to a greater extent from dmP than in the initial best structures of YdmPN, cNdmPY, and tNdmPY. The type VIa turn was maintained in both the YdmPN and cNdmPY peptides.

Comparison between Calculated Structures and Corresponding Stretches from the X-ray Structure of the Bovine RNase A. We superimposed the best structures of YdmPN and the cNdmPY from the calculations onto the corresponding stretches, 92–94 and 113–115, respectively, in the RNase A X-ray crystal structure. The RMSDs of the backbone atoms are 0.162 and 0.652 for YdmPN and cNdmPY, respectively. In YdmPN, the side chains of all residues preceding and following dmP have similar interactions and positions with respect to the side chains of the residue preceding and following Pro in wild-type RNase A, respectively (Figure 5B). In cNdmPY, the side chains of the Asn residue have a conformation similar to the corresponding X-ray conformation, but the side chains of the Tyr residues point in opposite directions. In the NMR structure of the cNdmPY tripeptide, the side chain of Tyr is close to that of dmP, whereas the Tyr115 side chain of RNase A is positioned far away from Pro114 and close to the side chain of Tyr73 in the X-ray structure. This is most likely due to the presence of additional interactions of the side chain of Tyr in NPY with neighboring residues in the whole protein which are absent in the tripeptides.

Conclusion

For the reasons stated in the Introduction, it is desirable to have a proline derivative for which the X-Pro peptide group is locked in the cis conformation. The experiments presented here demonstrate that L-dmP satisfies this requirement completely for YdmPN and largely (but not completely) for NdmPY. Therefore, YdmPN, but not NdmPY, is suitable for the incorporation into RNase A.

This investigation was facilitated by the development of procedures to separate L-dmP from its D-isomer, and to couple the sterically hindered L-dmP residue as the central residue between two naturally occurring amino acids in good yields. This will enable these tripeptides to be easily incorporated into RNase A by fragment coupling, using a synthetic procedure[64] that was developed for this purpose.

The solution structures of YdmPN and NdmPY have been determined by 2D ^1H NMR measurements. The ensembles of calculated 3D structures satisfy the available distance and dihedral angle constraints. YdmPN is observed to adopt a

(64) Welker, E.; Scheraga, H. A. *Biochem. Biophys. Res. Commun.* **1999**, *254*, 147.

completely cis conformation over the temperature range from 6 to 60 °C, whereas NdmPY is mostly cis, with the trans conformation increasing from about 10% to about 21% as the temperature increased from 6 to 80 °C. Based on $d_{\alpha N}(i,i+2)$ NOEs, both tripeptides have a high population of a type VIa reverse-turn conformation, similar to that in native RNase A; i.e., the dmP residue adopts the same backbone structure as proline and, therefore, can serve as a substitute for proline. Turn formation is thought to be an initial event in protein folding,[3] e.g., in chain-folding initiation sites.[13]

Compared to these dmP-related tripeptides, corresponding peptides containing proline have a lower percentage of cis conformation, e.g., YPN (29%),[65] SYPNDV (40%),[61] NPY (13%),[65] and SNPYDV (7.3%).[61] Presumably, the high preference for the cis conformation in the dmP peptides arises from steric interaction of the two methyl groups on the δ-carbon atom of the dmP residue with the preceding residue.

(65) Stimson, E. R.; Montelione, G. T.; Meinwald, Y. C.; Rudolph, R. K. E.; Scheraga, H. A. *Biochemistry* **1982**, *21*, 5252.

Finally, the NMR structure of YdmPN is compatible with that of YPN in RNase A, the NMR structure of cNdmPY is only partially compatible with that of NPY in RNase A (presumably because of interactions with other residues in the proteins), and the NMR structure of tNdmPY is compatible with the trans-NPY fragment.

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