Chain-Folding Initiation Structures in Ribonuclease A: Conformational Free Energy Calculations on Ac-Asn-Pro-Tyr-NHMe, Ac-Tyr-Pro-Asn-NHMe, and Related Peptides

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Abstract: In order to evaluate the role of the X-Pro peptide bond conformation in early forming *chain-folding initiation structures* of ribonuclease A, conformational free energy calculations were carried out on Ac-Tyr-Pro-Asn-NHMe and Ac-Asn-Pro-Tyr-NHMe, which correspond to residues 92-94 and 113-115 of bovine pancreatic ribonuclease A, and on related tri- and dipeptide sequences. These calculations show that, when the X-Pro peptide bond is *cis*, both Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe adopt primarily ($P_Z > 0.95$) type VI β-bends (at X-Pro), similar to those observed in the corresponding sequences of native ribonuclease A. When the Tyr-Pro peptide bond is *trans*, Ac-Tyr-Pro-Asn-NHMe almost exclusively adopts extended backbone conformations since most bend conformations of this ensemble have energetically favorable but entropically unfavorable Tyr/Asn side-chain/side-chain hydrogen bonds. On the other hand, when the Asn-Pro peptide bond is *trans*, Ac-Asn-Pro-Tyr-NHMe has a relatively high bend probability (at Pro-Tyr) when conformational entropy is included in the calculation. This is attributable primarily to Asn side-chain interactions with the Pro-Tyr-NHMe backbone which can stabilize β-bends at Pro-Tyr. Similar conclusions were drawn from spectroscopic studies[1] of the ensemble of solution conformations. Also, among the calculated low-energy conformations are type I β-bend conformations that are similar to those of the crystal structures.[1] From the combination of experimental[1] and theoretical results, it is concluded that either *cis*- or *trans*-Asn^{113}-Pro^{114} peptide bonds allow the formation of β-bend structures at Asn^{113}-Pro^{114}-Tyr^{115} of bovine ribonuclease A in the initial stage of polypeptide chain folding when local interactions dominate. Although *cis*-Tyr-Pro peptide bonds were found to be unfavorable under conditions in which short-range interactions predominate, when present the *cis*-Tyr^{92}-Pro^{93} peptide bond strongly favors β-bends at Tyr-Pro. Initiation structures with *trans*-Tyr^{92}-Pro^{93} peptide bonds and β-bends in the Tyr^{92}-Pro^{93}-Asn^{94} sequence can form only if longer-range interactions than those present in Ac-Tyr-Pro-Asn-NHMe contribute to their stabilization.

In the preceding paper,[1] and in a previous one,[2] we have reported the results of an experimental study of the solid state and solution conformations of the terminally blocked tripeptides Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe.[3-5] In native bovine pancreatic ribonuclease A, these sequences correspond to type VI[6] β-bends,[7-10] with *cis*-X-Pro peptide bonds within two proposed chain-folding initiation sites,[1,11,12] viz. Tyr^{92}-Pro^{93}-Asn^{94} (located within initiation site[12] E) and Asn^{113}-Pro^{114}-Tyr^{115} (located within initiation site[12] F). In this paper, we present the results of conformational free energy calculations on these two tripeptides and on several related terminally blocked peptides.

The spectroscopic[1] and crystallographic[1] measurements reveal a tendency of Ac-Asn-Pro-Tyr-NHMe to form a hydrogen-bonded β-bend at Pro-Tyr in water and in the solid state when the Asn-Pro peptide bond is *trans*. On the other hand, Ac-Tyr-Pro-Asn-NHMe adopts an extended conformation with a *trans*-Tyr-Pro peptide bond, in the solid state.[1] Local β-bend conformations, stabilized by short- and medium-range interactions, in these sequences of ribonuclease A are presumed to be important in the initial stages of folding.[1,12] These free energy calculations were undertaken both to identify and understand the interatomic interactions that lead to the β-bend-forming tendency of the ensembles of conformations with *trans*-X-Pro peptide bonds and to characterize the β-bend probabilities in the experimentally inaccessible sets of conformations with *cis*-X-Pro peptide bonds.

The conformational entropy,[13,14] calculated from the curvature of the potential energy surface near each energy-minimized conformation,[5,15-18] can be an important factor in determining the conformational free energy of a peptide. The contribution of entropy is likely to be especially important in conformations which contain intramolecular hydrogen bonds, because such interactions lead to a loss in entropy due to a restriction of the conformational space accessible to the peptide. In these calculations, we found that conformational entropy plays a crucial role in determining the relative stabilities of conformations, particularly in the terminally blocked tripeptides which have a greater tendency to form intramolecular hydrogen bonds than terminally blocked dipeptides or amino acids.

An additional aim of this study was to evaluate the ability of conformational free energy calculations (i.e., with inclusion of conformational entropy) to predict local structures that the polypeptide chain can adopt in the intial stages of protein folding. In this paper, we demonstrate that, when conformational entropy is accounted for, calculations can indeed predict the general bend forming tendency of short peptide fragments in relatively good agreement with solid-state and solution structure determinations,[1] even though solvent/solute interactions are not explicitly accounted for in the conformational potential energy function used here. Because of their ability to provide molecular details that are not

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accessible experimentally, and the relative ease with which the effects of amino acid substitutions can be evaluated and compared with substitutions observed in homologous proteins or introduced by genetic engineering techniques, theoretical calculations like those presented here will be of increasing importance in providing an understanding of the mechanisms of protein folding.

Methods

The nomenclature and conventions adopted are those recommended by an IUPAC-IUB nomenclature commission.[19] The calculations were carried out on terminally blocked peptides Ac-X-NHMe, i.e., those with Ac and NHMe terminal groups,[3] where X = Asn-Pro-Tyr, Ala-Pro-Tyr, Pro-Tyr, Pro-Phe, Tyr-Pro-Asn, Tyr-Pro-Ala, Phe-Pro-Ala, Pro-Asn, Pro-Ala, Tyr-Pro, and Phe-Pro.

All calculations were carried out with the computer program ECEPP (Empirical Conformational Energy Program for Peptides)[20] on an array processor,[21] using standard geometry[20] for bond lengths and bond angles, except that the C^{α}–H bond length was increased[22] from 1.00 to 1.09 Å (this involved essentially no change in the dihedral angles of single-residue minimum-energy conformations[5,23]). Minimization was carried out with the Powell algorithm[24] until the conformational energy (expressed in single precision) did not change by more than 1 cal/mol between successive iterations. Then a Newton–Raphson procedure[25] (expressed in double precision) was used to refine each dihedral angle to within less than 0.001% of its minimum-energy value. The second minimization procedure required the accurate calculations of the second derivatives of the energy at the minimum. During the minimization, all values of ϕ's, ψ's, and χ's were allowed to vary. For the tripeptides, all ω's were allowed to vary except the one for the *trans*-peptide bond between the acetyl group and the first amino acid residue. For the Pro-X dipeptides, the peptide bonds were all fixed at 180° (*trans*) except those preceding proline which were fixed at either 180° (*trans*) or 0° (*cis*). For the X-Pro dipeptides, the peptide bonds were all fixed at 180° (*trans*) except those preceding proline which were allowed to vary.

In the minimizations of the energies of the dipeptides, three sets of starting conformations were used: (1) All combinations of single-residue minima[5] with relative energy $\Delta E < 3$ kcal/mol (<5 kcal/mol for Ala and Phe). For Pro, the initial value of $\psi = -23°$ was also used, in addition to the three minimum-energy values; this value is not a minimum-energy one, but lies in the same broad potential energy well as the minimum at $\psi = -48°$ (see Figure 4 of ref 5). (2) Various types of β-bends.[6,15] Because ϕ of Pro is fixed at −75°, the only bends that were compatible with this value of ϕ_{Pro} in Pro-Y dipeptides were types I, II, III, and V; for the same reason only bend types I, II′, III, and V′ were used for X-Pro dipeptides. Bend types IV, VI, and VII were not used as starting conformations because they are not defined in terms of specific dihedral angles; however, these types of bends were observed among the final, energy-minimized structures. (3) Backbone–side chain hydrogen-bonded conformations constructed with molecular models. For Tyr-Pro, the conformation of Ac-Tyr-Pro-NHMe in the crystal[1] was also used as a starting conformation.

In the minimizations of the energies of the tripeptides, four sets of starting conformations were used: (1) Set A, all combinations of single-residue minima[5] with relative energy $\Delta E < 3$ kcal/mol (<5 kcal/mol for Ala and Phe); in addition, the initial value of $\psi = -23°$ for Pro was again used. (2) Set B, all combinations of minima of single residues[5] X with those of dipeptides Pro-Y.[15] Since the latter pertain to *trans*-peptide bonds preceding Pro, the dipeptide calculations were repeated for Ac-Pro-Y-NHMe (where Y = Tyr, Asn, and Ala) with *cis*-Ac-Pro peptide bonds. (3) Set C, all combinations of minima of X-Pro dipeptides[15] with those of single residues Y. (4) Set D, interresidue (backbone–side chain and side chain–side chain) hydrogen-bonded conformations constructed with molecular models. This set also included the conformations of Tyr^{92}-Pro^{93}-Asn^{94} and Asn^{113}-Pro^{114}-Tyr^{115} observed in the crystal structures of both ribonuclease S[7,8] and ribonuclease A,[9,10] the conformations of Tyr^{12}-Pro^{13}-Asn^{14} and Tyr^{67}-Pro^{68}-Asn^{69} in the crystal structure of concanavalin A,[26] the conformation of Ac-Asn-Pro-Tyr-NHMe[1] determined in its two crystal forms, and the conformation of crystalline Ac-Tyr-Pro-Asn-NHMe[1]. No separate β-bend conformations were selected for the tripeptides because they were already included in set D.

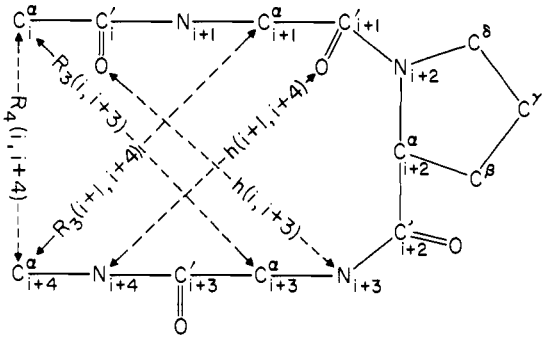

Figure 1. Schematic diagram specifying residues i to $i + 4$ and showing the definitions of the interatomic distances referred to in the text and in the supplementary tables.

Normalized statistical weights (w) and Boltzmann factors (v), relative free energies (ΔG) and conformational entropies (ΔS), distances [$h(i, i + 3)$ and $R_3(i, i + 3)$], bend types, and the presence of hydrogen bonds, for each conformation at 27 °C, as defined elsewhere,[15] were also computed and assessed (see Figure 1 for a representation of the distances and for the relevant nomenclature). In all peptides, the N-terminal acetyl group is considered as residue i.[27] A polar hydrogen atom and an oxygen or nitrogen atom with an interatomic distance of ≤2.3 Å are defined to be hydrogen bonded.

Vicinal HN–C^{α}H coupling constants $^3J_{HN-C^{\alpha}H}$ for each conformation were computed with the following expression of Bystrov et al.[28,29]

$$^3J_{HN-C^{\alpha}H} = 9.4 \cos^2 \theta - 1.1 \cos \theta + 0.4 \quad (1)$$

where, for L-amino acids,

$$\theta = \phi - 60° \quad (2)$$

Statistical averages over several conformations of any quantity A ($\langle A \rangle_Z$ and $\langle A \rangle_Q$), and the probabilities (P_Z and P_Q) of occurrence of various bend types or of particular backbone conformations, were defined and computed as in ref 15.

The minimum-energy conformations of the peptides are described by their backbone and side-chain dihedral angles (although only backbone dihedral angles are given in the tables). They are also represented in terms of a conformational letter code[4] assigned to each residue which specifies its location on a ϕ,ψ map (see Figure 1 of ref 5).

Results

A summary of the results of conformational energy calculations on the terminally blocked di- and tripeptides is given in Tables I–V. A complete listing of low-energy minima is available in the supplementary tables.[30]

Ac-Asn-Pro-Tyr-NHMe. There are many low-energy minima of this terminally blocked tripeptide listed in supplementary Table S1,[30] viz., 210, 92, and 31, with values of $\Delta E < 5$, <4, and <3 kcal/mol, respectively. They arise from all of the sets of starting conformations. Significantly, the set of conformations with the lowest values of ΔE do not correspond to those of highest statistical weight, since conformational entropy plays a major role in stabilizing the lowest free energy conformations. For example, the

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Table I. Conformations of Tripeptides[a] with Free Energies within 0.5 kcal/mol of Minimum Free Energy Conformation

	ΔG^b	ΔE	$-T\Delta S$	w	v	no. of H bonds[c] (i, j, k)	bend type $(i+1, i+2)$	bend type $(i+2, i+3)$	dihedral angles,[d] deg ϕ_{i+1}	ψ_{i+1}	ω_{i+1}	ψ_{i+2}	ϕ_{i+3}	ψ_{i+3}	conformation letter code
trans-Asn-Pro-Tyr	−1.53	0.01	−1.54	0.057	0.158	0, 0, 1			−159	77	−174	173	−142	155	DFE
	−1.32	3.97	−5.30	0.040	0.000	1, 0, 0			−160	86	176	78	−153	147	DCE[e]
	−1.31	4.00	−5.32	0.040	0.000	1, 0, 0			−160	86	176	78	−153	147	DCE[e]
	−1.14	3.09	−4.22	0.029	0.001	1, 2, 0		I	−159	104	175	−22	−95	72	DAC
	−1.12	3.08	−4.20	0.029	0.001	1, 2, 0		I	−159	104	175	−22	−95	72	DAC
	−1.09	0.14	−1.23	0.027	0.128	0, 0, 1			−141	156	165	84	−74	147	ECF
cis-Asn-Pro-Tyr	0.57	4.30	−3.72	0.002	0.000	1, 0, 0	VI	VII	−77	149	−6	−48	−145	70	FAD
	0.68	4.42	−3.74	0.001	0.000	1, 0, 0	VI	VII	−77	149	−6	−48	−145	71	FAD
	0.87	2.16	−1.29	0.001	0.004	0, 0, 1	VI		−71	153	−11	173	−119	151	FFE
	0.87	2.69	−1.82	0.001	0.002	0, 0, 1	VI		−74	152	−12	162	−106	75	FFC
	0.90	4.60	−3.71	0.001	0.000	0, 0, 0	VI		−84	151	−8	149	−90	147	FFF
trans-Ala-Pro-Tyr	−0.09	0.71	−0.80	0.113	0.057	0, 0, 0			−151	81	176	162	−144	154	DFE
	0.00	0.00	0.00	0.098	0.189	1, 0, 0			−152	78	178	76	−147	154	DCE
	0.03	0.02	0.01	0.093	0.182	1, 0, 0			−152	78	178	76	−147	154	DCE
	0.07	0.58	−0.51	0.087	0.071	1, 0, 0			−152	78	178	74	−146	52	DCD
	0.19	0.57	−0.38	0.071	0.072	1, 0, 0			−152	78	178	74	−146	50	DCD
	0.34	0.84	−0.50	0.055	0.046	0, 0, 0			−151	80	176	158	−146	154	DFE
cis-Ala-Pro-Tyr	3.09	2.66	0.43	0.001	0.002	1, 0, 0	VI	VII	−78	153	−8	−46	−144	67	FAD
	3.19	2.78	0.41	0.000	0.002	1, 0, 0	VI	VII	−78	153	−8	−46	−144	68	FAD
trans-Tyr-Pro-Asn	0.00	0.00	0.00	0.649	0.280	0, 0, 1			−151	91	172	89	−64	120	DCC
cis-Tyr-Pro-Asn	3.84	6.20	0.26	0.001	0.000	0, 0, 0	VI		−147	156	−6	156	−81	129	EFC
	3.95	6.36	0.32	0.001	0.000	0, 0, 0	VI		−147	155	−6	156	−80	130	EFF
trans-Tyr-Pro-Ala	−1.35	3.71	−5.06	0.074	0.001	1, 0, 0			−152	82	177	85	−68	140	DCF
	−1.34	3.73	−5.07	0.073	0.001	1, 0, 0			−152	82	177	85	−67	139	DCF
	−0.99	3.38	−4.37	0.041	0.002	2, 0, 0			−152	79	−179	84	−81	81	DCC
	−0.97	3.36	−4.33	0.039	0.002	2, 0, 0			−152	79	−179	84	−81	81	DCC
	−0.90	2.16	−3.06	0.035	0.016	2, 0, 0			−152	79	175	70	−163	160	DCE
	−0.88	3.17	−4.05	0.034	0.003	1, 0, 0			−151	84	179	−36	−157	164	DAE
	−0.85	4.49	−5.34	0.032	0.000	1, 0, 0			−151	85	174	164	−83	80	DFC
cis-Tyr-Pro-Ala	1.01	4.43	−3.42	0.001	0.000	1, 0, 0	VI	I	−147	156	−6	−43	−85	77	EAC
	1.05	4.48	−3.44	0.001	0.000	1, 0, 0	VI	I	−147	156	−6	−43	−85	77	EAC
trans-Phe-Pro-Ala	−0.54	2.48	−3.01	0.097	0.003	0, 0, 0			−153	85	174	164	−73	138	DFF
	−0.29	1.91	−2.20	0.064	0.008	1, 0, 0			−152	85	174	164	−83	80	DFC
	−0.19	1.96	−2.15	0.054	0.007	1, 0, 0			−146	81	177	78	−74	141	DCF
	−0.16	1.97	−2.13	0.051	0.007	1, 0, 0			−146	81	176	76	−152	66	DCD
	−0.14	0.92	−1.06	0.050	0.042	1, 0, 0			−152	84	179	−36	−157	164	DAE
cis-Phe-Pro-Ala	1.30	2.02	−0.72	0.004	0.007	1, 0, 0	VI	VII	−79	154	−6	−41	−151	68	FAD
	1.40	2.99	−1.59	0.004	0.001	1, 0, 0	VI	IV	−142	155	−5	−20	−135	82	EAD
	1.56	1.87	−0.31	0.003	0.009	1, 0, 0	VI	I	−147	155	−6	−43	−85	77	EAC
	1.65	2.71	−1.06	0.002	0.002	1, 0, 0	VI		−147	154	−4	153	−155	155	EFE
	1.70	2.97	−1.27	0.002	0.001	1, 0, 0	VI		−147	154	−5	150	−85	79	EFC

[a] *N*-Acetyl-*N'*-methyl tripeptide amides [b] A free energy of 0.00 is assigned to the conformation of lowest energy (see supplementary tables[30] for values of G_0 and E_0). [c] In each i, j, k set, i is the number of backbone–backbone hydrogen bonds, j is the number of backbone–side chain and side chain–backbone hydrogen bnds, and k is the number of side chain–side chain hydrogen bonds. [d] $\phi_{i+2} = -75°$. [e] Several minimum-energy conformations, such as these, were found to have identical backbone conformations, but different side-chain conformations and hence different overall energies.

Table II. Calculated Bend Probabilities of Tripeptides[a] with *trans*-X-Pro Peptide Bonds

tripeptide[a]	$P_{Z,total}$[b]	$P_{Z,I}$	$P_{Z,II}$	$P_{Z,III}$	$P_{Z,III'}$	$P_{Z,IV}$	$P_{Z,VII}$
Asn-Pro-Tyr	0.267	0.078		0.038		0.012	0.139
	(0.029)[c]	(0.011)		(0.005)		(0.011)	(0.003)
Ala-Pro-Tyr	0.106	0.037				0.025	0.044
	(0.069)	(0.006)				(0.043)	(0.021)
Tyr-Pro-Asn	0.026	0.003		0.002		0.021	
	(0.238)	(0.007)		(0.012)		(0.219)	
Tyr-Pro-Ala	0.197	0.067	0.000	0.012	0.004	0.032	0.082
	(0.340)	(0.279)	(0.000)	(0.038)	(0.003)	(0.015)	(0.004)
Phe-Pro-Ala	0.294	0.094	0.007	0.027		0.030	0.134
	(0.421)	(0.127)	(0.004)	(0.048)		(0.167)	(0.073)

[a] *N*-acetyl-*N'*-methyl tripeptide amides with a *trans*-peptide bond at X-Pro. [b] Bend probabilities in the Pro-Y portion. [c] The numbers in parentheses are bend probabilities in the Q space.

conformation with the highest statistical weight (w = 0.057; Figure 2B) is slightly higher in energy (ΔE = 0.01 kcal/mol) than the conformation of lowest energy (w = 0.004; Figure 2A), and the conformations with the second and third highest statistical weights

Table III. Calculated Bend Probabilities of Dipeptides

dipeptide[a]	$P_{Z,total}$[b]	$P_{Z,I}$	$P_{Z,II}$	$P_{Z,III}$	$P_{Z,IV}$	$P_{Z,VII}$
Pro-Tyr *t*	0.063	0.026			0.016	0.021
	(0.042)[c]	(0.008)			(0.017)	(0.017)
Pro-Phe[d] *t*	0.15	0.058		0.012	0.004	0.079
	(0.07)	(0.014)		(0.008)	(0.010)	(0.042)
Pro-Asn[d] *t*	0.17	0.069		0.031	0.003	0.066
	(0.16)	(0.055)		(0.042)	(0.021)	(0.045)
Pro-Ala[d] *t*	0.16	0.040	0.007	0.042	0.020	0.053
	(0.26)	(0.072)	(0.022)	(0.040)	(0.086)	(0.035)
Tyr-Pro[e] *t*	0.000					
	(0.000)					
Phe-Pro[e] *t*	0.000					
	(0.000)					
Pro-Tyr *c*	0.621	0.268		0.069	0.110	0.175
	(0.546)	(0.169)		(0.102)	(0.125)	(0.150)
Pro-Phe *c*	0.619	0.304		0.092		0.223
	(0.505)	(0.210)		(0.154)		(0.141)
Pro-Asn *c*	0.555	0.369		0.105		0.081
	(0.620)	(0.297)		(0.251)		(0.072)
Pro-Ala *c*	0.541	0.122	0.001	0.194		0.223
	(0.592)	(0.273)	(0.004)	(0.214)		(0.102)

[a] *N*-Acetyl-*N'*-methyl dipeptide amides. The designations *t* and *c* refer to the ensembles with *trans*- and *cis*-X-Pro peptide bonds, respectively. [b] Calculated bend probability. [c] The numbers in parentheses are bend probability of the Q space. [d] Results of Zimmerman and Scheraga, ref 15. [e] The calculated bend probabilities are for the set of conformations with *trans*-X-Pro peptide bonds only. There are 1 and 3 *cis* conformations of Tyr-Pro and Phe-Pro, respectively, with $\Delta E < 3$ kcal/mol. All of these *cis* conformations are type VI β-bends.

Table IV. Calculated Bend Probabilities of Tripeptides[a] with *cis*-X-Pro Peptide Bonds

tripeptide[a]	$P_{Z,VI}$[b]	N[c]
Asn-Pro-Tyr	0.98	17
Ala-Pro-Tyr	1.00	2
Tyr-Pro-Asn	0.97	9
Tyr-Pro-Ala	1.00	2
Phe-Pro-Ala	1.00	6

[a] *N*-Acetyl-*N'*-methyl tripeptide amides with a *cis*-peptide bond at X-Pro. [b] Type VI β-bend[6] probability of tripeptide (with a bend at X-Pro). [c] Number of minimum-energy conformations with *cis*-X-Pro peptide bonds used to calculate $P_{Z,VI}$.

($w = 0.040$ in both cases) are the 85th and 93rd lowest energy minima, with $\Delta E = 3.97$ and 4.00 kcal/mol, respectively.

The six *trans* conformations with free energy within 0.5 kcal of the *trans* conformation with lowest free energy are tabulated in Table I. These include both extended and type I β-bend conformations. The *trans* conformation of lowest free energy (and highest statistical weight) is an extended structure (DFE), characterized by a Tyr···Asn side chain–side chain $O^{\eta}H\cdots O^{\delta}$ hydrogen bond (see Figure 2B). While many conformations ($P_Z = 0.229$ for the set of conformations with *trans*-Asn-Pro peptide bonds) have this side chain–side chain hydrogen bond, it restricts their conformational fluctuations and thereby lowers their statistical weight, i.e., conformations with this side chain–side chain hydrogen bond are favored by enthalpy but unfavorable entropically.

In low-energy conformations of Ac-Asn-Pro-Tyr-NHMe and Ac-Pro-Tyr-NHMe with *trans*-X-Pro peptide bonds the Pro and Tyr residues generally occupy regions of the ϕ,ψ map which correspond to low-energy conformations of Ac-Pro-NHMe[5,23] (i.e., regions[4] F, C, and A) and Ac-Tyr-NHMe[5,23] (i.e., regions E, D, F, and C). This is because the short-range interactions inherent in these terminally blocked amino acids play an important role in limiting the conformational space accessible to the terminally blocked di- and tripeptides. The preferred conformations of Asn in Ac-Asn-Pro-Tyr-NHMe include D, E, and F (even though F does not appear in Table I), with normalized probabilities (in Z space) of 0.64, 0.23, 0.13 for Asn to adopt the D, E, and F conformations, respectively. The corresponding probabilities for these conformations in Ac-Asn-Pro-NHMe[15] and Ac-Asn-NHMe[5] are 0.76, 0.15, 0.03, and 0.00, 0.34, 0.01, respectively. These results indicate that the D backbone conformation of Asn is stabilized by interactions between the Asn and Pro residues and that the F backbone conformation of Asn is stabilized by medium-range (e.g., side chain/side chain or side chain/backbone) interactions in Ac-Asn-Pro-Tyr-NHMe.

Table V. Calculated Vicinal Coupling Constants $^3J_{NH-C^{\alpha}H}$ of *trans*-Tripeptides[a]

	$(^3J_{N_{i+1}H-C^{\alpha}_{i+1}H})_Z$			$(^3J_{N_{i+3}H-C^{\alpha}_{i+3}H})_Z$		
		exptl[b]			exptl[b]	
tripeptide	calcd	H_2O	Me_2SO	calcd	H_2O	Me_2SO
Asn-Pro-Tyr	7.21	6.8	7.9	8.26	7.8	8.9
	(8.24)[c]			(7.09)		
Ala-Pro-Tyr	8.00			8.37	6.7	8.2
	(7.94)			(8.44)		
Tyr-Pro-Asn	8.07	7.3	8.1	4.65	7.3	8.7
	(8.05)			(4.85)		
Tyr-Pro-Ala	8.38			6.70		
	(7.95)			(6.61)		
Phe-Pro-Ala	8.46			7.05		
	(7.95)			(6.78)		

[a] *N*-Acetyl-*N'*-methyl tripeptide amides. [b] Experimental values obtained from ^{1}H NMR at 27 °C (E. R. Stimson, unpublished results). [c] The numbers in parentheses are statistical average values in the Q space.

The bend probabilities for *trans*-Ac-Asn-Pro-Tyr-NHMe (i.e., with *trans*-Asn-Pro peptide bonds) are tabulated in Table II. No bend conformations at Asn-Pro[27] with $\Delta E < 5$ kcal/mol were observed. This is because the conformational space of residues preceding proline is limited to a narrow region which is inconsistent (except in Gly-Pro) with type I, II, or III β-bend (or α-helical) conformations.[15,31] This conformational restriction is due primarily to atomic overlaps involving the side-chain atoms (par-

(31) Pincus, M. R.; Gerewitz, F.; Wako, H.; Scheraga, H. A. *J. Protein Chem.* **1983**, *2*, 131.

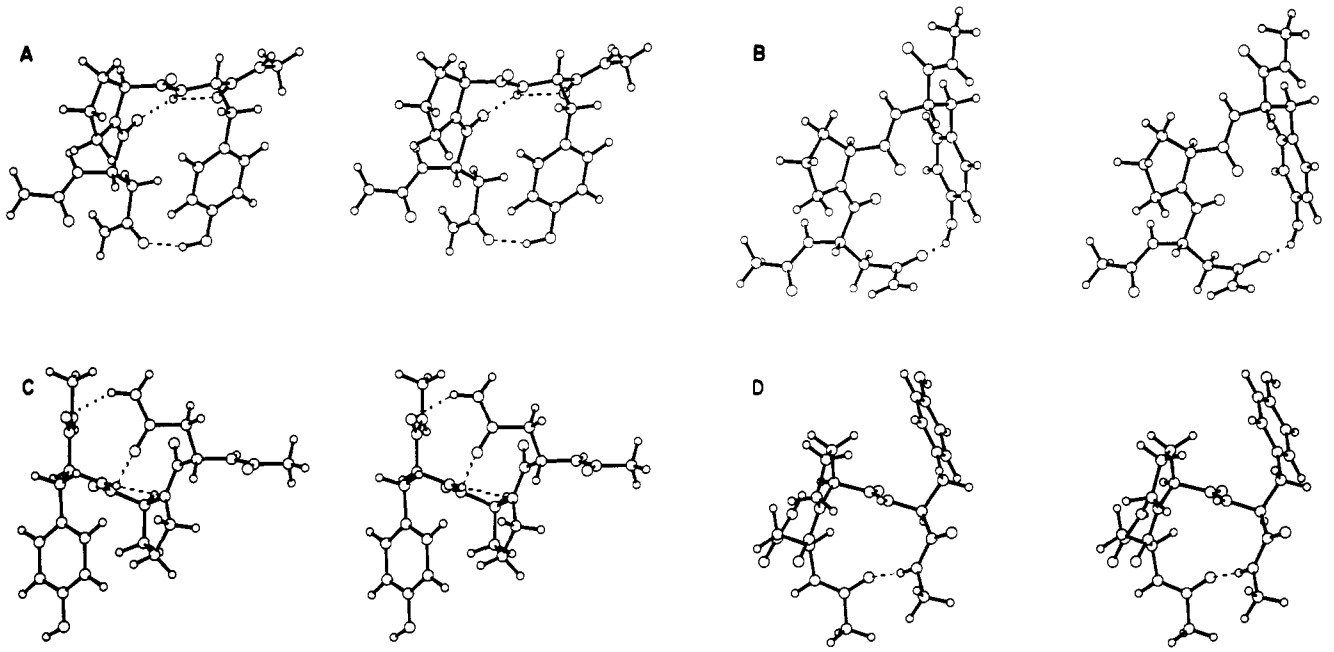

Figure 2. Minimum-energy conformations of Ac-Asn-Pro-Tyr-NHMe. (A) Lowest energy conformation with a *trans*-Asn-Pro peptide bond. ΔE = 0.00 kcal/mol, ΔG = 0.00 kcal/mol. The ϕ and ψ of the three residues are (−145°, 77°, −75°, 68°, −167°, 160°), and the corresponding conformational letter code is DCE. (B) Lowest free energy conformation with a *trans*-Asn-Pro peptide bond. ΔE = 0.01 kcal/mol, ΔG = −1.53 kcal/mol. The ϕ and ψ of the three residues are (−159°, 77°, −75°, 173°, −142°, 155°), and the conformational letter code is DFE. (C) Lowest free energy β-bend conformation with a *trans*-Asn-Pro peptide bond. ΔE = 3.09 kcal/mol, ΔG = −1.14 kcal/mol. The ϕ and ψ of the three residues are (−159°, 104°, −75°, −22°, −95°, 72°), and the conformational letter code is DAC. This conformation is a type I β-bend. (D) Lowest free energy conformation with a *cis*-Asn-Pro peptide bond. ΔE = 4.30 kcal/mol, ΔG = 0.57 kcal/mol. The ϕ and ψ of the three residues are (−77°, 149°, −75°, −48°, −145°, 70°), and the conformational letter code is FAD. This conformation is a type VI/type VII double bend.

ticularly C^{δ}) of proline with the side-chain atoms or backbone NH group[15] of the residue preceding proline. For this reason, β-bend (and α-helical) conformations at *trans*-Asn-Pro should not be energetically accessible even in the presence of long-range interactions.

While the bend probability of *trans*-Ac-Asn-Pro-Tyr-NHMe (with the bend at Asn-Pro) is quite low, the probability of bend formation in this tripeptide (with the bend at Pro-Tyr) is relatively high when conformational entropy is included (i.e., P_Z = 0.267, P_Q = 0.029). In almost all of these bend conformations, Asn adopts the D or F conformation, both of which are preferentially stabilized by Asn/Pro or Asn/Tyr interactions, as pointed out above. Furthermore, the bend probability of *trans*-Ac-Pro-Tyr-NHMe (with the bend at Pro-Tyr) is fairly low (P_Z = 0.063, Table III). Taken together, these observations indicate that the bend-forming tendency at Pro-Tyr of *trans*-Ac-Asn-Pro-Tyr-NHMe is primarily attributable to interactions between Asn and Pro-Tyr. A similar conclusion was drawn from spectroscopic measurements,[1] where both Raman and NMR data demonstrate the presence of a significant fraction of molecules with a β-bend at Pro-Tyr in *trans*-Ac-Asn-Pro-Tyr-NHMe but not in *trans*-Ac-Pro-Tyr-NHMe.

Calculated bend probabilities for the various types of β-bends in *trans*-Ac-Asn-Pro-Tyr-NHMe are also presented in Table II. The most probable β-bend conformations for the tripeptide are type VII (P_Z = 0.139) and type I (P_Z = 0.078) (with the bend at Pro-Tyr), both of which have *trans*-Asn-Pro peptide bonds. It should be noted that *each* type VII β-bend conformation has a low value of P_Z but, because there are *many* such conformations in the ensemble, the type VII β-bend was found to be the most probable. The *trans*-Ac-Asn-Pro-Tyr-NHMe β-bend conformation of highest statistical weight is type I at Pro-Tyr (see Table I and Figure 2C).

The majority of low-energy bend conformations of *trans*-Ac-Asn-Pro-Tyr-NHMe have Asn in the D backbone conformation and similar backbone conformations at Pro-Tyr. In fact, the set of DAC (type I at Pro-Tyr), DAA (type I or III at Pro-Tyr), and DAE (type VII at Pro-Tyr) backbone conformations of *trans*-Ac-Asn-Pro-Tyr-NHMe may be considered as a single class of conformers with a statistical weight of 0.23 (supplementary Table[30] S1). One of these bend conformers is shown in Figure 2C. Type I (e.g., AC or AA) and type III (e.g., AA or AE) β-bends are actually two (somewhat arbitrarily distinguished) subtypes of a continuous category of bend types,[32] while the AE backbone conformation at Pro-Tyr may be thought of as a distorted type I β-bend, similar to the AC backbone conformation of Pro-Tyr in Figure 2C. Since the overall bend probability for *trans*-Ac-Asn-Pro-Tyr-NHMe is 0.27 (Table II), these similar DAC, DAA, and DAE bend conformations account for about 85% of the bend probability for this peptide. Hence, although the bend probability at Pro-Tyr of *trans*-Ac-Pro-Tyr-NHMe is quite low (Table III), the presence of Asn in the D backbone conformation in *trans*-Ac-Asn-Pro-Tyr-NHMe leads to an enhancement of the bend-forming tendency at Pro-Tyr.

These results reveal that the D backbone conformation of Asn, which is favored by the conformational constraints imposed by Pro on a preceding Asn residue,[15] allows interactions between the Pro-Tyr-NHMe backbone and the Asn side chain (and backbone) which stabilize otherwise unfavorable type I, III, and VII β-bends at Pro-Tyr. In fact, 34% of the low-energy β-bend conformations have intramolecular hydrogen bonds between the NHMe amide and the asparagine side-chain (or backbone) amide carbonyl oxygen. The DAB backbone conformation of the two crystal structures[1] (a type I β-bend at Pro-Tyr) also has an NHMe amide···Asn backbone carbonyl oxygen intramolecular hydrogen bond (as well as a Tyr NH···Asn side-chain oxygen hydrogen bond).

While type I and similar β-bends at Pro-Tyr were found to have a relatively high probability, no type II β-bends were observed among the low-energy minima of either *trans*-Ac-Pro-Tyr-NHMe or of *trans*-Ac-Asn-Pro-Tyr-NHMe.

The preceding analysis pertains only to the ensemble of conformations with *trans*-Asn-Pro peptide bonds. When the Asn-Pro peptide bond of Ac-Asn-Pro-Tyr-NHMe is *cis*, β-bends are observed both at Asn-Pro and at Pro-Tyr. All of the *cis*-Ac-Asn-

(32) Némethy, G.; Scheraga, H. A. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 320.

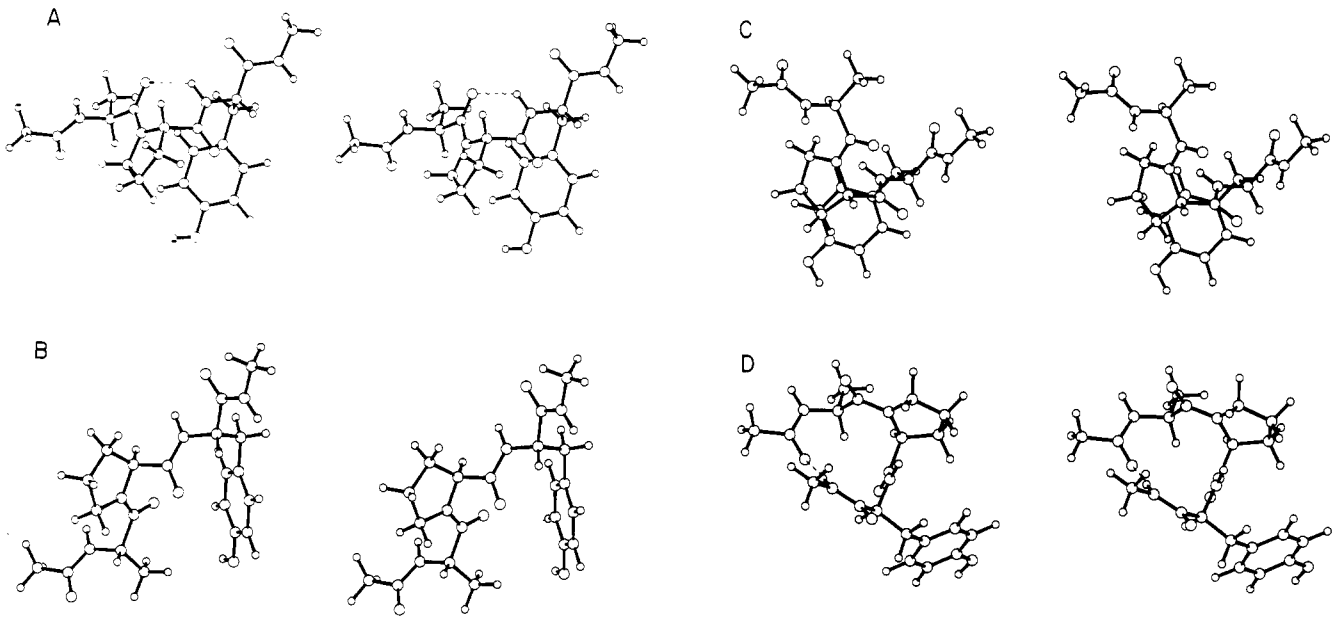

Figure 3. Minimum-energy conformations of Ac-Ala-Pro-Tyr-NHMe. (A) Lowest energy conformation with a *trans*-Ala-Pro peptide bond. ΔE = 0.00 kcal/mol, ΔG = 0.00 kcal/mol. The ϕ and ψ of the three residues are (−152°, 78°, −75°, 76°, −147°, 154°), and the corresponding conformational letter code is DCE. (B) Lowest free energy conformation with a *trans*-Ala-Pro peptide bond. ΔE = 0.71 kcal/mol, ΔG = −0.09 kcal/mol. The ϕ and ψ of the three residues are (−151°, 81°, 75°, 162°, −144°, 154°), and the conformational letter code is DFE. (C) Lowest free energy β-bend conformation with a *trans*-Ala-Pro peptide bond. ΔE = 2.92 kcal/mol, ΔG = 0.97 kcal/mol. The ϕ and ψ of the three residues are (−151°, 80°, −75°, −44°, −89°, 73°), and the conformational letter code is DAC. This conformation is a type I β-bend. (D) Lowest free energy conformation with a *cis*-Ala-Pro peptide bond. ΔE = 2.66 kcal/mol, ΔG = 3.09 kcal/mol. The ϕ and ψ of the three residues are (−78°, 153°, −75° −46°, −144°, 67°), and the conformational letter code is FAD. This conformation is a type VI/type VII double bend.

Pro-Tyr-NHMe conformations (i.e., with a *cis*-Asn-Pro peptide bond) within 0.5 kcal/mol of the lowest free energy conformation have type VI β-bends at Asn-Pro (see Table I). In fact, the two lowest free energy conformations are double bends,[33] with a type VI bend at Asn-Pro and a type VII bend at Pro-Tyr. The β-bend probability for *cis*-Ac-Asn-Pro-Tyr-NHMe is 0.98 (Table IV). This suggests that, in the initial stages of folding, all ribonuclease A molecules with *cis*-Asn[113]-Pro[114] peptide bonds will have a local β-bend structure within chain-folding initiation site[1,12] F. (This does *not* necessarily mean that, in the initial stages of folding, the majority of ribonuclease A molecules have *cis*-Asn[113]-Pro[114] peptide bonds.)

The strong β-bend forming tendency of *cis*-Ac-Asn-Pro-Tyr-NHMe arises from the *cis*-peptide bond conformation of Asn-Pro and is relatively independent of Asn/Tyr interactions. This conclusion is supported by conformational free energy calculations on Ac-Asn-Pro-NHMe[15] for which all five minimum-energy conformations (ΔE < 3 kcal/mol) with a *cis*-Asn-Pro peptide bond are β-bend conformations, and by the very high β-bend probability calculated for *cis*-Ac-Pro-Tyr-NHMe (P_Z = 0.62, Table III). These conformational preferences arise predominantly from steric and electrostatic interactions in conformations with *cis*-X-Pro peptide bonds, since many of these β-bend conformations of *cis*-Ac-Asn-Pro-NHMe,[15] *cis*-Ac-Pro-Tyr-NHMe, and *cis*-Ac-Asn-Pro-Tyr-NHMe are not stabilized by hydrogen bonds.

Ac-Ala-Pro-Tyr-NHMe. In the conformational analysis of *trans*-Ac-Asn-Pro-Tyr-NHMe presented above, it was observed that hydrogen bonds involving the Asn side-chain primary amide play an important role in stabilizing β-bend conformations in the Pro-Tyr part of the molecule. In order to provide supporting evidence for this conclusion, we have also calculated the low-energy conformations of Ac-Ala-Pro-Tyr-NHMe. There are 61 low-energy minima with ΔE < 3 kcal/mol listed in supplementary Table[30] S2.

As in *trans*-Ac-Asn-Pro-Tyr-NHMe, the lowest energy conformation of *trans*-Ac-Ala-Pro-Tyr-NHMe (w = 0.098, v = 0.189, Figure 3A) does not correspond to that of lowest free energy (w = 0.113, v = 0.057, Figure 3B).

Entropic considerations in Ac-Ala-Pro-Tyr-NHMe, which cannot form entropically unfavorable side chain/side chain interactions, are less important than those of Ac-Asn-Pro-Tyr-NHMe in determining conformational statistic weights. For example, while entropic factors change their relative order, the six structures with the highest statistical weight are also the six of lowest energy, all having ΔE < 1 kcal/mol (see Table I).

Because of the absence of the side-chain amide, medium-range interactions in *trans*-Ac-Ala-Pro-Tyr-NHMe are less effective in stabilizing β-bend conformations than those in *trans*-Ac-Asn-Pro-Tyr-NHMe. When such interactions do occur, however, they can stabilize β-bend conformations at Pro-Tyr. Hence, while β-bend conformations at Ala-Pro are unfavorable for steric reasons (i.e., bends with proline in position[27] 3 are of high energy[15,31] for reasons discussed above), the β-bend probability of Pro-Tyr is higher in *trans*-Ac-Ala-Pro-Tyr-NHMe than in *trans*-Ac-Pro-Tyr-NHMe (cf. Tables II and III). This is due mostly to backbone/backbone hydrogen bonds in the tripeptide between the Ac-Ala peptide carbonyl oxygen and the Pro-Tyr-NHMe portion of the molecule which enhance the statistical weights of the DAC (P_Z = 0.032), DAD (P_Z = 0.019), and DAE (P_Z = 0.044) conformations (corresponding to type I, IV, and VI β-bends, respectively, at Pro-Tyr) relative to the corresponding conformations at Pro-Tyr of *trans*-Ac-Pro-Tyr-NHMe. Nevertheless, the β-bend probability (at Pro-Tyr) of *trans*-Ac-Ala-Pro-Tyr-NHMe is much lower than that of *trans*-Ac-Asn-Pro-Tyr-NHMe (Table II), demonstrating the essential role of the Asn side chain of the latter compound in stabilizing β-bends.

It should be noted that, while *trans*-Ac-Asn-Pro-Tyr-NHMe has a *higher* β-bend tendency than *trans*-Ac-Ala-Pro-Tyr-NHMe, the latter is still expected to adopt the β-bend conformation to some extent (i.e., P_Z = 0.106). As in *trans*-Ac-Asn-Pro-Tyr-NHMe, the most (free) energetically favorable β-bend of *trans*-Ac-Ala-Pro-Tyr-NHMe is the DAC conformation (type I; Figure 3C), which is 1.06 kcal/mol in free energy above the minimum free energy extended conformation.

We next consider the ensemble of Ac-Ala-Pro-Tyr-NHMe conformations with *cis*-Ala-Pro peptide bonds (i.e., *cis*-Ac-Ala-Pro-Tyr-NHMe). From the 844 starting conformations with *cis*-Ala-Pro peptide bonds, only two distinct conformational energy

(33) Isogai, Y.; Némethy, G.; Rackovsky, S.; Leach, S. J.; Scheraga, H. A. *Biopolymers* **1980**, *19*, 1183.

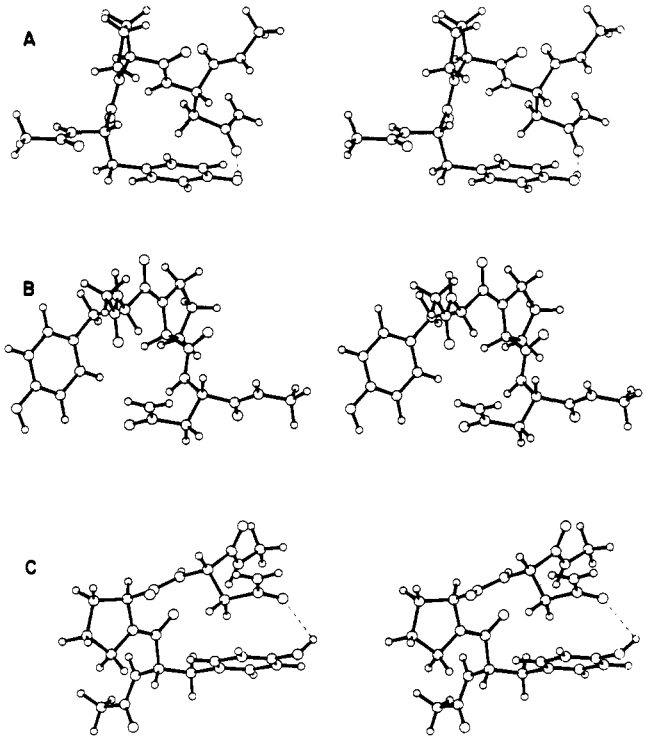

Figure 4. Minimum-energy conformations of Ac-Tyr-Pro-Asn-NHMe. (A) Lowest free energy conformation with a *trans*-Tyr-Pro peptide bond. ΔE = 0.00 kcal/mol, ΔG = 0.00 kcal/mol. The ϕ and ψ of the three residues are (−151°, 91°, −75°, 89°, −64°, 120°), and the corresponding conformational letter code is DCC. (B) Lowest free energy conformation with a *cis*-Tyr-Pro peptide bond. ΔE = 6.20 kcal/mol, ΔG = 3.84 kcal/mol. The ϕ and ψ of the three residues are (−147°, 156°, −75°, 156°, −81°, 129°), and the conformational letter code is EFC. This conformation is a type VI β-bend at Tyr-Pro. (C) Lowest free energy β-bend conformation with a *trans*-Tyr-Pro peptide bond. ΔE = 0.15 kcal/mol, ΔG = 2.08 kcal/mol. The ϕ and ψ of the three residues are (−152°, 81°, −75°, 86°, −172°, −50°), and the conformational letter code is DCG. This conformation is a type IV β-bend at Pro-Asn.

minima were found within 3 kcal/mol of the lowest energy *trans*-Ac-Ala-Pro-Tyr-NHMe conformation. Both of these have FAD double bend[33] backbone conformations (a type VI bend at Ala-Pro and a type VII bend at Pro-Tyr) with a methyl N*H*··· acetyl carbonyl oxygen backbone/backbone hydrogen bond (Figure 3D). Since both *cis*-Ac-Ala-Pro-Tyr-NHMe and *cis*-Ac-Asn-Pro-Tyr-NHMe have strong β-bend forming tendencies (both at X-Pro and at Pro-Tyr), the bend-forming tendency of the latter cannot be attributed to essential Asn side-chain hydrogen bonds. While energetically favorable, the methyl N*H*···acetyl carbonyl oxygen hydrogen bond (which does not occur in any of the *trans* conformations) restricts conformational flexibility and destabilizes these *cis* conformers entropically; i.e., $T\Delta S$ = −0.43 and −0.41 kcal/mol at 27 °C for the two *cis* minima.

Ac-Tyr-Pro-Asn-NHMe There are 50, 28, and 15 low-energy minima of this terminally blocked tripeptide with values of ΔE < 5, <4, and <3 kcal/mol, respectively (see supplementary Table[30] S3). In the ensemble of conformations with *trans*-Tyr-Pro peptide bonds, there is a very strong tendency for the molecule to adopt the extended DCC backbone conformation. For example, the conformation of lowest energy (Figure 4A), which also is that of lowest free energy (w = 0.65, Table I), has an extended DCC backbone conformation. In fact, the combined statistical weight for all DCC backbone conformations (each with different side-chain conformations) is 0.74. Moreover, the combined statistical weights of all non-bend *trans* conformations is 0.97. Therefore, this tripeptide preferentially adopts extended backbone conformations.

This preference for the extended backbone conformation is attributable both to short-range interactions inherent in the amino acid residues themselves and to medium-range Tyr side-chain/Asn side-chain interactions. Essentially all low-energy structures have Tyr $O^{\eta}H\cdots O^{\delta}$ Asn and/or Asn $N^{\delta}H\cdots O^{\eta}$ Tyr side-chain/side-chain hydrogen bonds (with normalized probabilities of 0.92 and 0.03, respectively), even though these interactions restrict conformational flexibility and are entropically unfavorable. Significantly, while side-chain/side-chain interactions are a common conformational feature of these extended (DCC) conformations, the DC conformation at Tyr-Pro and the CC conformation at Pro-Asn also are the most energetically favorable conformations of *trans*-Ac-Tyr-Pro-NHMe (supplementary Table S12) and *trans*-Ac-Pro-Asn-NHMe.[15] Hence, the Tyr side-chain/Asn side-chain interactions in *trans*-Ac-Tyr-Pro-Asn-NHMe act to stabilize conformations which are the most energetically favored by interactions already present in these dipeptide sequences.

In both *trans*-Ac-Tyr-Pro-Asn-NHMe and *trans*-Ac-Tyr-Pro-NHMe, no conformations (ΔE < 5 kcal/mol) with β-bends at Tyr-Pro were observed. Again, this is attributed primarily to steric conformational constraints on the residue preceding proline[5,23] (as discussed above for *trans*-Ac-Asn-Pro-Tyr-NHMe) which make the energies of β-bend conformations very high when the X-Pro peptide bond is *trans*. These interactions would also prevent the formation of ribonuclease folding intermediates with *trans*-Tyr^{92}-Pro^{93} peptide bonds and β-bends at Tyr-Pro.

The β-bend probability of *trans*-Ac-Tyr-Pro-Asn-NHMe (with a bend at Pro-Asn), however, is also very low (Table II), the most energetically favorable β-bend conformation (type IV at Pro-Asn) having a free energy that is 2.08 kcal/mol greater than that of the lowest energy conformation (Figure 4C). We ascribe this low β-bend tendency predominantly to entropic effects, arising from Tyr side-chain/Asn side-chain interactions, which favor extended (e.g., DCC) conformations of the backbone. This conclusion is supported by the observation that *trans*-Ac-Pro-Asn-NHMe has a significantly higher β-bend probability (at Pro-Asn) than *trans*-Ac-Tyr-Pro-Asn-NHMe (cf. Tables II and III). In addition, the bend probability of *trans*-Ac-Tyr-Pro-Asn-NHMe at Pro-Asn is diminished by an order of magnitude when conformational entropy is incorporated into the calculation (i.e., P_Z = 0.026, while P_Q = 0.238).

This entropic effect on bend probability is exactly opposite that observed for *trans*-Ac-Asn-Pro-Tyr-NHMe, for which inclusion of conformational entropy leads to a *higher* calculated β-bend probability (at Pro-Tyr). It is instructive to identify the structural properties of the sequences which are responsible for these entropic differences. In *trans*-Ac-Asn-Pro-Tyr-NHMe, there is competition between side-chain Asn/backbone Pro-Tyr-NHMe hydrogen bonds (which stabilize β-bend conformations at Pro-Tyr) and side-chain Asn/side-chain Tyr hydrogen bonds (which correspond mostly to extended backbone conformations). In these extended conformations of *trans*-Ac-Asn-Pro-Tyr-NHMe, the Asn side-chain, Tyr side-chain, and backbone conformations are restricted by the side-chain/side-chain interactions between Asn and Tyr residues, while most of the β-bend conformations lack side-chain/side-chain interactions and are therefore more conformationally flexible, especially with respect to rotation of the Tyr ring. For this reason, the bend conformers (with Asn side-chain/Pro-Tyr-NHMe backbone interactions) are entropically favored. In *trans*-Ac-Tyr-Pro-Asn-NHMe, on the other hand, it is more difficult for the Tyr side chain to form hydrogen bonds to the Pro-Asn-NHMe backbone or for the Asn side chain to form hydrogen bonds to the Ac-Tyr-Pro backbone [e.g., in the absence of entropic considerations, the probability of a side-chain/backbone hydrogen bond (P_Q) in the tripeptide is 0.007]. Hence, the side-chain/backbone hydrogen bonds do not compete effectively with Tyr side-chain/Asn side-chain hydrogen bonds which are present in nearly all of the calculated conformations. Because extended backbone conformations (with Tyr side-chain/Asn side-chain hydrogen bonds) are more conformationally flexible and have fewer backbone/backbone hydrogen bonds than β-bend conformations (with Tyr side-chain/Asn side-chain hydrogen bonds), the extended conformations are entropically favored. This conclusion is examined further below in the conformational analysis of Ac-Tyr-Pro-Ala-NHMe, which lacks these side-chain/side-chain interactions.

The lowest energy conformations of Ac-Tyr-Pro-Asn-NHMe with a *cis*-Tyr-Pro peptide bond has a relative energy of 6.20 kcal/mol and a relative free energy of 3.84 kcal/mol (Table I). The energy is so high because the *cis* conformers cannot adopt the hydrogen bonds that are found among the *trans* conformers. The β-bend probability among the *cis* conformers, however, is very high (Table IV). Of the nine *cis*-Ac-Tyr-Pro-Asn-NHMe conformations with $\Delta E_{min,cis} < 1$ kcal/mol (i.e., $6.2 < \Delta E < 7.2$ kcal/mol), seven have type VI β-bends in the Tyr-Pro portion. Two of these are double bends,[33] with a type VI bend at Tyr-Pro and a type I bend at Pro-Asn. Similarly, very high bend probabilities were calculated for *cis*-Ac-Tyr-Pro-NHMe and *cis*-Ac-Pro-Asn-NHMe (Table III and footnote *e* therein). In the initial stages of folding, any ribonuclease A molecules with *cis*-Tyr^{92}-Pro^{93} peptide bonds will have local β-bend structure within proposed chain-folding initiation site[1,12] E. This does not mean that the majority of ribonuclease A molecules have *cis*-Tyr^{92}-Pro^{93} peptide bonds in the initial stages of folding. Indeed, these calculations show that the *trans*-Tyr-Pro peptide bond is much more energetically favorable than the *cis*-peptide bond in the absence of long-range interactions.

Ac-Tyr-Pro-Ala-NHMe. In order to examine further the role of side-chain/side-chain hydrogen bonds in entropically destablizing β-bend conformations at Pro-Asn of *trans*-Ac-Tyr-Pro-Asn-NHMe, conformational energy calculations were also carried out for Ac-Tyr-Pro-Ala-NHMe. In this tripeptide, there are 81 and 10 low-energy minima with values of $\Delta E < 5$ and <3 kcal/mol, respectively (Table[30] S4). In this molecule, conformational entropy factors play a crucial role in determining the conformations of highest statistical weight; e.g., the Tyr O$^{\eta}$H···OC′Ala hydrogen bond of the lowest energy conformation restricts both backbone and side-chain conformational flexibility, making the free energy of this conformation 1.35 kcal/mol greater than the conformation of lowest free energy which lacks the Tyr side-chain hydrogen bond. The dramatic role of conformational entropy is clearly seen in Table I in which the seven conformations listed (with free energies within 0.5 kcal/mol of the minimum free energy conformation) all have $\Delta E \geq 2.16$ and $\Delta G < -0.85$ kcal/mol relative to the conformation of lowest energy. None of these conformations have entropically unfavorable hydrogen bonds involving the Tyr side chain.

The backbone conformations of highest statistical weight are extended DCF ($P_Z = 0.199$) and DCC ($P_Z = 0.119$) conformations. The DC conformation of Tyr-Pro, as pointed out above, is the backbone conformation of lowest free energy (and lowest energy) for *trans*-Ac-Tyr-Pro-NHMe (Table[30] S12), while the CC and CF backbone conformations at Pro-Ala are the lowest energy and lowest free energy conformations, respectively, of *trans*-Ac-Pro-Ala-NHMe. Hence, as in *trans*-Ac-Tyr-Pro-Asn-NHMe, the short-range interactions inherent in the dipeptide sequences dominate in determining the conformation of this tripeptide.

The remaining minima of *trans*-Ac-Tyr-Pro-Ala-NHMe, however, correspond to a wide range of backbone conformations (Table[30] S4). Thus, unlike *trans*-Ac-Tyr-Pro-Asn-NHMe which adopts predominantly the DCC extended backbone conformation, *trans*-Ac-Tyr-Pro-Ala-NHMe may be regarded as a statistical ensemble of many different low-energy backbone conformations.

The absence of entropically unfavorable Tyr side-chain/Asn side-chain hydrogen bonds among bend conformations of *trans*-Ac-Tyr-Pro-Ala-NHMe causes the β-bend probability (Table II) of Ac-Tyr-Pro-Ala-NHMe (with a bend at Pro-Ala) to be significantly higher than that of *trans*-Ac-Tyr-Pro-Asn-NHMe (with a bend at Pro-Asn), as anticipated above. While *trans*-Ac-Pro-Ala-NHMe and *trans*-Ac-Pro-Asn-NHMe have nearly identical bend probabilities ($P_Z = 0.16$ and 0.17, respectively), addition of the Tyr residue on the N-terminal side has little effect on the bend probability at Pro-Ala ($P_Z = 0.197$ for *trans*-Ac-Tyr-Pro-Ala-NHMe) but decreases it significantly at Pro-Asn ($P_Z = 0.026$ for *trans*-Ac-Tyr-Pro-Asn-NHMe). Hence, the low β-bend tendency of *trans*-Ac-Tyr-Pro-Asn-NHMe is attributable to Tyr/Asn interactions, rather than to a predisposition of Pro-Asn to adopt the extended conformation. In addition, the absence of a competing side-chain/side-chain hydrogen bond in *trans*-Ac-Tyr-Pro-Ala-NHMe leaves the Tyr OH free to interact with the backbone of Ala, energetically stabilizing type I and type III β-bends (see Supplementary Table S4) which are predominant β-bend conformations in the absence of conformational entropy (Table II). The Tyr O$^{\eta}$H···OC′ Ala side-chain/backbone hydrogen bond of these two conformations, however, are entropically unfavorable leading to a lower bend probability when entropy is included in the calculation (Table II). The β-bend conformations which are not destabilized entropically generally correspond to those which involve Tyr backbone/Pro-Ala-NHMe backbone interactions. As one might expect, the entropic destabilization of β-bend conformations of *trans*-Ac-Tyr-Pro-Ala-NHMe due to their side-chain/backbone interactions is much less drastic than the β-bend destabilization effects of side-chain/side-chain hydrogen bonds observed for *trans*-Ac-Tyr-Pro-Asn-NHMe bend conformations.

Only two conformations of Ac-Tyr-Pro-Ala-NHMe with *cis*-Tyr-Pro peptide bonds were observed with $\Delta E < 5$ kcal/mol. Both of these are double β-bends,[33] with a type VI β-bend at Tyr-Pro and a type I β-bend at Pro-Ala (Table I). Similarly, *cis*-Ac-Pro-Ala-NHMe has a very high β-bend probability (Table III). As in the other molecules studied here, the high β-bend forming tendency (Table IV) of the *cis*-X-Pro sequence is independent of side-chain interactions.

Ac-Phe-Pro-Ala-NHMe. In the previous section, it was pointed out that, while Tyr side-chain/Ala backbone hydrogen bonds are present in the lowest energy β-bend conformations of *trans*-Ac-Tyr-Pro-Ala-NHMe, these conformers have little conformational flexibility and are entropically unfavorable. The moderate bend-forming tendency of this sequence therefore arises predominantly from backbone/backbone (rather than side-chain/backbone) hydrogen bonds as well as from interactions inherent in *trans*-Ac-Pro-Ala-NHMe. To provide supporting evidence for this conclusion, conformational energy calculations were also carried out for Ac-Phe-Pro-Ala-NHMe, which is almost identical to Ac-Tyr-Pro-Ala-NHMe except in its ability to form side-chain hydrogen bonds.

trans-Ac-Phe-Pro-Ala-NHMe has a significantly higher bend probability (at Pro-Ala) than either *trans*-Ac-Tyr-Pro-Ala-NHMe or *trans*-Ac-Pro-Ala-NHMe (Tables II and III). Furthermore, because it lacks the ability to form side-chain hydrogen bonds, the bend probability of *trans*-Ac-Phe-Pro-Ala-NHMe is relatively independent of whether or not conformational entropy is included in the calculation (Table II). Comparison of the bend probability of *trans*-Ac-Phe-Pro-Ala-NHMe and *trans*-Ac-Pro-Ala-NHMe (with the bend at Pro-Ala; cf. Tables II and III) reveals that interactions between Ac-Phe and Pro-Ala-NHMe are important in stabilizing β-bends at Pro-Ala. Presumably, similar interactions also would stabilize β-bends in *trans*-Ac-Tyr-Pro-Ala-NHMe (or in *trans*-Ac-Tyr-Pro-Asn-NHMe) except for the fact that entropically unfavorable side-chain/side-chain and side-chain/backbone hydrogen bonds are generally associated with these bends. The potential for solvent to suppress intramolecular side-chain/side-chain hydrogen bonds and enhance the bend-forming tendency of these Tyr-containing sequences is addressed in the Discussion section.

In addition to the conformations with *trans*-Phe-Pro peptide bonds, we observed six minimum-energy conformations with *cis*-Phe-Pro peptide bonds (Table IV). The five of lowest free energy are described in Table I. Four of these six are double bends[33] which are type VI at Phe-Pro and either type I, IV, or VII at Pro-Ala. Similarly, all conformations of *cis*-Ac-Phe-Pro-NHMe ($\Delta E < 3$ kcal/mol) are type VI β-bends (see footnote *e* of Table III).

Calculation of Backbone Vicinal Coupling Constants. In order to evaluate the role of solvent effects on the distribution of molecular conformations, we also calculated backbone vicinal coupling constants for the ensembles of tripeptides with *trans*-X-Pro peptide bonds. The values, obtained by statistically averaging the corresponding coupling constants of the individual conformers, are

presented in Table V. Where available, we also cite the spectroscopically determined values. The significance of these data are addressed in the Discussion section.

Additional Conformation-Dependent Properties. Additional calculated conformational properties for the ensembles of energy-minimized structures are given in supplementary tables for the dipeptides (supplementary Table S13) and tripeptides (supplementary Table S14). In these tables, we have listed the number of starting conformations (N_{sc}), the number of low-energy (ΔE < 5 or 3 kcal/mol) minima (N_m), the energy of the global minimum (E_0), and selected ensemble-averaged internuclear distances (as defined in Figure 1) for each of Ac-Pro-Tyr-NHMe, Ac-Pro-Phe-NHMe, Ac-Pro-Asn-NHMe, Ac-Pro-Ala-NHMe, Ac-Tyr-Pro-NHMe, Ac-Phe-Pro-NHMe, Ac-Asn-Pro-Tyr-NHMe, Ac-Ala-Pro-Tyr-NHMe, Ac-Tyr-Pro-Asn-NHMe, Ac-Tyr-Pro-Ala-NHMe, and Ac-Phe-Pro-Ala-NHMe. In addition, the calculated backbone coupling constants for the ensembles of terminally blocked dipeptides with *trans*- or *cis*-X-Pro peptide bonds are also tabulated in supplementary Table S13.

Discussion

1. Ac-Asn-Pro-Tyr-NHMe: Summary of Experimental Results and Comparison with Conformational Free Energy Calculations. At this point, it is useful to summarize the experimental results presented in the preceding paper[1] and to compare them with the results of these conformational free energy calculations. In both crystalline forms,[1] Ac-Asn-Pro-Tyr-NHMe was found to have a type I β-bend conformation at Pro-Tyr (with a *trans*-Asn-Pro peptide bond) and intramolecular hydrogen bonds involving both the N*H*Me and Tyr N*H* amide protons. These correspond to DAB backbone conformations[4] with (ϕ_{Asn}, ψ_{Asn}, ϕ_{Pro}, ψ_{Pro}, ϕ_{Tyr}, ψ_{Tyr}) values of (−101°, 106°, −58°, −27°, −80°, −8°) and (−108°, 108°, −60°, −28°, −90°, −5°) for crystal forms I and II, respectively. A similar β-bend conformation, in which both the Tyr N*H* and N*H*Me amide protons are intramolecularly hydrogen bonded, is the predominant backbone conformation in aqueous[1] solution. Spectroscopic evidence for this conclusion includes NOE measurements, indicating short interproton distances characteristic of β-bends, identification of hydrogen bond donors by solvent spin-saturation transfer measurements in water, measurements of vicinal coupling constants,[2] and comparison of the Raman spectra of crystals and aqueous solutions, demonstrating a similar (but not identical) backbone conformation for *trans*-Ac-Asn-Pro-Tyr-NHMe in these two environments. No evidence for a β-bend conformation of *trans*-Ac-Pro-Tyr-NHMe was observed in water.

The general conclusion of these conformational free energy calculations indicates a relatively high tendency for *trans*-Ac-Asn-Pro-Tyr-NHMe to adopt a β-bend conformation at Pro-Tyr (P_Z = 0.27) and is in good agreement with the experimental results. The stabilization of β-bends at Pro-Tyr is due to interactions of both the backbone and side chain of Ac-Asn with the Pro-Tyr-NHMe backbone. For this reason, β-bends at Pro-Tyr are more probable for *trans*-Ac-Asn-Pro-Tyr-NHMe than for *trans*-Ac-Ala-Pro-Tyr-NHMe or for *trans*-Ac-Pro-Tyr-NHMe. β-Bend conformations of *trans*-Ac-Asn-Pro-Tyr-NHMe are also stabilized entropically relative to extended structures, since the latter tend to be less flexible conformations with Asn side-chain/Tyr side-chain hydrogen bonds. Although the most probable calculated β-bend conformation is type VII (with a bend at Pro-Tyr), the bend conformation with highest statistical weight is type I (with a bend at Pro-Tyr; see Figure 2C). Several other low-energy DAC and DAA type I β-bend conformations, as well as conformationally similar DAF (type III), DAA (type III), and DAE (type VII) β-bends were observed among the energy-minimized structures.

The low-energy structure with Pro-Tyr in a conformation most like that of the crystal structures is a DAA conformation (ΔG = 1.50 kcal/mol; see Table S1) with (ϕ_{Asn}, ψ_{Asn}, ϕ_{Pro}, ψ_{Pro}, ϕ_{Tyr}, ψ_{Tyr}) of (−142°, 80°, −75°, −18°, −79°, −17°). In this calculated structure the N*H*Me amide proton is intramolecularly hydrogen bonded to the Asn backbone carbonyl, as in the crystal structure.[1] The calculated conformation, however, has a side-chain/side-chain hydrogen bond while, in the crystal structure, the Asn side chain is hydrogen bonded to the Tyr N*H* backbone and the Tyr side chain is part of the intermolecular hydrogen bond network.

It is also instructive to compare the calculated conformations with those of the Asn-Pro-Tyr sequence in the structures of crystalline proteins. For example, Asn[67]-Pro[68]-Tyr[69] has an EAA backbone conformation, with a type III β-bend at Pro-Tyr (and a *trans*-Asn-Pro peptide bond) in the crystal structure[34] of bovine pancreatic phospholipase A_2. As was pointed out in the Results section, type I and III β-bends are two (somewhat arbitrarily distinguished) subtypes of a continuous category of bend types,[32] so that the tendencies of a sequence to form type I and type III β-bends are not different. Several similar type III β-bends (with DAA backbone conformations) were observed among the calculated structures, those of lowest free energy having (ϕ_{Asn}, ψ_{Asn}, ϕ_{Pro}, ψ_{Pro}, ϕ_{Tyr}, ψ_{Tyr}) of (−159°, 83°, −75°, −20°, −79°, −34°; ΔG = −0.67 kcal/mol) and (−160°, 81°, −75°, −24°, −71°, −37°; ΔG = +0.03 kcal/mol) which compare well, especially in the Tyr-Pro portion, with the corresponding backbone dihedral angles in the phospholipase A_2 crystal structure,[34] viz. (−121°, 119°, −57°, −32°, −69°, −25°).

In the Asn[113]-Pro[114]-Tyr[115] sequence of the crystal structure[9,10] of ribonuclease A, the Asn-Pro peptide bond is *cis* and there is a type VI β-bend at Asn-Pro. These calculations reveal that, when the Asn-Pro peptide bond is *cis*, the type VI bend probability at Asn-Pro is 0.98. In the isolated Asn-Pro-Tyr sequence, however, the lowest free energy *cis* conformation is 2.1 kcal/mol higher in energy than the lowest free energy *trans* conformation, indicating that the preference for a *cis*-Asn-Pro peptide bond conformation in the native folded protein arises from long-range interactions. Similar conclusions have been drawn from spectroscopic measurements.[2]

2. Ac-Tyr-Pro-Asn-NHMe: Summary of Experimental Results and Comparison with Conformational Free Energy Calculations. In their crystal structures,[1] both Ac-Tyr-Pro-Asn-NHMe and Ac-Pro-Asn-NHMe have extended backbone conformations, with *trans*-X-Pro peptide bonds. A similar extended conformation was observed for crystalline H-Tyr-Pro-Asn-Gly-OH.[35] Except for a hydrogen bond involving the Asn N*H* backbone amide proton in a fraction of the *trans*-Ac-Tyr-Pro-Asn-NHMe molecules, the conformational analysis in water[1] provides no definitive evidence for a β-bend.

These conformational free energy calculations indicate a very low ($P_Z \sim 0.03$) tendency of Ac-Tyr-Pro-Asn-NHMe to adopt a β-bend when the Tyr-Pro peptide bond is *trans* This is due to the fact that nearly all β-bends of Ac-Tyr-Pro-Asn-NHMe form entropically unfavorable Tyr side-chain/Asn side-chain hydrogen bonds. Hence, extended conformations, like those of the crystal structures,[1,35] are preferred when the Tyr-Pro peptide bond is *trans*.

In the crystal structure[26] of concanavalin A, the Tyr-Pro-Asn sequence occurs twice; Tyr[12]-Pro[13]-Asn[14] has a CCC extended structure while Tyr[67]-Pro[68]-Asn[69] is in the ECB* conformation, with a type II β-bend at Pro-Asn. While CC is a favorable conformation for Pro-Asn of *trans*-Ac-Tyr-Pro-Asn-NHMe and *trans*-Ac-Pro-Asn-NHMe, the C conformation is a high-energy conformation for residues preceding proline.[36] Similarly, while the EC backbone conformation at Tyr-Pro is a stable one in *trans*-Ac-Tyr-Pro-NHMe and *trans*-Ac-Tyr-Pro-Asn-NHMe, the B* backbone conformation of Asn is a very high energy one in Ac-Asn-NHMe,[5] *trans*-Ac-Pro-Asn-NHMe,[15] *trans*-Ac-Tyr-Pro-Asn-NHMe, and Ac-Asn-Ala-NHMe,[17] Ala being residue 70 in concanavalin A. Hence both the CCC and ECB* (type II β-bend) conformations of Tyr-Pro-Asn in concanavalin A must be stabilized by interactions involving residues outside these sequences.

In the crystal structure of ribonuclease A,[9,10] Tyr[92]-Pro[93]-Asn[94] has a type VI β-bend at Tyr-Pro with a *cis*-Tyr-Pro peptide bond.

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(35) Prëcigoux, G.; Geoffre, S.; Hospital, M.; Leroy, F. *Acta Crystallogr., Sect. B*, **1982**, *B38*, 2172.
(36) Schimmel, P. R.; Flory, P. J. *J. Mol. Biol.* **1968**, *34*, 105.

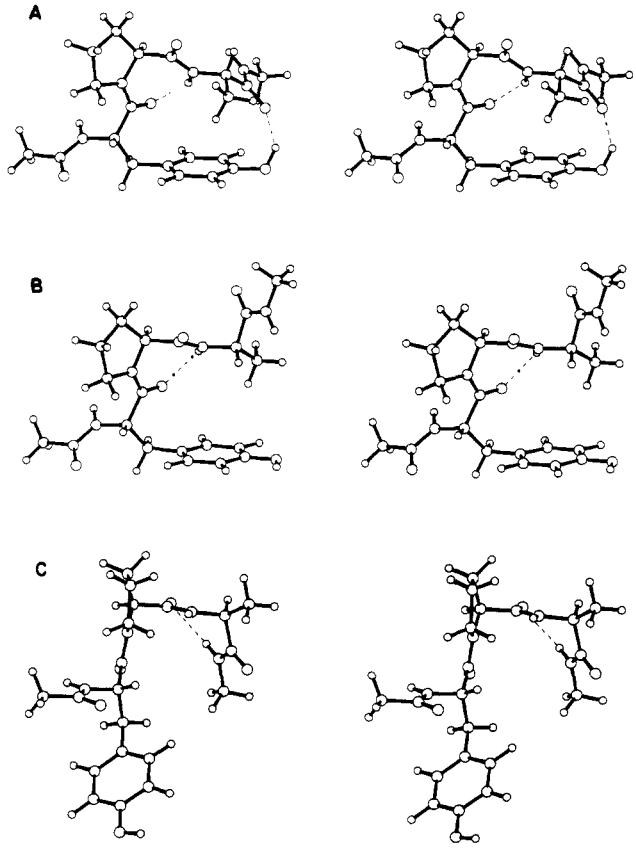

Figure 5. Minimum-energy conformations of Ac-Tyr-Ala-NHMe. (A) Lowest energy conformation with a *trans*-Tyr-Pro peptide bond. ΔE = 0.00 kcal/mol, ΔG = 0.00 kcal/mol. The ϕ and ψ of the three residues are (−153°, 78°, −75°, 71°, −165°, 51°), and the corresponding conformational letter code is DCD. (B) Lowest free energy conformation with a *trans*-Tyr-Pro peptide bond. ΔE = 3.71 kcal/mol, ΔG = −1.35 kcal/mol. The ϕ and ψ of the three residues are (−152°, 82°, −75°, 85°, −68°, 140°), and the conformational letter code is DCF. (C) Lowest free energy β-bend conformation with a *trans*-Tyr-Pro peptide bond. ΔE = 4.47 kcal/mol, ΔG = −0.42 kcal/mol. The ϕ and ψ of the three residues are (−145°, 81°, −75°, −48°, −84°, 76°), and the conformational letter code is DAC. This backbone conformation corresponds to a type I β-bend at Pro-Ala.

In these calculations we have found that, when the peptide bond is *cis*, the type VI bend probability at Tyr-Pro of Ac-Tyr-Pro-Asn-NHMe is very high ($P_Z > 0.95$). However, the lowest free energy *cis* conformation is about 2.4 kcal/mol less stable than the lowest free energy *trans* conformation. Hence, the *cis*-Tyr^{92}-Pro^{93} peptide bond conformation of native ribonuclease A is stabilized by interactions outside the Tyr-Pro-Asn sequence.

In view of this discussion, it is interesting to speculate on the conformation of the sequence Tyr^{23}-Pro^{24}-Asn^{25} of adrenocorticotropic hormone (ACTH), whose solution structure has yet to be determined. Proton NMR studies[37,38] indicate that this Tyr-Pro peptide bond is *trans*. In spite of the fact that statistical compilations[39] indicate a strong tendency for Pro and Asn to occupy positions[27] 2 and 3 of β-bends, these conformational free energy calculations predict a low bend-forming tendency at either Tyr^{23}-Pro^{24} or Pro^{24}-Asn^{25} of ACTH unless these bends are stabilized by interactions outside the Tyr-Pro-Asn sequence or by the effects of solvent.

3. Role of Entropy and Solvent in Peptide Conformational Energy Calculations. In these calculations, we observed that the conformational entropy has a large influence on the relative stabilities of the various low-energy conformations and on statistically averaged conformation-dependent quantities (e.g., bend probabilities). The assumption that each potential well has the same shape around its local energy minimum does not hold for these terminally blocked tripeptides. This phenomenon was also demonstrated previously in calculations of free energies of dipeptides.[15-18] The role of entropy becomes especially important in molecules which can form side-chain/side-chain or side-chain/backbone hydrogen bonds, a feature which was not obvious from earlier calculations on dipeptides.[15-18] The results reported here indicate that the effect of conformational entropy on the relative stability of peptide conformation becomes more important as the length of the peptide chain is increased, and is especially important when side-chain/side-chain interactions (e.g., hydrogen bonds) are possible. While the quantitative aspects of the entropy calculations are sensitive to the form of the empirical potential functions, the results of the computations (indicating that hydrogen bonds restrict conformational freedom) are physically reasonable.

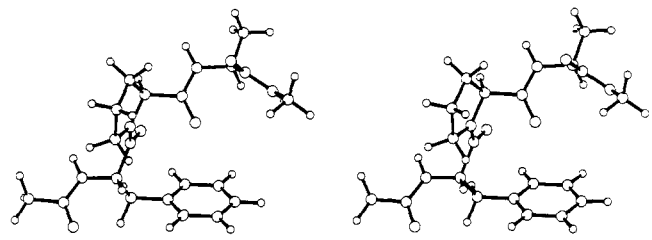

Figure 6. Minimum-energy conformation of Ac-Phe-Pro-Ala-NHMe. Lowest free energy conformation with a *trans*-Phe-Pro peptide bond. ΔE = 2.48 kcal/mol, ΔG = −0.54 kcal/mol. The ϕ and ψ of the three residues are (−153°, 85°, −75°, 164°, −73°, 138°), and the conformational letter code is DFF.

In a separate study (E. R. Stimson, unpublished results), we demonstrated that the distribution of conformations adopted both by *trans*-Ac-Asn-Pro-Tyr-NHMe and *trans*-Ac-Tyr-Pro-Asn-NHMe is solvent dependent. Solvent/solute interactions have not been included in the conformational free energy calculations undertaken here. Because the conformations accessible to these molecules are determined principally by steric factors, we believe that the solvent will act to stabilize particular low-energy conformations among the energy-minimized set, rather than to stabilize high-energy structures not tabulated in the supplementary tables. For example, while these conformational free energy calculations identify the basic physical tendency of *trans*-Ac-Asn-Pro-Tyr-NHMe to form type I or III β-bends at Pro-Tyr, the combined probability for these bend types is much lower than the estimated fraction of β-bend conformations in water, determined experimentally.[1] Similarly, because the strong bend-forming tendency of peptides containing *cis*-X-Pro peptide bonds arises primarily from steric factors, it is unlikely that solvent effects will disrupt the bend-forming tendency, even though they do effect the *cis*/*trans* equilibrium constants.

In Table V, we compare backbone vicinal coupling constants calculated from the low-energy ensembles of conformations with experimentally measured values. Where data are available, these calculated values of $^3J_{NH-C^\alpha H}$ are in quite good agreement with the spectroscopically measured values, particularly those measured in dimethyl sulfoxide (see Table V). One exception is the relatively poor agreement of the calculated and measured values for the Pro-Asn-NHMe portion of *trans*-Ac-Tyr-Pro-Asn-NHMe. β-Bends in this part of the molecule were found to be unfavorable in these conformational free energy calculations because they are generally associated with the entropically destabilizing Tyr side-chain/Asn side-chain hydrogen bond. Selective suppression of this intramolecular hydrogen bond by the solvent could, however, lead to an enhanced β-bend tendency; e.g., relatively high bend probabilities (at Pro-Ala) are observed for *trans*-Ac-Tyr-Pro-Ala-NHMe and *trans*-Ac-Phe-Pro-Ala-NHMe which cannot have side-chain/side-chain hydrogen bonds.

In the preceding paper,[1] we pointed out that sequences corresponding to β-bends in chain-folding initiation sites should be evolutionally conserved, although substitutions of amino acids with structural properties that retain the bend-forming tendency of the sequence should be tolerated. In this study, we found that the bend-forming tendency of Asn-Pro-Tyr (with a bend at Pro-Tyr, when the Asn-Pro peptide bond is *trans*) arises primarily from stabilization of the bend backbone conformation at Pro-Tyr-NHMe by hydrogen bond interactions with the Asn side chain

and backbone. With the assumption that local bend conformations with *trans*-Asn[113]-Pro[114] peptide bonds are parts of chain-folding initiation structures of ribonuclease A (see below), it follows from these arguments that homologous ribonuclease sequences will generally have amino acids preceding Pro[114] which retain the ability to form bend stabilizing side-chain hydrogen bonds with the Pro[114]-Y[115] backbone even though Asn[113] is not intramolecularly hydrogen bonded in the crystal structure[9,10] (which has a *cis*-Asn-Pro peptide bond). The variations observed at position 113 among homologous pancreatic ribonuclease sequences[40] are Gln, Asp, Ser, and Lys, all of which retain the side-chain hydrogen-bonding capability of Asn, while Val[109]-Pro[110]-Tyr[111] is not in a β-bend in the crystal structure[34] of phospholipase A_2. However, as has been pointed out elsewhere,[31] porcine ribonuclease A has the sequence Asn[113]-Pro[114]-Pro[115] for which β-bends (with the Asn-Pro peptide bond *trans*) at Pro[114]-Pro[115] are energetically unreasonable.[31,36]

4. Role of Proline Peptide Bond Conformation in Chain-Folding Initiation Structures of Bovine Ribonuclease A. The refolding of disulfide-intact bovine pancreatic ribonuclease A from thermally and/or guanidine denatured conditions exhibits multiple kinetic phases,[41,42] suggesting that different unfolded forms of the molecule follow different folding pathways. One difference between these unfolded forms has been attributed to proline peptide bond *cis*/*trans* isomerization,[43] since the rate-limiting step for their interconversion shows several features characteristic of proline peptide bond isomerization in model compounds.[43-46] Several kinetic studies indicate that folding to an intermediate enzymatically active conformation (I_N) may not require the native peptide bond conformation at all X-Pro sequences of the protein.[47-50] Conformational energy calculations[31] also indicate that starting conformations of residues 106–124 of bovine ribonuclease A with either *cis*- or *trans*-Asn[113]-Pro[114] peptide bonds can attain minimum-energy β-sheet structures of comparable energy. These minimum-energy conformations can then be interconverted by proline *cis*/*trans* isomerization, keeping the ends of the molecule fixed. The activation energy calculated for this process is 16.5 kcal/mol,[31] since attractive non-bonded interactions in these structures can stabilize the transition state. In these calculations[31] it was found that, when the Asn[113]-Pro[114] peptide bond is *trans*, β-bends may form either at Gly[112]-Asn[113] or at Pro[114]-Tyr[115]. Significantly, in the low-energy structure with the bend at Gly[112]-Asn[113], Gly adopts the C* backbone conformation which is the most energetically favorable in Ac-Gly-NHMe[5,23] and Asn adopts the D backbone conformation which is the most favorable for Asn in *trans*-Ac-Asn-Pro-NHMe[15] and in *trans*-Ac-Asn-Pro-Tyr-NHMe. There is no experimental evidence, however, that short-range interactions will stabilize this type II′ β-bend conformation at Gly[112]-Asn[113].

In this work, and in the preceding paper,[1] we demonstrate that, in populations of Ac-Asn-Pro-Tyr-NHMe with *trans*-Asn-Pro peptide bonds, short- and medium-range interactions favor local β-bend conformations at Pro-Tyr. Furthermore, in populations with the Asn-Pro peptide bond *cis*, β-bend conformations are even more strongly favored. *cis*-Ac-Tyr-Pro-Asn-NHMe is also predominantly a β-bend conformation, but we cannot make a definitive statement regarding the β-bend tendency of *trans*-Ac-Tyr-Pro-Asn-NHMe in water; amide proton saturation transfer measurements in H_2O do not indicate a large fraction of hydrogen-bonded bends, and conformational free energy calculations predict a low bend-forming tendency.

In the absence of long-range interactions (e.g., disulfide bonds), ensembles of ribonuclease conformations with *cis*- and *trans*-Asn[113]-Pro[114] peptide bonds are of similar free energy under conditions of temperature and solution composition for which the native protein is folded, as we have demonstrated elsewhere[2] with a proteolytic fragment corresponding to residues 105–124 of performic acid oxidized ribonuclease. Similarly, the *cis*- and *trans*-Tyr[92]-Pro[93] peptide bond conformers of Ac-Tyr-Pro-Asn-NHMe[1,2] and performic acid oxidized ribonuclease[51] are of comparable free energy. These experimental findings are not consistent with the calculations which show a strong preference for the *trans*-X-Pro conformers of Ac-Asn-Pro-Tyr-NHMe and Ac-Tyr-Pro-Asn-NHMe. This discrepancy may be due to the absence of solvent effects in the calculations.

With regard to the folding of reduced ribonuclease, these calculations and the spectroscopic measurements presented in the preceding paper[1] predict that β-bend conformations may be anticipated within chain-folding initiation site F[12] at Pro[114]-Tyr[115] (and possibly[31] also at Gly[112]-Asn[113]) in the (major) population of molecules with *trans*-Asn[113]-Pro[114] peptide bonds. Molecules which have *cis*-Asn[113]-Pro[114] peptide bonds also have a strong tendency to form native-like type VI β-bends at Asn[113]-Pro[114]. Similarly, molecules which have *cis*-Tyr[92]-Pro[93] peptide bonds have a strong tendency to form (native-like) type VI β-bends at Tyr[92]-Pro[93] within proposed chain-folding initiation site E,[12] but those with *trans*-Tyr[92]-Pro[93] peptide bonds cannot initiate folding with structures having β-bends within Tyr[92]-Pro[93]-Asn[94].

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Supplementary Material Available: Tables of minimum-energy conformations of the *N*-acetyl-*N*′-methylamide of Asn-Pro-Tyr, Ala-Pro-Tyr, Tyr-Pro-Asn, Tyr-Pro-Ala, Phe-Pro-Ala, Pro-Ala, Pro-Asn, Pro-Phe, Pro-Tyr, Pro-Tyr, Phe-Pro, and Tyr-Pro and conformation-dependent quantities of dipeptides and tripeptides (28 pages). Ordering information is given on any current masthead page.

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