Equilibrium Ratios of *cis*- and *trans*-Proline Conformers in Fragments of Ribonuclease A from Nuclear Magnetic Resonance Spectra of Adjacent Tyrosine Ring Resonances†

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ABSTRACT: In a preliminary attempt to acquire direct spectroscopic evidence for the involvement of cis–trans isomerism about X–Pro peptide bonds in protein folding, we have obtained the 600-MHz proton nuclear magnetic resonance spectrum of a tryptic fragment of performic acid oxidized bovine pancreatic ribonuclease A. This fragment, O-T-16, corresponds to residues 105–124 of ribonuclease A and contains a proposed hydrophobic "nucleation" site, i.e., a site that initiates and directs protein folding. It also contains two proline residues, one of which, in the sequence Asn^{113}-Pro^{114}-Tyr^{115} (sequence numbers for fragments refer to the corresponding sequence numbers in intact ribonuclease A), may be involved in cis–trans isomerism during folding. To facilitate interpretation of the spectrum of O-T-16, we examined the conformational properties of Asn^{113}-Pro^{114}-Tyr^{115} and the reverse sequence Tyr^{92}-Pro^{93}-Asn^{94}, which also occurs in ribonuclease A. For this purpose, various terminally blocked model compounds related to these sequences were synthesized and studied by ^{13}C and ^{1}H NMR (nuclear magnetic resonance) spectroscopy. In all of these model compounds, the resonances of the Tyr phenyl ring have chemical shifts that reflect the same cis content of X–Pro peptide groups as that determined from the resonances of the pyrrolidine ring of proline. Using the Tyr ring protons as probes, we found the equilibrium cis–trans content of Asn^{113}-Pro^{114} in O-T-16 to be ~12% cis; in native ribonuclease A, the Asn^{113}–Pro^{114} peptide bond is exclusively in the cis conformation. The cis content about the Asn–Pro peptide bond in O-T-16 (12 ± 1% cis) is similar to that measured in Ac-Asn-Pro-NHMe (14 ± 2% cis) and Ac-Asn-Pro-Tyr-NHMe (13 ± 1% cis). The same short-range interactions that determine the cis–trans equilibrium in these model compounds appear to determine this equilibrium in the 20-residue fragment O-T-16. In native ribonuclease A, both Asn^{113}-Pro^{114}-Tyr^{115} and Tyr^{92}-Pro^{93}-Asn^{94} occur in type VI β bends, with a *cis*-proline peptide bond at Asn-Pro and Tyr-Pro, respectively. The small amounts of cis isomer detected in the corresponding model compounds and in the Asn^{113}-Pro^{114} peptide bond of O-T-16 indicate that there is little or no overall native structure in these fragments. Long-range interactions involving additional residues other than 105–124 apparently are required to convert the Asn^{113}–Pro^{114} peptide bond completely to the cis conformation of native ribonuclease A. Preliminary measurements, however, suggest that one or both of the *trans forms* of Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe have β-bend conformations in water. This suggests that proline may be in either the cis or trans conformation for folding since either may allow the formation of the β bend that is necessary for a compact β-sheet structure to form in the hydrophobic initiation site(s).

Cis–trans isomerism at X–Pro[1,2] peptide bonds may be involved in protein folding (Brandts et al., 1975), but the evidence to support this hypothesis is largely circumstantial (Brandts et al., 1977; Lin & Brandts, 1978; Nall et al., 1978; Schmid & Baldwin, 1978; Babul et al., 1978; Stellwagen, 1979; Creighton, 1980; Garel, 1980a,b; Henkens et al., 1980; Schmid, 1981; Schmid & Blaschek, 1981). While these studies show that a rate-determining step in the folding kinetics of several proteins has the characteristics of proline isomerization, in none of these published studies has the equilibrium population of cis and trans conformers in an unfolded protein been measured directly.[3] This paper describes the first part of an attempt to obtain direct evidence for cis–trans isomerization of X-Pro in the folding of bovine pancreatic ribonuclease A.

By assuming that the important initial interactions on the refolding pathway(s) of ribonuclease A are short range and medium range and predominantly hydrophobic, Matheson & Scheraga (1978) suggested that Ile^{106}-Val^{118} and Lys^{61}-Glu^{111} are the primary and secondary segments, respectively, to form local structures that may direct subsequent folding events. Native ribonuclease contains four proline residues; those at positions 42 and 117 have trans conformations whereas those at positions 93 and 114 have cis conformations (Wyckoff et al., 1970; Richards & Wyckoff, 1971; Wlodawer et al., 1982). Whereas proline occurs in the cis conformation to some (small)

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[1] Abbreviations: Ac, acetyl; Me, methyl; HOBT, 1-hydroxybenzotriazole; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; AcONp, (4-nitrophenyl)acetate; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; MA, mixed anhydride; DME, 1,2-dimethoxyethane; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate; O-T-16, C-terminal 20 amino acid tryptic fragment of performic acid oxidized ribonuclease; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; T_1, spin–lattice relaxation time; δ, chemical shift; $\Delta\delta_{\alpha_1}$, chemical shift difference between cis and trans isomers; SSB, spinning side band; X-Pro, a dipeptide sequence containing any amino acid residue X and proline.

[2] Sequence numbers for fragments refer to the corresponding sequence numbers in intact ribonuclease A.

[3] Recently, Lin & Brandts [1981; summarized by Ooi & Scheraga (1982)] presented preliminary data on the cis/trans ratio for Pro^{93} in unfolded ribonuclease A by selective proteolytic cleavage of the trans form.

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extent in *short* peptides, it is unusual for it to adopt this conformation in proteins (Lewis et al., 1973; Chou & Fasman, 1977); presumably long-range interactions force it into the cis conformation in those proteins where it is found in this form. Both *cis*-prolines occur in type VI β bends (Lewis et al., 1973) in the native structure of ribonuclease A and are in the suggested "nucleation" (i.e., chain-folding initiation) sites. Furthermore, cis–trans isomerization about the peptide bonds at Asn^{113}-Pro^{114} and/or Tyr^{92}-Pro^{93} has been proposed as the rate-limiting step in the refolding of ribonuclease (Garel, 1980b; Schmid, 1981; Schmid & Blaschek, 1981).

Both ^{13}C and ^{1}H NMR spectroscopies have been used to investigate the cis–trans equilibrium about tertiary amide bonds (Madison & Schellman, 1970; Stewart & Siddall, 1970; Wüthrich & Grathwohl, 1974; Stimson et al., 1977). In small proline-containing peptides, the cis–trans equilibrium is affected by the nature of the residues preceding proline and by the solvent (Evans & Rabenstein, 1974; Grathwohl & Wüthrich, 1976a) and also by specific sequence-dependent interactions (Grathwohl & Wüthrich, 1976b). It is therefore reasonable to suspect that the cis/trans ratio may differ for Tyr^{92}-Pro^{93} and Asn^{113}-Pro^{114} in unfolded ribonuclease A. In this paper we use the NMR spectroscopic properties of adjacent tyrosine ring resonances as a probe for measuring the relative amounts of cis and trans conformers about peptide bonds involving specific prolines in fragments of ribonuclease that are large enough to exhibit medium-range interactions. This technique may also be applied to measurements on the whole protein at various stages of folding.

An easily obtainable and suitable segment of ribonuclease is O-T-16, a tryptic fragment that corresponds to residues 105–124 of performic acid oxidized ribonuclease A. It contains prolines in the sequences Asn^{113}-Pro^{114}-Tyr^{115} and Val^{116}-Pro^{117}-Val^{118}, both of which are included in the proposed nucleation site Ile^{106}-Val^{118}, with cis–trans isomerism of one of them (Pro^{114}) having been proposed as a rate-limiting step in folding (Garel, 1980b). The only tyrosyl residue in this fragment is Tyr^{115}, and an NMR spectroscopic probe of Tyr^{115} can provide information about cis–trans isomerism about the Asn^{113}–Pro^{114} peptide bond.

Toma et al. (1978, 1981) have observed that ^{1}H and ^{13}C nuclear magnetic resonances of tyrosyl side chains in Tyr-Pro-containing fragments of adrenocorticotropin can be used to measure the proline cis–trans equilibrium. In this paper, we demonstrate the utility of both ^{13}C and ^{1}H NMR spectroscopies of tyrosine ring nuclei for determining the cis–trans equilibrium in synthetic peptides and in O-T-16. We test this spectroscopic probe on the *N*-acetyl-*N'*-methyl amides of several peptides and then apply it to the proteolytic fragment O-T-16 to determine the cis–trans ratio about the Asn^{113}–Pro^{114} peptide bond. The corresponding peptide is terminally blocked Asn-Pro-Tyr. Although not present in O-T-16, we also examined Tyr-Pro-Asn because it is part of the secondary nucleation site (in Lys^{61}-Glu^{111}) and is possibly involved in a rate-limiting isomerization (at Tyr^{92}-Pro^{93}-Asn^{94}); in addition it has the reverse sequence of Asn-Pro-Tyr, and both tripeptides might have significant conformational similarities. The study of these tripeptides might also provide information about the tendencies toward incipient formation of specific structures that may be important in their roles in the nucleation sites. We did not prepare terminally blocked Val-Pro-Val, although it is present in O-T-16, because of its anticipated insolubility in water. This direct examination of X-Pro cis–trans isomerization by NMR spectroscopy (using the nuclear magnetic resonances of tyrosine as a probe) is being applied to the whole ribonuclease A molecule.

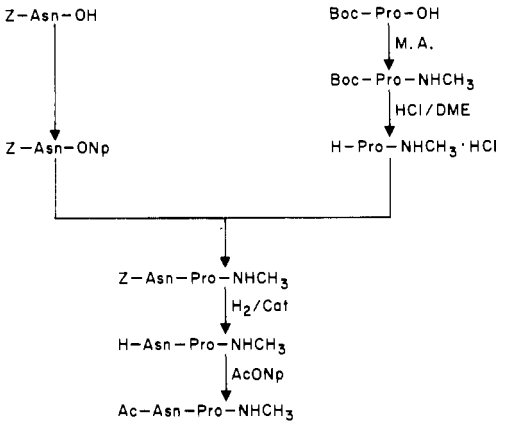

FIGURE 1: Schematic representation of synthesis of Ac-Asn-Pro-NHMe.

Materials and Methods

Materials. All reagents and solvents were of either analytical or spectroscopic grade. All amino acids were of the L configuration. HOBT, DCC, AcONp, and *N*-ethylmorpholine were obtained from Aldrich Chemical Co. and were purified before use. Boc-Tyr-OH was purchased from Bachem, Inc. Boc-Pro-OH was prepared by the method of Schnabel (1967). Z-Asn-OH was prepared by Dr. Francis Cardinaux, using the method of Boissonnas et al. (1955). Inorganic salts were reagent grade or better and were used without further purification. Dowex 50W-X2 was purchased from Sigma Chemical Co. Deuterated solvents were purchased from Aldrich Chemical Co.

Methods. The purity of the amino acid derivatives and peptides was checked routinely by TLC on Merck silica gel plates (F 254, 0.25 mm) in the following solvent systems: (a) chloroform–methanol, 9:1, (b) chloroform–methanol–acetic acid, 95:20:3, and (c) 1-butanol–pyridine–acetic acid–water, 4:1:1:2.

Melting points (uncorrected) were determined in a Thomas-Hoover apparatus. Specific rotations were measured with a Perkin-Elmer 141 polarimeter. NMR spectra of intermediates were obtained with a Varian 390 spectrometer and found to be in agreement with the proposed structures. Amino acid analyses were carried out with a Technicon TSM-1 autoanalyzer on samples that had been hydrolyzed in 6 N HCl in evacuated sealed ampules at 105 °C for 20–68 h. Microanalyses for C, H, and N compositions were carried out by Galbraith Laboratories.

Synthetic Methods. The peptides Ac-Asn-Pro-NHMe, Ac-Pro-Tyr-NHMe, Ac-Asn-Pro-Tyr-NHMe, Ac-Tyr-Pro-NHMe, and Ac-Tyr-Pro-Asn-NHMe were synthesized according to the schemes outlined in Figures 1–4. The final products were purified by chromatography on a Dowex 50W-X2 column (H^+ cycle), lyophilized from water, and recrystallized (except for Ac-Asn-Pro-NHMe, which was used as a foam directly after lyophilization). Whenever possible, all intermediates were isolated and characterized. The properties of the peptides are summarized as follows (see also paragraph at end of paper regarding supplementary material).

Ac-Asn-Pro-NHMe: foam; R_f 0.27 (solvent system b). Anal. Calcd for $C_{12}H_{20}N_4O_4 \cdot 0.5H_2O$: C, 49.18; H, 7.25; N, 19.12. Found: C, 49.50; H, 7.16; N, 19.04. Amino acid analysis: Asp, 1.00; Pro, 0.99.

Ac-Pro-Tyr-NHMe: mp 219–220 °C; $[\alpha]_D$ −60.3° (*c* 0.5, MeOH); R_f 0.32 (solvent system b). Anal. Calcd for $C_{17}H_{23}N_3O_4 \cdot 0.5H_2O$: C, 59.63; H, 7.06; N, 12.27. Found:

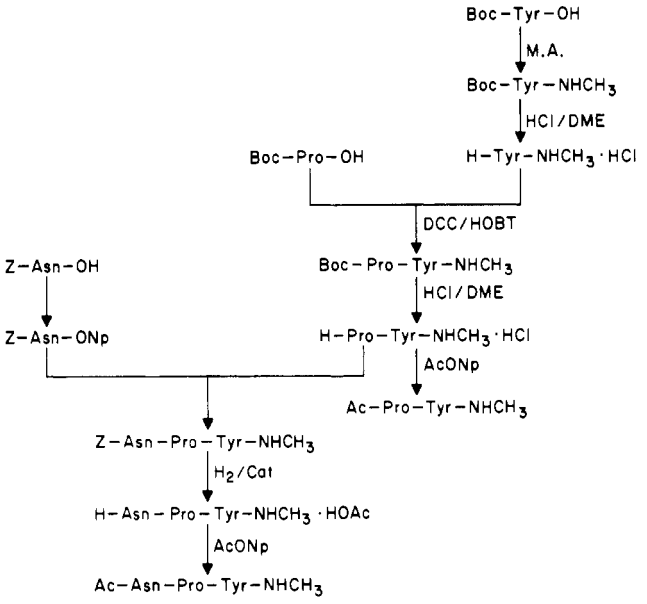

FIGURE 2: Schematic representation of synthesis of Ac-Pro-Tyr-NHMe and Ac-Asn-Pro-Tyr-NHMe.

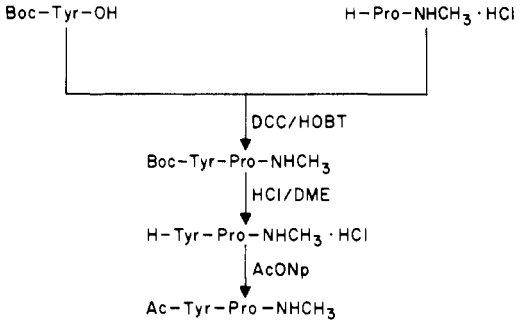

FIGURE 3: Schematic representation of synthesis of Ac-Tyr-Pro-NHMe.

C, 59.67; H, 7.31; N, 12.28. Amino acid analysis: Pro, 0.98; Tyr, 0.97; Cl-Tyr, 0.05.

Ac-Asn-Pro-Tyr-NHMe: mp 246–247 °C (recrystallized from EtOH), 256–257 °C (recrystallized from H_2O); $[\alpha]_D$ −102° (*c* 0.5, MeOH); R_f 0.12 (solvent system b), 0.54 (solvent system c). Anal. Calcd for $C_{21}H_{29}N_5O_6$: C, 56.36; H, 6.53; N, 15.65. Found: C, 56.09; H, 6.83; N, 15.39. Amino acid analysis: Asp, 0.99; Pro, 0.99; Tyr, 0.99; Cl-Tyr, 0.04.

Ac-Tyr-Pro-NHMe: mp 205–207 °C; $[\alpha]_D$ −33.7 (*c* 0.5, MeOH); R_f 0.48 (solvent system b). Anal. Calcd for $C_{17}H_{23}N_3O_4 \cdot H_2O$: C, 58.10; H, 7.17; N, 11.95. Found: C, 58.16; H, 7.23; N, 11.71. Amino acid analysis: Pro, 0.99; Tyr, 1.01.

Ac-Tyr-Pro-Asn-NHMe: mp 145 °C; R_f 0.22 (solvent system b); $[\alpha]_D$ −48.0° (*c* 0.3, MeOH). Anal. Calcd for $C_{21}H_{29}N_5O_6 \cdot 2.5H_2O$: C, 51.23; H, 6.95; N, 14.22. Found: C, 51.48; H, 7.11; N, 13.97. Amino acid analysis: Asp, 0.99; Pro, 0.97; Tyr, 1.04.

Preparation of O-T-16. Bovine pancreatic ribonuclease (type IIA) was purchased from Sigma Chemical Co. and was purified on carboxymethylcellulose columns by the method of Taborsky (1959). For prevention of formation of chlorotyrosine during the subsequent oxidation of ribonuclease, the purified protein was desalted in two steps; the pooled fractions were first passed through an Amberlite MB-1 anion/cation-exchange resin and then lyophilized at a concentration less than 10 mg/mL to avoid aggregation (Crestfield et al., 1963). Residual salts were then removed by gel filtration through Sephadex G-50. The pooled and lyophilized ribonuclease A

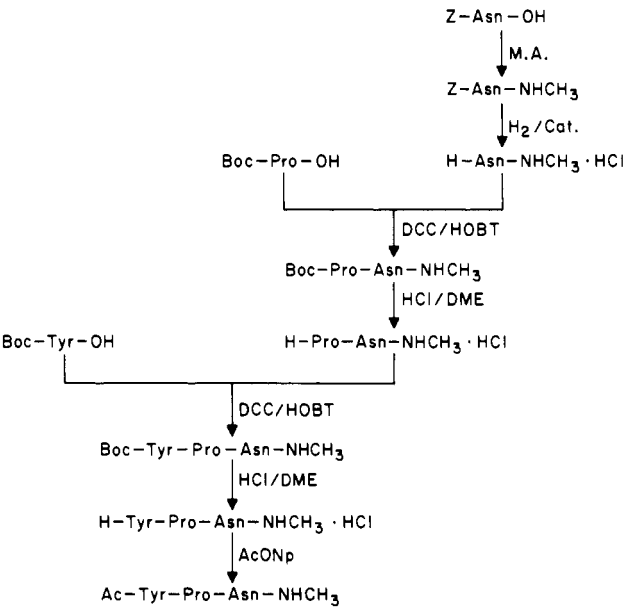

FIGURE 4: Schematic representation of synthesis of Ac-Tyr-Pro-Asn-NHMe.

was chloride free on the basis of a silver nitrate test. It exhibited a single peak when rechromatographed on carboxymethylcellulose and migrated as a single band on NaDodSO$_4$–polyacrylamide gel electrophoresis (Laemmli, 1970). This material was then oxidized at 0 °C with performic acid by the method of Hirs (1956) and lyophilized.

O-T-16 was prepared by a modification of the method of Hirs (Hirs et al., 1956; Hirs, 1960; Brown, 1962). About 800 mg of the oxidized protein was treated for 24 h with chymotrypsin-free trypsin (Sigma) in 0.2 M phosphate buffer, pH 7.0, at 25 °C. The ribonuclease and trypsin concentrations were 5 and 0.025 mg/mL, respectively. Proteolysis was quenched by lowering the pH to 2.3 with HCl. The 150-mL digest was then applied to a 39 × 2.5 cm jacketed column of Dowex 50W-X2 cation-exchange resin (in the NH_4^+ form) that had been equilibrated with 0.2 M ammonium formate at pH 3.08 and 35 °C. Most of the tryptic fragments were eluted by washing the column at ~1 mL/min with 750 mL of 2 M ammonium acetate at pH 5.1 and 35 °C, followed by a second wash with 750 mL of deaerated 2 M ammonium acetate at pH 5.6 and 25 °C. During these washes, the column bed shrank by ~15%. The column was then thermally reequilibrated at 50 °C, and the tightly bound O-T-16 was eluted at 1 mL/min with deaerated 2 M ammonium acetate buffer at pH 6.6 and 50 °C. The major peak from this last elution was pooled, diluted with distilled water, and lyophilized. This material was then desalted on a 44 × 2.4 cm Sephadex G-50 column, equilibrated with distilled water, and the material from the single peak was pooled and lyophilized. This fluffy lyophilizate obtained in ~45% yield (from purified ribonuclease A) exhibited a single spot on TLC with an R_f of 0.30 (solvent system c) and had the following amino acid composition: Cys-OH, 1.01 ± 0.10 (1); Asx, 2.04 ± 0.11 (2); Ser, 1.01 ± 0.07 (1); Glx, 1.04 ± 0.04 (1); Pro, 1.88 ± 0.08 (2); Gly, 1.09 ± 0.03 (1); Ala, 1.87 ± 0.06 (2); Val, 3.70 ± 0.1 (4); Ile, 1.96 ± 0.17 (2); Tyr, 0.95 ± 0.05 (1); Phe, 1.02 ± 0.01 (1); His, 1.92 ± 0.08 (2). The values for serine and tyrosine were obtained from timed acid hydrolyses on the assumption of first-order kinetics for their degradation, and the value for Ile was obtained by extrapolating to infinite hydrolysis time to take account of the unusual stability of the Ile–Ile peptide bond. The reported average deviations represent the ranges

of values about the mean or extrapolated values. There was *no* detectable cystine or chlorotyrosine.

Analytical Methods. Unless noted otherwise, concentrations of peptides were determined by micro-Kjeldahl nitrogen analysis (Lang, 1958; Noel & Hambleton, 1976a,b). Gel filtration of O-T-16 to determine its molecular weight was performed at 20 °C with a Sephadex G-100 superfine column (1.6 × 95 cm) (in 0.01 M sodium phosphate buffer, pH 7.0, at an initial peptide concentration of 3.5×10^{-4} M) with downward flow, calibrated with appropriate peptides of known molecular weight; a molecular weight of 2090 was obtained (theoretical 2213). A sedimentation equilibrium run (at the same concentration in the same solvent, at 17 °C) using a titanium rotor at 68 000 rpm gave a molecular weight of 1850. These results indicate that O-T-16 is unaggregated at the concentrations used in spectroscopic measurements ($\sim 2.5 \times 10^{-4}$ M in H_2O).

^{13}C NMR Spectroscopy. Peptides were prepared for natural abundance ^{13}C NMR measurements by dissolving them in D_2O at concentrations (by weight) in the range of 25–50 mg/mL (under N_2 to exclude O_2 where appropriate). The spectra were independent of concentration in this range. Unless described otherwise, all ^{13}C NMR measurements were made in 8 mm diameter tubes in a Varian CFT-20 spectrometer. At 20 MHz, 8000 data points over a 4000-Hz sweep width were sufficient to resolve most peaks. For chemical shift data, a 30° pulse was routinely followed by a 1-s accumulation time with no delay time; 40 000–100 000 transients were summed to obtain each spectrum.

The ^{13}C NMR measurements of Ac-Asn-Pro-Tyr-NHMe, Ac-Tyr-Pro-NHMe, and Ac-Tyr-Pro-Asn-NHMe were also made in 10 mm diameter tubes at 75.45 MHz on a Brüker WM-300 spectrometer to resolve peaks from the Tyr ring carbons of the cis and trans forms of these compounds. The same conditions were used as at 20 MHz, except that a large number of data points (16 000 over a 26 000-Hz sweep width) were taken.

At both 20 and 75.45 MHz, the probe temperature was 23 ± 2 °C (except where noted otherwise) and 0–1 Hz of exponential line broadening was used in the Fourier transformation. Wide-band-modulated proton decoupling, centered in the aliphatic region, was also used in all spectra. The approximate cis/trans ratios in the carbon spectra were determined by relative peak heights. In general, proton-decoupled ^{13}C NMR spectra cannot be used as a reliable quantitative measure of relative populations because of NOE and T_1 differences (Wüthrich, 1978); as indicated by consistency with cis/trans ratios computed from ^1H NMR spectra, however, these NOE and T_1 differences are small for the small peptides studied here. Chemical shifts were referenced externally to dioxane, which was assigned the value of δ 67.40 in aqueous solution.

600-MHz ^1H NMR Spectroscopy. Proton NMR spectra at 25 ± 2 °C in either D_2O or H_2O were obtained with 5-mm tubes on the 600-MHz spectrometer at Carnegie-Mellon University. Generally, 50–1000 scans were accumulated for each spectrum. The concentration range of model compounds in water was from 0.4 to 5 mg/mL, while that of O-T-16 was ~0.5 mg/mL. The spectra were independent of concentration in this range. The lock was the HOD signal from the solvent, which is 4.8 ppm from DSS at pD 6.6. Assignments were made by decoupling procedures.

In the case of the aliphatic protons, areas were integrated manually with a compensating polar planimeter on expanded spectra, while, in the aromatic and amide regions, ratios were determined directly from relative peak heights. Because it was often necessary to use resolution enhancement (with ~1 Hz of line broadening) to show the small shoulders on the main peaks in the tyrosine region, ratios of these areas are not as accurate as those obtained for the upfield (aliphatic) resonances. Nevertheless, agreement in cis/trans ratios calculated from enhanced aromatic resonances and nonenhanced aliphatic resonances was good.

Results

^{13}C NMR Data. The natural abundance ^{13}C chemical shifts of the carbon atoms of several terminally blocked model peptides, measured at 20 and 75.45 MHz in D_2O, pD ~6.6 and 25 °C, are presented in Table I. The chemical shifts of the carbonyl carbons were not assigned to individual residues. Those of the hydrogen-bearing carbons were assigned by comparison with other peptides in the nonordered conformation (Wüthrich, 1978), except for the nearly overlapping Asn C^α and Pro C^δ resonances of Ac-Asn-Pro-Tyr-NHMe, which appear in reverse order from those of Wüthrich (1978) and were assigned by comparison within the present series of compounds. The assignments for the Asn C^α and Pro C^δ resonances, however, are somewhat arbitrary and conceivably could be reversed.

The splitting of most resonances is attributed to cis–trans isomerism about the X–Pro peptide bond, and the percent of cis conformers in the model peptides, estimated from ^{13}C NMR, is given in Table II. These values are only estimates since there may be differences in T_1's and NOE's between the various carbons, and such differences would affect their relative areas (see, however, Materials and Methods).

The differences in chemical shifts between the β and γ carbons of proline, $\Delta\delta_{\beta\gamma}$, for the compounds of Table I are 5–6 ppm for *trans*-prolines and 9 ppm for *cis*-prolines.[4] Since these values are characteristic of *trans*- and *cis*-prolines, respectively (Siemion et al., 1975), it appears that the observed splittings are due to cis–trans isomerism about the X–Pro peptide bond and not, for example, to two populations of trans conformers.

As indicated in Tables I and II, at 20 MHz nearly every carbon of the terminally blocked peptides reflects the X-Pro cis–trans equilibrium. At 75.45 MHz the chemical shifts of the Tyr ring carbons of Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe also reflect the cis–trans equilibrium. An example of this splitting of aromatic carbon resonances is presented in Figure 5. When observed with a sufficient signal-to-noise ratio, the cis/trans ratio computed from the Tyr ring carbon resonances is in good agreement with that computed from other carbon resonances. Since these aromatic resonances fall in a relatively uncrowded region of the ^{13}C NMR spectrum of a typical protein, they may provide a relatively accessible probe of the cis conformation in unfolded ribonuclease A.

Since O-T-16 contains a large percentage of nonpolar residues [and, hence, according to Matheson & Scheraga (1978), may contain the primary nucleation site for the folding of ribonuclease], its solubility in water is very low (<1 mg/mL) and lower at an ionic strength of 0.1 M. Hence, no measurements of the natural abundance ^{13}C NMR spectrum could be made for O-T-16.

[4] The magnitude of $\Delta\delta_{\beta\gamma}$ varies linearly with the dihedral angle ψ of proline (Siemion et al., 1975). This might suggest that the values of ψ_{Pro} are the same for all cis compounds, and likewise for all trans compounds, in Table I. Shielding by the aromatic ring of tyrosine, however, may render the relation of Siemion et al. (1975) inapplicable to these compounds.

Table I: ^{13}C Chemical Shift Data (in ppm) for Terminally Blocked Model Peptides at 20 MHz in D_2O at pD ~6.6 and 25 °C

assignment	Ac-X-NHMe[a]	Ac-Asn-Pro-NHMe	Ac-Pro-Tyr-NHMe	Ac-Asn-Pro-Tyr-NHMe	Ac-Tyr-Pro-NHMe[b]	Ac-Tyr-Pro-Asn-NHMe[b]
C=O	174.44[c]	175.20[e]	174.97[e]	175.25[e]	n.o.[e]	n.o.[e]
	173.88[d]	174.52	174.68	174.64		
		172.02	174.34	174.44		
		174.86	174.13	174.05		
		173.64	174.01	172.55		
		171.92				
Tyr Ph C_1 (γ)	129.15[c]		129.07	129.33 (t), 128.85 (c)[f]	n.o.	129.08 (t), 128.18 (c)
$C_{2,6}$ (δ)	131.20[c]		131.18	131.16 (t), 131.27 (c)[f]	131.42[g]	131.36 (t), 131.43 (c)
$C_{3,5}$ (ϵ)	116.19[c]		116.19	116.19	116.34 (t), 116.54 (c)	116.21 (t), 116.54 (c)
C_4 (ζ)	152.22[c]		155.20	155.18	n.o.	n.o.
Pro C^α (t)[h]	61.13[d]	61.82[g]	60.96	61.67	61.79	61.64
(c)[h]	62.53[d]		61.98	~61.45	61.63	61.42
Tyr C^α (t)	56.34[c]		55.71[g]	55.66	53.83	53.97
(c)				~55.43	53.94	54.31
Pro C^δ (t)	49.38[d]	49.33	49.41	49.17[g]	47.80	48.70
(c)	47.78[d]	48.88	47.78		47.86	47.84
Asn C^α (t)		48.74		48.74		51.47
(c)		48.30		48.16		51.74
Tyr C^β (t)	37.10[c]		36.55[g]	36.85[f]	36.56	37.00
(c)				37.77[f]	38.31	38.06
Asn C^β (t)		36.92		36.33[g]		36.32
(c)		37.96				37.38
Pro C^β (t)	30.66[d]	30.12	30.26	29.85	29.96	29.88
(c)	32.29[d]	32.34	32.25	32.34	31.82	31.84
NMe (t)	26.45[c]	26.62	26.49[g]	26.62	26.80[g]	26.81[g]
(c)		26.82		~26.85		
Pro C^γ (t)	24.81[d]	25.10	24.81	24.85[g]	25.19	25.37
(c)	23.17[d]	22.56	23.13		22.29	22.38
acetyl CH_3 (t)	22.11[d]	22.27	22.19	22.25[g]	22.48	22.29
(c)	21.92[d]	22.36	21.60		26.68	26.74

[a] X is Tyr or Pro. [b] These spectra were obtained at 75.45 MHz and 30 °C. [c] Values from Ac-Tyr-NHMe. [d] Values from Ac-Pro-NHEt. [e] It was not possible to assign the carbonyl resonances in this column to specific amino acid residues; n.o., not observed. [f] At 75.45 MHz these peaks (due to cis and trans forms) could be resolved. [g] Cis and trans resonances could not be distinguished because of either overlap or insufficient signal-to-noise ratio. [h] *All* symbols (t and c) in this column refer to trans-cis isomerism about the X-Pro peptide bond.

Table II: Estimated Percent of Cis Conformers in Terminally Blocked Model Peptides from ^{13}C Data at 20 and 75.45 MHz in D_2O at pD ~6.6 and 25 °C

	percent of cis conformer about X-Pro peptide bond					
assignment	Ac-Pro-NHEt	Ac-Asn-Pro-NHMe	Ac-Pro-Tyr-NHMe	Ac-Asn-Pro-Tyr-NHMe	Ac-Tyr-Pro-NHMe[b]	Ac-Tyr-Pro-Asn-NHMe[b]
Asn C^α		*a*		14		30
Asn C^β		14		*a*		28
Pro C^α	24	*a*	25	13	35	28
Pro C^β	24	16	*a*	14	34	27
Pro C^γ	*a*	12	25	*a*	36	31
Pro C^δ	23	*a*	22	13	36	25
Tyr C^α			*a*	14	38	29
Tyr C^β			*a*	*a*	36	26
Tyr C^γ			*a*	*a*	*a*	32
Tyr C^δ				13	*a*	33
Tyr C^ϵ				13	43	29
acetyl C		13	25	*a*	27	29
NHMe C		11	*a*	11	*a*	*a*
average	24 ± 1	13 ± 1	24 ± 1	13 ± 1	36 ± 3	29 ± 2

[a] Cis and trans peaks are not sufficiently resolved or signal-to-noise ratio is too low for estimating these values. The percent cis calculated from ^{13}C data for Ac-Pro-NHEt agrees with that reported earlier (Stimson et al., 1977), viz. 24% from ^1H data. [b] Spectra obtained at 75.45 MHz, 30 °C.

^1H NMR Data. The ^1H NMR data acquired at 600 MHz are given in Table III for all compounds. The percent of cis conformers in the model compounds, estimated from ^1H nuclear magnetic resonances of proline, is given in Table IV. Where the data are available, the average values agree with those obtained by ^{13}C NMR (given in Table II).

As seen in Tables III and IV, all of the tyrosine and asparagine proton resonances, and in particular those of the ring protons, are sensitive to the cis–trans isomerism about the neighboring X–Pro peptide bond. Even though the tyrosine ring resonances for the cis and trans isomers overlap (see Figure 6B–D), resolution enhancement clears up this region somewhat. Hence, the percent of cis conformer could be computed from the tyrosine ring proton resonances and led to values in agreement with those determined from the proline ^1H and ^{13}C resonances.

The resonances of the tyrosine ring protons in Tyr-Pro-Y and X-Pro-Tyr sequences can be used to detect cis–trans isomerism about the X–Pro peptide bond, and the splitting pattern reflects the position of the Tyr residue relative to that of Pro. In Tyr-Pro-Y peptides (in which Tyr is the X group in the X–Pro peptide bond), the splitting between *cis*- and

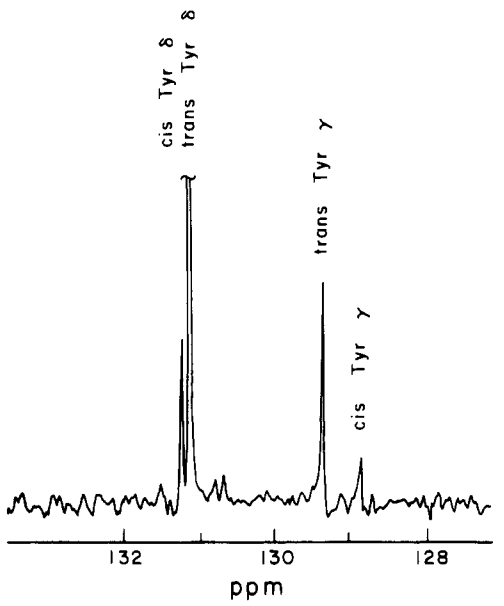

FIGURE 5: Tyr phenyl C^{δ} and C^{γ} region of the ^{13}C NMR spectrum (at 75.45 MHz) of Ac-Asn-Pro-Tyr-NHMe in D_2O at 25 °C.

trans-Tyr C^{δ} resonances, $\Delta\delta_{ct}$, is ~40 Hz at 600 MHz, while in the X-Pro-Tyr peptides $\Delta\delta_{ct}$ is only ~20 Hz. Although a similar correlation between the sequence and the magnitude of $\Delta\delta_{ct}$ cannot be made for the Tyr C^{ϵ} resonances, in Tyr-Pro-Y peptides the *cis*-Tyr C^{ϵ} resonances are at lower field than the *trans*-Tyr C^{ϵ} resonances, while in X-Pro-Tyr sequences they are at higher field. Thus, the *magnitude* of the Tyr C^{δ} splitting and the *direction* of the Tyr C^{ϵ} splitting can each be correlated with the position of Tyr relative to that of Pro [Figure 6; see also Figure 1 of Toma et al. (1978)]. This empirical observation may also be useful in assigning Tyr aromatic resonances in unfolded ribonuclease A, which contains both Tyr-Pro-Y and X-Pro-Tyr sequences.

Another region of the proton spectrum that is sensitive to the cis–trans equilibrium is that of the amide protons (Tables III and IV). In the terminally blocked tripeptides, the amide protons of both the peptide groups preceding and following the X–Pro peptide bond reflect the X-Pro cis–trans equilibrium. This is most clearly illustrated by Ac-Asn-Pro-Tyr-NHMe, where even the Asn side-chain amides accurately reflect the equilibrium about the Asn–Pro peptide bond.

With the information from the model compounds, we now consider the 600-MHz proton spectrum of O-T-16. The regions of the Tyr ring and Pro C^{δ} resonances are illustrated in Figures 7 and 8B, respectively. As in the model compounds, the aromatic proton resonances of a Tyr following Asn–Pro are useful probes of the cis–trans equilibrium. Both the chemical shifts and the relative intensities of the Tyr ring proton resonances of O-T-16 are the same as those in Ac-Asn-Pro-Tyr-NHMe (see Tables III and IV). Hence, we assign the larger downfield Tyr C^{δ} and Tyr C^{ϵ} doublets to conformations with *trans*-Pro^{114} and the upfield set of doublets to those with *cis*-Pro^{114}. In O-T-16 the relative positions of the cis and trans resonances of Tyr C^{ϵ}, and the ~20-Hz chemical shift difference between the *cis*- and *trans*-Tyr C^{δ} resonances, are characteristic of a Tyr in a position following Pro; i.e., in the sequence Asn-Pro-Tyr (cf. Figure 7 and Figure 6B). On this basis, the Tyr^{115} aromatic resonances of O-T-16 reflect the cis–trans equilibrium about the Asn^{113}–Pro^{114} peptide bond, rather than about the Val^{116}–Pro^{117} peptide bond.

In the aliphatic region of the proton spectrum of O-T-16, the signal-to-noise ratio is too low to enable us to distinguish *cis*-Pro C^{δ} (or minor-peak) resonances (Figure 8B). Since the spectra of O-T-16 (Figure 8B) and of Ac-Asn-Pro-Tyr-NHMe (Figure 8C) resemble each other in the Pro C^{δ} region, we tentatively assign the downfield Pro C^{δ} multiplets of O-T-16 primarily to the trans conformer(s) of Pro^{114}. The remaining two sets of Pro C^{δ} multiplets are tentatively assigned to *trans*-Pro^{117}.

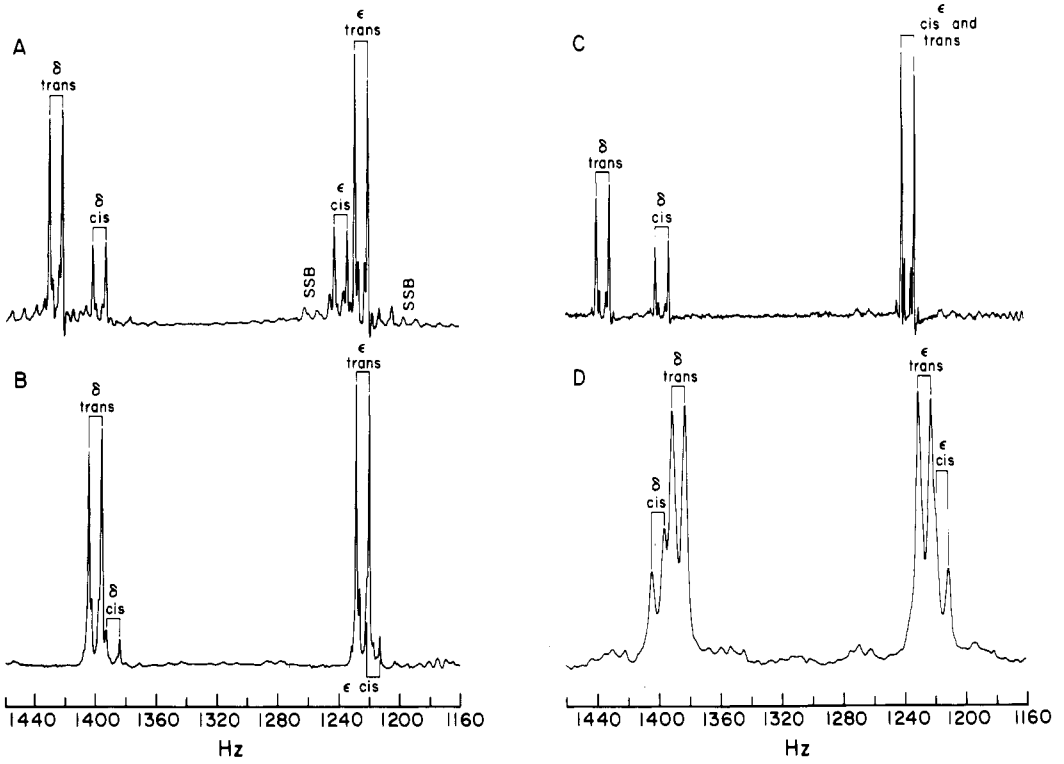

FIGURE 6: ^1H NMR spectra at 600 MHz (in hertz downfield from HOD lock) of Tyr ring proton region, in D_2O at 25 °C, of (A) Ac-Tyr-Pro-Asn-NHMe, (B) Ac-Asn-Pro-Tyr-NHMe, (C) Ac-Tyr-Pro-NHMe, and (D) Ac-Pro-Tyr-NHMe. The spectra in (A–C) but not in (D) were resolution enhanced.

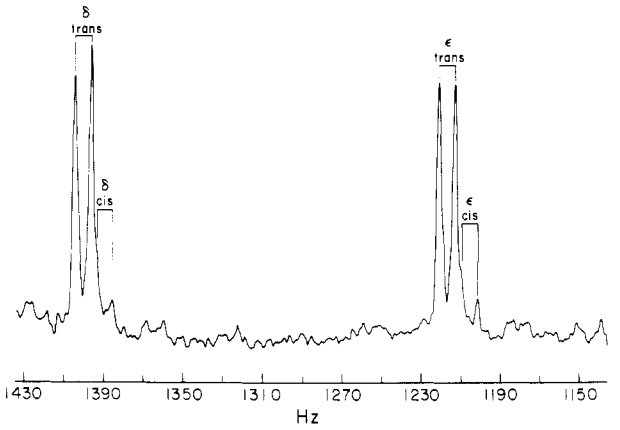

FIGURE 7: ^{1}H NMR spectrum at 600 MHz (in hertz downfield from HOD lock) of the Tyr ring proton region of O-T-16 in D_2O at 25 °C.

Discussion

Both ^{13}C and ^{1}H NMR spectra of nuclei in the neighborhood of proline can detect cis–trans isomerism about the X–Pro peptide bond in the model compounds studied here. Furthermore, the percentage of cis isomer, estimated from both the ^{13}C and ^{1}H NMR spectra, is the same. The observed percentages are independent of concentration and therefore do not reflect intermolecular interactions. This information is obtainable not only from the proline resonances but also from those of neighboring asparagine and tyrosine residues. Toma et al. (1978, 1981) previously used the Tyr ring as a probe of the cis–trans equilibrium in Tyr-Pro-Y fragments. Recently, Grathwohl & Wüthrich (1981) studied the kinetics of the pH-induced cis–trans isomerization of H-Tyr-Pro-OH using the tyrosine ring protons. Here we have extended these observations to X-Pro-Tyr sequences and have compared the values of the cis content in model compounds measured by Tyr aromatic resonances and by Pro resonances.

The kinetics of cis–trans isomerization of an X–Pro peptide bond have been followed spectrophotometrically by the change in pK_a of an adjacent ε-nitrotyrosine (Garel & Siffert, 1979; Garel, 1980a,b) and by the quenching of Tyr fluorescence (Henkens et al., 1980; Schmid, 1981) during folding. The relative amplitudes of bands corresponding to fast- and slow-folding species in these kinetics experiments provide an indirect estimate of the equilibrium cis–trans ratio in the unfolded protein. In NMR spectroscopy (in the slow-exchange limit), the cis/trans ratio is given by the ratio of resonance intensities. In contrast to the kinetic methods, NMR spectroscopy thus provides a direct method for determining cis/trans ratios about particular X–Pro peptide bonds either in the whole (unfolded) protein or in fragments thereof.

While di- and tripeptides containing proline generally consist of a mixture of cis and trans isomers, several larger peptides are devoid of cis conformers in water, e.g., oxytocin, lysine-vasopressin, [Ile^3]angiotensin II, tuftsin, luteinizing hormone-releasing hormone, and the fibrinogen fragment H-Gly-Val-Arg-Gly-Pro-Ala-NH_2 (Smith et al., 1972; Blumenstein et al., 1979; Deslauriers et al., 1973; Von Dreele et al., 1978). On the other hand, as demonstrated here, O-T-16 contains some cis isomer in D_2O. Similarly, fragments of corticotropin as large as 32 residues contain measurable amounts of cis conformer (Toma et al., 1978, 1981). The essential absence of cis conformers from several oligopeptides cannot be attributed to a dependence of the cis–trans ratio on chain length. Instead, it appears that specific interactions among the neighboring groups of X-Pro (and/or longer range interactions) determine the cis content in these various peptides.

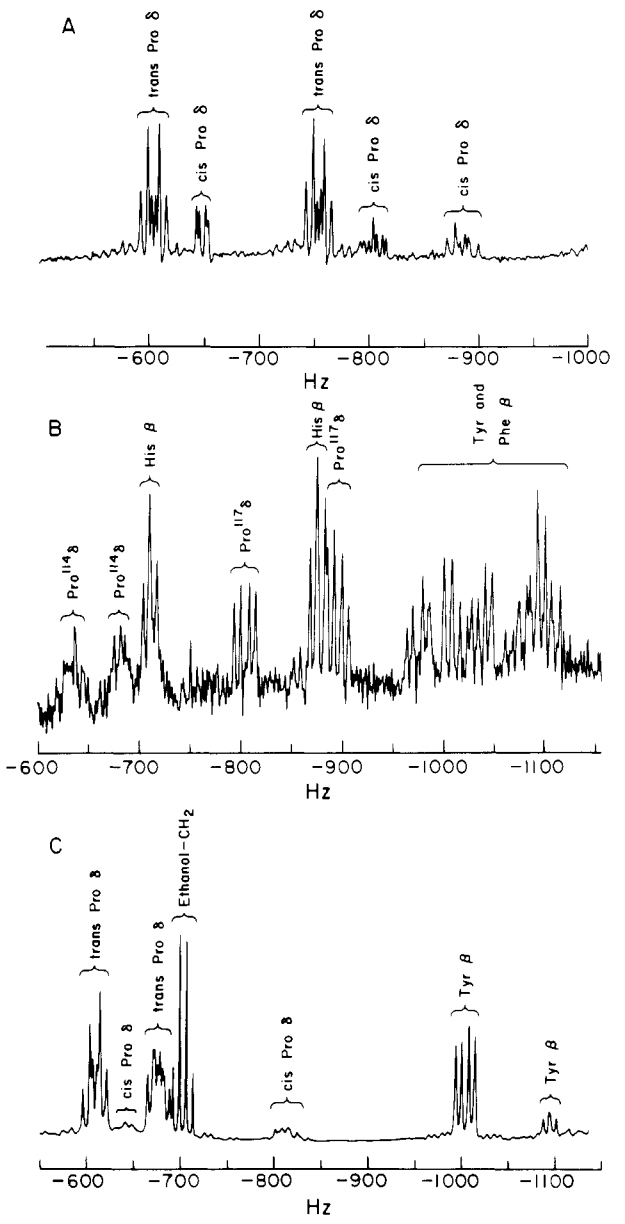

FIGURE 8: ^{1}H NMR spectra at 600 MHz (in hertz downfield from HOD lock) of the Pro C$^\delta$ region of (A) Ac-Tyr-Pro-Asn-NHMe, (B) O-T-16, and (C) Ac-Asn-Pro-Tyr-NHMe in D_2O at 25 °C. In (C), the ethanol resonance arises from residual solvent used in recrystallization.

In water the percentage of cis conformer in Ac-Tyr-Pro-Asn-NHMe (27 ± 2%) is significantly lower than in Ac-Tyr-Pro-NHMe (36 ± 3%); local interactions due to the incorporation of Asn into the Tyr-Pro-Y sequence favor the trans conformer. For the X-Pro-Tyr peptides Ac-Pro-Tyr-NHMe (24 ± 1%), Ac-Asn-Pro-Tyr-NHMe (13 ± 1%), and Ac-Asn-Pro-NHMe (14 ± 2%), the cis–trans equilibrium is determined predominantly by the nature of the residue preceding Pro, as has been observed with other X-Pro peptides (Grathwohl & Wüthrich, 1976a). In particular, incorporation of Tyr in position Y of the Asn-Pro-Y sequence has little effect on the Asn-Pro cis–trans equilibrium.

Several features of the proton chemical shifts and coupling constants for Ac-Asn-Pro-Tyr-NHMe (Table III) suggest some degree of structural order in the major (i.e., trans) conformer in water. For example, the NHMe amide proton resonance (2.68 ppm, relative to HOD) is upfield from that

Table III: ^{1}H Chemical Shift Data at 600 MHz for Terminally Blocked Peptides in D_2O at pD ~6.6 and 25 °C[a]

proton assignment	Ac-Asn-Pro-NHMe δ[b]	cp[b]	^3J[b]	Ac-Pro-Tyr-NHMe δ	cp	^3J	Ac-Asn-Pro-Tyr-NHMe δ	cp	^3J	O-T-16[c] δ	cp	^3J	Ac-Tyr-Pro-NHMe δ	cp	^3J	Ac-Tyr-Pro-Asn-NHMe δ	cp	^3J
from D_2O																		
Asn H^α (t)[b]	0.16	t					0.14	t		*d*						*d*		
(c)[b]	*d*						*d*			*d*						*d*		
Asn H^β (t)	−2.15	dd	7.83				−1.99	dd	9.72	*d*						−2.02	m	
	−2.00	dd	6.53				−2.11	dd	6.84							−2.07	m	
(c)	−2.22	dd	6.53				−2.21	m		*d*						−2.14	m	
	−2.05	dd	8.09															
Pro H^α (t)	−0.44	dd	7.05	−0.49	dd	4.59	−0.46	dd	4.68	*d*			−0.48	dd	5.48	−0.41	dd	
															5.22			
(c)	*d*			−0.40	dd	3.06	−0.43	t	5.04	*d*			−0.31	dd	6.03	−0.32	dd	
Pro H^β (t)	−2.56	m		−2.64	m		−2.65	m		*d*			−2.61	m		−2.55	m	
				−3.00	m		−3.20	m										
(c)	−2.50	m		~−2.50	m		−2.57	m		*d*			−3.16	m		*d*		
Pro H^γ (t)	−2.85	m		−3.05	m		−2.89	m		*d*			−2.87	m		−2.87	m	
				−2.89	m		−3.11	m										
(c)	*d*			*d*			*d*			*d*			*d*			*d*		
Pro H^δ (t)	−0.98	dt	7.05	−1.23	t	6.63	−1.00	m	7.56	−1.06[e]	m		−1.00	dt	7.05	−1.26	dt	6.69
	−1.07	dt	6.53				−1.12	m		−1.14[e]	m		−1.30	dt		−1.00	dt	
																−1.08	m	
(c)	−1.21	m		−1.37	dd	4.59	−1.07	m	5.40	−1.34[f]	m		−1.18	m		−1.34	m	
	−1.32	m					−1.35	m		−1.48[f]	m		−1.49	m		−1.47	m	
																−1.26	dt	5.78
Tyr H^α (t)				−0.33	t		−0.32	dd		*d*			*d*			−0.21	dd	
(c)				−0.22	dd		~−0.15			*d*			*d*			−0.16	m	
Tyr H^β (t)				−1.75	dd	6.89	−1.66	dd	6.48	*d*			−1.77	dd	6.26	−1.73	dd	5.54
				−1.86	dd	8.16	−1.93	dd	7.92				−1.95	dd		−1.97	dd	
(c)				−1.71	dd	6.12	−1.81	t	7.56	*d*			−1.86	dd	6.26	*d*		
				−1.93	dd	9.69												
Tyr H^δ (t)				2.31	d	8.38	2.34	d	8.46	2.34	d	8.50	2.39	d	8.67	2.38	d	8.45
(c)				2.33	d	8.38	2.32	d	8.46	2.31	d		2.33	d	8.67	2.33	d	8.45
Tyr H^ϵ (t)				2.04	d	8.38	2.05	d	8.46	2.03	d	8.26	2.06	d	8.67	2.04	d	8.45
(c)				2.03	d	8.38	2.03	d	8.46	2.01	d		*d*			2.06	d	8.45
acetyl (t)	−3.13	s		−2.71	s		−2.81	s					−2.89	s		−2.90	s	
(c)	−2.85	s		−3.18	s		−2.88	s					−2.84	s		−2.83	s	
NMe (t)	−2.09	s		−2.15	s		−2.12	s					−2.10	s		−2.06	s	
(c)	−2.03	s		−2.11	s		−2.18	s					−2.13	s		−2.11	s	
from H_2O																		
N*H*Me (t)	3.09	q		2.86	q	~4.4	2.68	m	4.64				2.58	x		2.99	q	
(c)	3.23	q		3.11	q	~4.4	2.80	m					2.17	x		3.11	x	
Asn-N*H* (t)	3.60	d	7.18				3.57	d	6.78	*d*						3.52	d	7.31
(c)	3.38	d	7.35				3.47	d	6.78	*d*						3.68	d	7.03
Asn-CONH_1 (t)	2.17	s					2.19	s		*d*						2.12	s	
(c)	2.08	s					*c*			*d*						*d*		
Asn-CONH_2 (t)	2.87	s					2.86	s		*d*						2.78	s	
(c)	2.79	s					3.02	s		*d*						2.66	s	
Tyr-N*H* (t)				3.31	d	7.45	3.15	d	7.85	*d*			3.43	x		3.40	d	7.31
(c)				3.67	d	8.54	3.29	d	7.14	*d*			3.19	x		3.08	x	
Tyr-O*H*				*g*			4.85	s		*g*								

[a] An internal reference standard was not added to these solutions because it would have interacted with the solute. Hence, for the conversion of the reported chemical shifts to values relative to DSS, 4.80 ppm should be added. Here (−) designates upfield from the proton resonance of HOD. [b] δ, chemical shift in ppm; cp, coupling pattern; ^3J, three-bond coupling constant, in hertz; t, trans conformer; c, cis conformer. The coupling patterns are s (singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), m (multiplet), and x (broadened by amide exchange). In D_2O, the observed coupling pattern assignments are as follows: Asn H^β, $^3J_{\alpha\beta}$; Pro H^α, $^3J_{\alpha\beta}$; Pro H^δ, $^3J_{\gamma\delta}$; Tyr H^β, $^3J_{\alpha\beta}$; Tyr H^δ, $^3J_{\delta\epsilon}$; Tyr H^ϵ, $^3J_{\delta\epsilon}$. In H_2O, the coupling constants for Tyr-N*H* and Asn-N*H* are $^3J_{NH-C^\alpha H}$ and are characteristic of the corresponding average dihedral angles, ϕ. [c] The pD of O-T-16 was 5.2. [d] Not observed because peak was obscured by other overlapping peaks. [e] Assigned to *trans*-Pro^{114} by analogy with Ac-Asn-Pro-Tyr-NHMe. [f] Although these are listed on the cis line, they are tentatively assigned to *trans*-Pro^{117}. [g] Not observed because of exchange broadening.

of Ac-Tyr-NHMe (2.80 ppm, not shown in Table III) and of Ac-Pro-Tyr-NHMe (2.86 ppm). The Tyr amide proton resonance (3.15 ppm) of Ac-Asn-Pro-Tyr-NHMe is also upfield from that of Ac-Pro-Tyr-NHMe (3.31 ppm). These observations suggest that the backbone amide protons of the Tyr and NHMe groups are shielded from the solvent and possibly are involved in hydrogen bonds. Also, the resonances of the methyl protons of the acetyl group (−2.81 ppm) are downfield from those of Ac-Asn-Pro-NHMe (−3.13 ppm), suggesting that they are in the local magnetic field of a carbonyl or phenyl ring in Ac-Asn-Pro-Tyr-NHMe. Taken together, these observations *suggest* that the NHMe and/or Tyr-NH amide protons may be hydrogen bonded to the carbonyl group of the Ac-Asn peptide bond in the same molecule. In addition, the coupling constant $^3J_{NH-C^\alpha H}$ of Tyr in Ac-Asn-Pro-Tyr-NHMe (7.85 Hz) indicates that the average dihedral angle ϕ_{Tyr} is −155°, −85°, 45°, or 75° (Bystrov, 1976). Since ϕ_{Pro} is constrained to be −75° (Momany et al., 1975), this value of $^3J_{NH-C^\alpha H}$ for Tyr is consistent with the presence of a type I or II β bend with ϕ_{Tyr} being approximately −90° or 80°, respectively (Lewis et al., 1973), in the Pro-Tyr portion of *trans*-Ac-Asn-Pro-Tyr-NHMe.

The behavior of the side chains of Ac-Asn-Pro-Tyr-NHMe also indicates some degree of ordered structure in the trans

Table IV: Percent of Cis Conformers in Water Determined by 600-MHz Proton NMR at pD ~6.6 or pH ~6.3 and 25 °C

proton assignment	percent of cis conformer about X-Pro peptide bond					
	Ac-Asn-Pro-NHMe	Ac-Pro-Tyr-NHMe	Ac-Asn-Pro-Tyr-NHMe	Ac-Tyr-Pro-NHMe	Ac-Tyr-Pro-Asn-NHMe	O-T-16[a]
from D_2O						
Asn H^{α}	*b*		*b*		*b*	
Asn H^{β}	13		*b*		*b*	
Pro H^{α}	*b*	28	*b*	35	25	
Pro H^{β}	14	23	*b*	*b*	*b*	
Pro H^{γ}	*b*	*b*	*b*	*b*	*b*	
Pro H^{δ}	13	25	12	36	*b*	
Tyr H^{α}		22	*b*	*b*	*b*	
Tyr H^{β}		25	12	36	*b*	
Tyr H^{δ}		25	14	37	25	11
Tyr H^{ϵ}		25	14	*b*	25	13
acetyl	16	25	14	38	25	
*N*Me	14	26	12	35	25	
from H_2O						
N*H*Me	16	26	12	30	26	
Asn-CON*H*	16		*b*		*b*	
Asn-CON*H*	16		13		15[c]	
Asn-N*H*	16		12		26	
Tyr-N*H*		24	12	33		
average	15 ± 1	25 ± 1	13 ± 1	35 ± 2	25 ± 1	12 ± 1

[a] The pD was 5.2 for this compound. [b] Peaks obscured by other overlapping peaks and therefore not observed. [c] Low value, attributed to line-broadening effects of exchange, not included in average.

conformer. The Tyr C^{β} protons (with resonances at −1.66 and −1.93 ppm) are nonequivalent (Figure 8C), suggesting that rotation about the C^{α}–C^{β} bond of the Tyr side chain is restricted. Conformational analysis (Pachler, 1964) based on the observed coupling constants ($^3J_{C^{\alpha}H\text{–}C^{\beta}H_1}$ = 6.48 Hz and $^3J_{C^{\alpha}H\text{–}C^{\beta}H_2}$ = 7.92 Hz) indicates that χ^1 is predominantly (~85%) gauche (±60°). The Asn C^{β} protons (with resonances at −1.99 and −2.11 ppm) are also nonequivalent, and conformational analysis based on their different $^3J_{C^{\alpha}H\text{–}C^{\beta}H}$ coupling constants (9.72 and 6.84 Hz) indicates that χ^1 of Asn is also ±60°. Thus, the proton NMR data provide evidence for restricted rotation about the C^{α}–C^{β} bonds of both the Asn and Tyr side chains of Ac-Asn-Pro-Tyr-NHMe. Similar restricted rotation of the side chains was observed in Ac-Pro-Tyr-NHMe and in Ac-Asn-Pro-NHMe. Further, while the Tyr phenolic proton is not observed in Ac-Pro-Tyr-NHMe (because of broadening due to rapid exchange with H_2O), it is observed in Ac-Asn-Pro-Tyr-NHMe; presumably, intramolecular interactions in the latter compound decrease the rate of exchange of the phenolic proton. This observation and the restricted mobility of the Tyr and Asn side chains suggest that there is a side-chain–side-chain hydrogen bond between the Tyr phenolic proton (donor) and the Asn side-chain amide (acceptor). All of the above conclusions can be accommodated by a molecular model that involves a β bend.

Because of the low propensity of open-chain peptides to form intramolecular hydrogen bonds in water, we must regard this proposed β-bend structure, with both side-chain–side-chain and backbone–backbone hydrogen bonds, for *trans*-Ac-Asn-Pro-Tyr-NHMe as tentative.

The NMR data suggest that Ac-Tyr-Pro-Asn-NHMe also has some degree of ordered structure in water. Both the cis and trans conformers have at least three Pro C^{δ} proton resonances; in each conformer, one of the Pro C^{δ} protons gives rise to two resonances (Table III). This may be due to conformational restrictions that reduce the rate of puckering of the pyrrolidine ring. The Asn C^{β} protons are nonequivalent in *trans*-Ac-Tyr-Pro-Asn-NHMe (−2.02 and −2.07 ppm), and the Tyr C^{β} protons are nonequivalent in the trans forms of both Ac-Tyr-Pro-NHMe (−1.77 and −1.95 ppm) and Ac-Tyr-Pro-Asn-NHMe (−1.73 and −1.97 ppm), indicating that the mobility of these side chains is restricted. These observations, and the fact that the cis content decreases from 36 to 27% in going from Ac-Tyr-Pro-NHMe to Ac-Tyr-Pro-Asn-NHMe, suggest that there is some degree of ordered structure in the cis and/or trans conformers of Ac-Tyr-Pro-Asn-NHMe in water. Further studies of the conformation(s) of cis and trans conformers of Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe in water are currently in progress (G. T. Montelione, E. R. Stimson, S.-G. Huang, Y. C. Meinwald, J. B. Denton, and H. A. Scheraga, unpublished results).

In O-T-16, the cis content of the Asn^{113}-Pro^{114} peptide group is the same as that observed in the Asn-Pro model compounds, viz. 12%. The same short-range interactions that determine the cis/trans ratio in the Asn-Pro-Y model compounds appear to dominate in determining the equilibrium ratio in the 20-residue tryptic fragment. Theoretical calculations (M. Oka and H. A. Scheraga, unpublished results) indicate that the Asn-Pro-Tyr sequence, with a cis peptide bond, has a very high probability of adopting a β-bend conformation at Pro-Tyr. On this basis, it appears that O-T-16 has some (≤12%) local native (i.e., type VI) β-bend structure at Asn^{113}-Pro^{114} but little or no overall native structure.

Because of its low solubility, no NMR measurements could be made on the ribonuclease A fragment 105–124 containing reduced cysteine in place of cysteic acid. Hence, we cannot rule out the possibility that the presence of cysteic acid in O-T-16 disrupts the native β-bend structure with a cis peptide bond. Since, however, Ac-Asn-Pro-Tyr-NHMe and Asn^{113}-Pro^{114} in O-T-16 have the same cis/trans equilibrium ratio, it is unlikely that cysteic acid at Cys^{110} disrupts cis structure that would otherwise be present.

The observation of only a small amount (≤12%) of native β-bend structure in the Asn^{113}-Pro^{114}-Tyr^{115} portion of O-T-16 does not rule out the possible presence of nonnative β bends (with *trans*-Pro^{114}). Indeed, both theoretical calculations and the X-ray structure of Ac-Asn-Pro-Tyr-NHMe (M. Oka, E. Arnold, E. R. Stimson, G. T. Montelione, J. C. Clardy, and H. A. Scheraga, unpublished results) reveal that, in the absence of solvent interactions, the peptide forms a β bend at

Pro-Tyr with a trans Asn–Pro peptide bond. Furthermore, the preliminary conformational analysis discussed above, based on proton chemical shifts and coupling constants, is consistent with the existence of a reverse-turn conformation in the trans form of Ac-Asn-Pro-Tyr-NHMe in water. If, as is presumed, the same interactions determine the cis/trans ratio in Asn^{113}-Pro^{114} in O-T-16 as in Ac-Asn-Pro-Tyr-NHMe, then it is likely that this tryptic fragment also contains a β bend at Pro-Tyr with a trans peptide bond. This implies that neither cis nor trans proline conformations are essential for the formation of the β bend at Asn^{113}-Pro^{114}-Tyr^{115}, since either may allow the formation of the β bend that is necessary to form a compact β-sheet structure in the hydrophobic nucleation site.

Since the Asn^{113}-Pro^{114} peptide group is ~12% cis in O-T-16 and 100% cis in native ribonuclease A, long-range interactions must be involved in converting the rest of Pro^{114} from trans to cis during folding. Theoretical calculations on fragments of ribonuclease A with nativelike conformations demonstrate that the isomerization of Pro^{114} can be accomplished with little deformation of the chain or motion of remote residues (M. R. Pincus, F. Gerewitz, H. Wako, and H. A. Scheraga, unpublished results). The experimental evidence presented here indicates that the trans conformer(s) is (are) more energetically favored in the solvated 105–124 fragment than is the cis conformer(s). Interactions with other parts of the chain must cause the cis conformation of Pro^{114} to become more favorable during the folding process. Such long-range interactions may arise from the F, K, and E contacts (i.e., interactions of residues 81–102 with residues 104–124) described by Némethy & Scheraga (1979). Studies in progress on fragments and on folding intermediates of ribonuclease A may provide further information about the initiation site(s) for chain folding and about the stage of folding at which proline isomerization occurs.

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Supplementary Material Available

Experimental procedures and properties of peptide intermediates (7 pages). Ordering information is given on any current masthead page.

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Kinetic and Hydrodynamic Analysis of Blood Clotting Factor V–Membrane Binding†

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ABSTRACT: The kinetics and hydrodynamic properties of factor V–membrane interaction were characterized. Factor V bound to membranes containing acidic phospholipids with a high collisional efficiency. For membranes of 20% phosphatidylserine–80% phosphatidylcholine, an association rate constant of $(1.13 \pm 0.10) \times 10^8$ M^{-1} s^{-1} was obtained. These membranes contained about 20 factor V binding sites per vesicle of 3.6×10^6 daltons. This association rate represented about a 30% collisional efficiency. Dissociation of factor V was measured by a fluorescence energy transfer method with a dissociation rate constant of 0.0055 s^{-1} at 10 °C. The equilibrium dissociation constant for binding to these membranes at 10 °C and 0.14 M ionic strength was 5×10^{-11} M. Ionic strength, pH, calcium, and charge density in the membrane had large effects on the rate of factor V–membrane dissociation, indicating a strongly ionic interaction between protein and membrane. In contrast, the association rate was nearly insensitive to ionic strength. The membrane-binding properties were relatively unchanged after thrombin digestion of factor V or after long-term protein storage which resulted in loss of procoagulant activity. Other proteins of the prothrombinase reaction greatly decreased the rate of factor Va–membrane dissociation. At protein saturation, factor V increased the hydrodynamic radius of phospholipid vesicles by 11.4 nm. In contrast, factor Va increased the hydrodynamic vesicle radius by only about 5 nm. The mass of membrane-bound protein was comparable for both proteins.

Blood clotting factors Va and Xa plus phospholipids form the enzymatic complex, prothrombinase, which converts prothrombin to thrombin [see Jackson & Nemersen (1980) for a review]. The substrate and enzyme of this reaction, prothrombin and factor Xa, are vitamin K dependent proteins and, in the presence of calcium, bind to membranes containing acidic phospholipids. Both the equilibrium and dynamic properties of these protein–membrane interactions have been studied extensively [see Wei et al. (1982) and references therein].

Factor V also associates with membranes. It is known that the factor V–membrane interaction requires acidic phospholipids but not calcium (Jobin & Esnouf, 1967; Bloom et al., 1979). Factor V binding to purified phospholipids (Bloom et al., 1979) appeared quite different from binding to platelets (Tracy et al., 1981), the presumed relevant biological membrane. Proteolysis by thrombin converts factor V to factor Va, a much more active form of the protein. The membrane-binding properties of factor V appeared to differ from those of factor Va (Bloom et al., 1979). Factor Va has been shown to enhance the binding of factor Xa to platelets or phospholipid vesicles (Miletich et al., 1978; Dahlbäck & Stenflo, 1978; Tracy et al., 1979). This tighter binding probably arises from simultaneous protein–protein and protein–membrane interactions.

The properties of factors V– and Va–membrane interaction were investigated further in order to better characterize this interaction and to determine the relative affects of protein activation on this association. Kinetic analysis revealed a rapid association, suggesting a facile protein–phospholipid recognition. No major differences between factors V and Va were apparent. Hydrodynamic measurements showed a major difference in the degree to which these proteins protrude from the membrane surface.

Materials and Methods

Highly purified phospholipids were purchased from Sigma Chemical Co. Single bilayer vesicles of defined composition were prepared by sonication and gel filtration according to standard procedures (Nelsestuen & Lim, 1977; Huang, 1969). Phospholipid concentration was determined by phosphate assay (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 25. Unless indicated, the buffer used was 0.05 M Tris–0.1 M NaCl (pH 7.5).

Dansyl-PE[1] was formed from 3 equiv of dansyl chloride and 1 equiv of dipalmitoylphosphatidylethanolamine in chloroform

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[1] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl, 8-(dimethylamino)-1-naphthalenesulfonyl; dansyl-PE, *N*-dansylphosphatidylethanolamine; Tris, tris(hydroxymethyl)aminomethane; Dnp, dinitrophenyl; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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