Cyclic Peptides. III. Solution Conformations of *cyclo*(Serylprolylglycylserylprolylglycyl) from Nuclear Magnetic Resonance

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Abstract: *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) [*retrocyclo*(Pro-Ser-Gly-Pro-Ser-Gly)] has been synthesized and studied by 220-MHz nuclear magnetic resonance spectroscopy. The spectra, obtained in DMSO-d_6 and in aqueous solution, clearly show two sets of resonances having unequal areas for individual α protons and NH protons, indicating that the cyclic hexapeptide exists in two conformations in solution, designated β_{LT} and Ω_C. The H–N–C_α–H vicinal couplings, NH exchange rates, and temperature dependence of the NH chemical shifts suggest that β_{LT}, the predominant structure in aqueous solution, has all peptide bonds in the trans conformation and contains two Ser–Ser intramolecular hydrogen bonds with Ser (ϕ, ψ) angles corresponding to an L-residue β structure. The major conformation in DMSO-d_6, Ω_C, was found to contain no intramolecular hydrogen bonds and has both Ser–Pro peptide bonds in the cis conformation. By examination of spectra obtained in mixed DMSO-d_6–aqueous solvent systems, the two conformations were shown to be in dynamic equilibrium. It was concluded that the cyclic peptide undergoes a solvent-induced change in relative populations of β_{LT} and Ω_C with these populations dependent only upon solvent composition at a given temperature.

We have reported[2] the synthesis and conformational analysis (by 220-MHz nmr) of *cyclo*(Pro-Ser-Gly-Pro-Ser-Gly) (abbreviated herein *c*-$(PSG)_2$). The major resonances present in the nmr spectra of *c*-$(PSG)_2$ were assigned to two rapidly interconverting β-type structures, each containing two intramolecular Gly–Gly hydrogen bonds and all-trans peptide bonds; an asymmetric conformation containing one cis and one trans Gly–Pro peptide bond was proposed to account for the minor resonances.

We report here the synthesis and a 220-MHz nmr study of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) (abbreviated herein *c*-$(SPG)_2$). Our interest in this cyclic hexapeptide was stimulated by the results obtained for *c*-$(PSG)_2$, since *c*-$(SPG)_2$ is its "retroisomer" and in fact is its only possible C_2-symmetric isomer with L-Ser, L-Pro, and Gly components. The previous study[2] suggested that in cyclic hexapeptides of the form *cyclo*(X-Pro-Y-X-Pro-Y), which contain all-trans peptide bonds, Pro C_α–C=O bonds are trans', and only the residues preceding the prolines form intramolecular hydrogen bonds.[3] Thus, while the presence of a conformation containing two intramolecular Ser–Ser hydrogen bonds in *c*-$(SPG)_2$ could be anticipated, it was further desired to examine the spectra of a second proline-containing cyclic hexapeptide for evidence of conformations having cis, as well as trans, X–Pro peptide bonds.

Synthesis. *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) (*c*-$(SPG)_2$) was synthesized according to the scheme outlined in Scheme I (below). The precursor Ser-Pro-Gly-*p*-nitrophenyl ester hydrobromide (III) was prepared as shown, and subjected to the action of triethylamine in pyridine at high dilution. The product of cyclodimerization, *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) (V), was isolated in 4% yield. The details of the synthesis and characterization of this cyclic peptide are contained in the Experimental Section.

Results and Discussion

Nmr Spectra in Water and Dimethyl Sulfoxide. The 220-MHz nmr spectrum, in the τ 4.7–6.9 region, of *c*-$(SPG)_2$ in D_2O is shown in Figure 1. The assignments indicated were checked by spin decoupling at 100 and 220 MHz. In Figure 1 two sets of resonances of unequal areas, designated T (major) and C (minor), are seen for Ser and Pro α-protons, immediately suggesting the presence of at least two conformations of this cyclic peptide. Resonances corresponding to the (accidentally) equivalent (T) Gly $C_\alpha H_2$ protons (singlet at τ 6 in Figure 1) and one of the nonequivalent (C) Gly $C_\alpha H_2$ protons are also observed. Due to overlapping of the (T and C) Ser $C_\beta H_2$, Pro $C_\delta H_2$, and upfield (C) Gly $C_\alpha H$ resonances, it was not possible to make precise assignments in the τ 6–6.5 region. Similar features are seen in the partial *c*-$(SPG)_2$ spectrum recorded in DMSO-d_6 shown in Figure 2. Note that the major resonances in DMSO-d_6 are labeled C, a reversal of the designations in aqueous solution.

The NH region of the spectrum obtained in H_2O–CH_3COOH (98:2 by volume) is presented in Figure 3a. It is seen that four sets of resonances, two T and two C, are found for each pair of Ser and Gly NH protons of each conformer, with relative areas the same as the area ratios found for the T and C α-proton resonances in Figure 1. Further, in Figure 3a–e, it is seen that increasing the mole fraction[4] M_f, of DMSO-d_6, results in an increase in the area of the C resonances relative to the T resonances. In Figure 3c, where the mole fraction of DMSO-d_6 is 0.7, the T and C res-

(1) (a) Bell Laboratories; (b) Harvard Medical School.

(2) D. A. Torchia, A di Corato, S. C. K. Wong, C. M. Deber, and E. R. Blout, *J. Amer. Chem. Soc.*, 94, 609 (1972).

(3) R. Schwyzer and U. Ludescher (*Helv. Chim. Acta*, 52, 2033 (1969)) have suggested a β-type conformation (designated IIIa by these authors) for *cyclo*(Gly-Pro-Gly-Gly-Pro-Gly), in which the Gly's *following* the Pro's are internally hydrogen bonded. We have found that it is possible to make a model of such a conformation, only if (1) Pro C_α-C=O bonds are cis', and (2) the Pro's are preceded by a Gly or D residue.

(4) The mole fraction, M_f, is defined as the ratio of moles of DMSO-d_6 to total moles of (DMSO-d_6 plus aqueous) solvent.

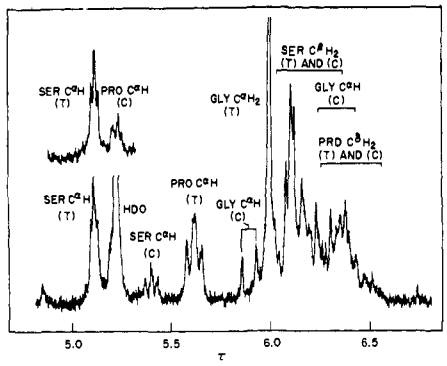

Figure 1. The 220-MHz nmr spectrum of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) in the τ 4.7–6.9 region. Solvent, D_2O, T = 23°. Upper left, T = 45°. Concentration: 15 mg/ml. Chemical shifts (τ scale) given in parts per million downfield from internal *tert*-butyl alcohol (taken at τ 8.77 from DSS).

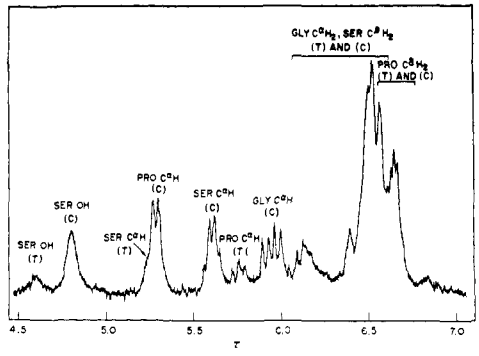

Figure 2. The 220-MHz nmr spectrum of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) in the τ 4.5–7.0 region. Solvent, DMSO-d_6, T = 23°. Concentration: 15 mg/ml. Chemical shifts (τ scale) given in parts per million downfield from internal TMS.

onances have about equal areas. In DMSO-d_6 (M_f = 1.0), Figure 3e, the major resonances in water (T) have become the minor resonances, explaining the *apparent* reversal in assignments mentioned earlier.

These solvent effects are reversible. Starting with either an aqueous or DMSO-d_6 solution, the relative areas of the T and C resonances are found to depend only upon the composition of the solvent mixture at 23°. Also at this temperature, the changes in the areas of the T and C resonances accompanying a change in solvent occurred in less than the time (*ca.* 2–3 min) required to obtain a single scan spectrum.

The H–N–C_α–H coupling constants, designated $J_{N\alpha}$, corresponding to the T and C resonances, are summarized in Table I. Since the (T) Gly α-protons are

Table I. Experimentally Determined H–N–C_α–H Coupling Constants[a,d] in the 220-MHz Nmr Spectra of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly)

Solvent	(T) Gly $J_{N\alpha}$	(T) Ser $J_{N\alpha}$	(C) Gly $J_{N\alpha}$	(C) Ser $J_{N\alpha}$
H_2O–CH_3COOH[b]	13.0[c]	7.0	7.5, 4.0	6.0
DMSO-d_6	12.0[c]	7.0	8.0, 3.5	6.0

[a] In hertz. [b] 98:2 by volume. [c] Value is the sum of the two Gly $J_{N\alpha}$. [d] Uncertainty: $< \pm 0.5$ Hz.

equivalent (as mentioned above), only the sum of the (T) Gly $J_{N\alpha}$ could be determined. To within ±0.5

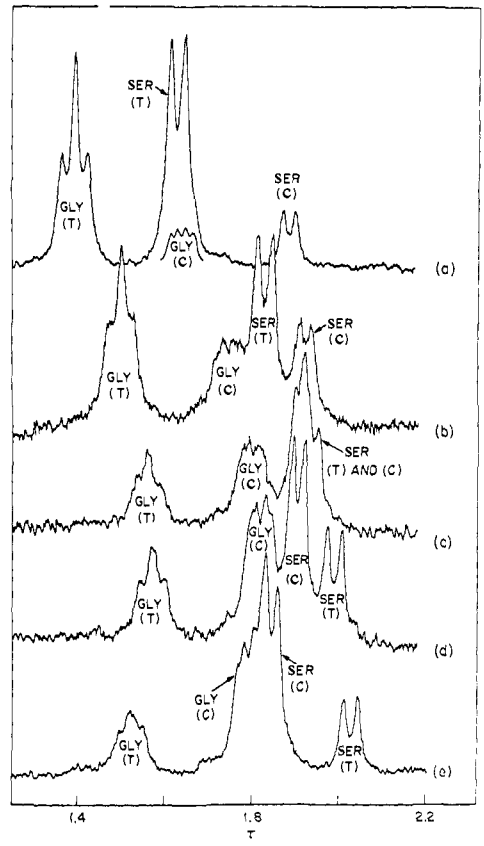

Figure 3. The 220-MHz nmr spectrum of the NH protons of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly). (a) In H_2O–CH_3COOH (98:2 by volume); (b, c, and d) in DMSO-d_6, H_2O–CH_3COOH (98:2 by volume); (e) in DMSO-d_6. In a through e M_f = 0.0, 0.5, 0.7, 0.8, and 1.0, respectively, where M_f is the mole fraction of DMSO-d_6. To achieve the signal to noise ratio shown in these spectra, 15 to 30 scans were accumulated in a CAT. Concentrations: 5–15 mg/ml.

Hz the couplings of the T and C resonances are independent of solvent. In addition, relative areas of T and C resonances are independent of concentration in both solvents over a range 4×10^{-3} M to 3×10^{-2} M.

Temperature and Exchange Studies of NH Resonances. The temperature dependence of the NH chemical shifts is shown in Figure 4a and b. In both H_2O–CH_3COOH, Figure 4a, and DMSO-d_6, Figure 4b, the (T and C) Gly NH resonances and the (C) Ser NH resonances show significant upfield shifts as the temperature increases; by contrast, the chemical shift of the (T) Ser NH resonance is almost independent of temperature. Separate T and C resonances are observed in the NH region of the spectrum over the entire temperature range studied, 3–80°.

In H_2O (pH ~6, 100 MHz, Temp = 3°) only a single broad resonance (line width *ca.* 50 Hz), corresponding to the (T) Ser NH proton, is observed. On lowering the pH to *ca.* 5, the (T) Ser NH resonance sharpens to a doublet with individual lines having widths of 3 to 4 Hz, but resonances corresponding to the other NH protons are not observed, indicating their line widths are still greater than 50 Hz. Therefore, the rate for exchange of the (T) Ser NH protons with solvent is *at least* an order of magnitude *smaller* than the exchange rates of the other NH protons. This result, along with the temperature independence of the (T) Ser NH chemical shift, strongly suggests that only these protons

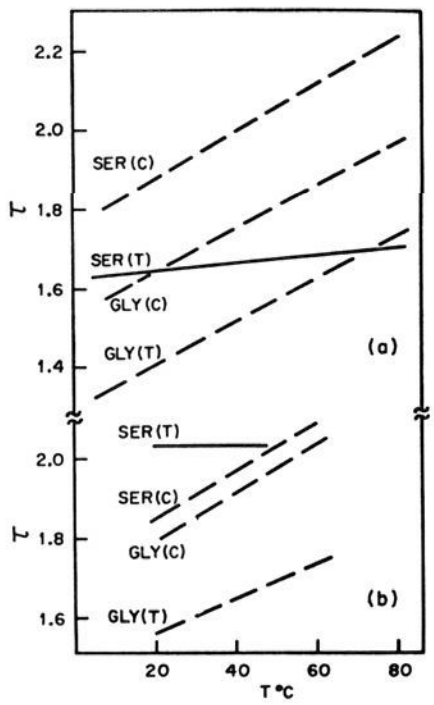

Figure 4. The temperature dependence of the chemical shifts of the NH resonances; (a) in H_2O–CH_3COOH, 98:2 by volume; (b) in DMSO-d_6.

are involved in intramolecular hydrogen bonds. On further lowering the pH, broad (T) Gly and (C) Ser NH resonances are seen[5a] which sharpen to the multiplets shown in Figure 3a at pH $\lesssim$ 4.

Exchange rates for individual NH protons were not determined in DMSO-d_6, since both (T and C) Ser and Gly NH resonances disappear in less than the time required to obtain a spectrum on adding D_2O (20 μl) to a DMSO-d_6 solution (5 mg of *c*-$(SPG)_2$ in 0.35 ml of solvent) at 23°.[5b]

Conformations of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) in Solution. The results above suggest that the cyclic hexapeptide exists in two distinct conformations in solution, designated β_{LT} and Ω_C, corresponding to the double sets of resonances T and C, respectively, seen in Figures 1–3. In aqueous solution about 75% of the *c*-$(SPG)_2$ conformations are β_{LT}, while in DMSO-d_6 80% of the hexapeptide conformations are Ω_C. The coupling constants for the T and C resonances are the same in both solvents, suggesting that the (ϕ, ψ) angles of the conformations β_{LT} and Ω_C are solvent independent, even though their relative populations are not.

On increasing the temperature in either solvent, the areas of the smaller resonances in each case increase relative to the areas of the larger. Since the higher energy state (*i.e.*, the smaller resonances in each case at room temperature) is increasingly populated with increasing temperature, it is evident that the β_{LT} and Ω_C conformers are in dynamic equilibrium.

Figures 1–3 show that both conformers have C_2-symmetry since protons in every *pair* of Ser, Pro, and Gly residues in each conformation have identical chemical shifts. Therefore, both β_{LT} and Ω_C contain two trans ($\omega = 0°$) or two cis ($\omega = 180°$)[6] Ser–Pro peptide bonds.[7]

(5) (a) The (C) Gly NH resonance was obscured by the larger (T) Ser NH resonance. (b) Note that with *c*-$(PSG)_2$ under similar conditions (in DMSO-d_6 with added D_2O), the internal Gly and external Ser NH's each exchanged with a half-life of ~30 min.[2] The results with the two closely related cyclic hexapeptides are illustrative of (a) the complexities in the interpretation of exchange rate data involving multiple conformations, and (b) the fact that correlations of such data between different systems may be difficult.

(6) For explanation of conventions used in rotation angle nomenclature, see J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Nemethy, G. N. Ramachandran, and H. A. Scheraga, *Biopolymers*, **4**, 121 (1966); *J. Biol. Chem.*, **241**, 1004 (1966); *J. Mol. Biol.*, **15**, 399 (1966).

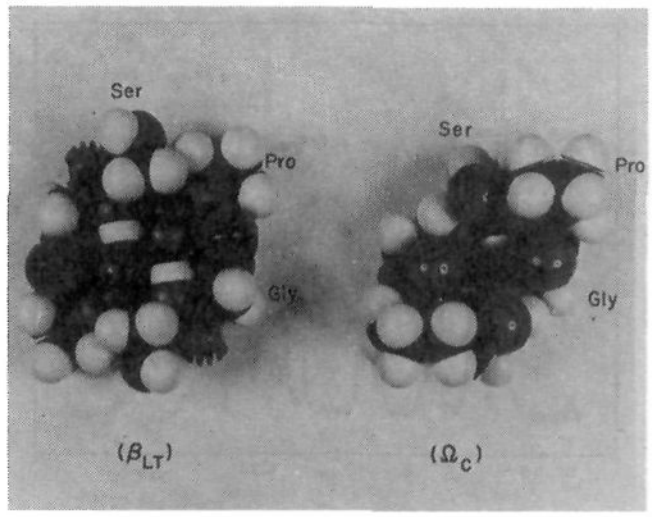

Figure 5. Corey–Pauling–Koltun models of the two proposed solution conformations of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly). Left, β_{LT}; right, Ω_C.

It is proposed that the β_{LT} structure contains all trans peptide bonds since Corey–Pauling–Koltun (CPK) model studies reveal that conformations having two trans Ser–Pro peptide bonds have both Gly NH's exposed to solvent, while both Ser NH's are directed into the center of the molecule. Such a conformation is in accord with the firm evidence presented above for the involvement of (T) Ser NH's in strong internal hydrogen bonds. A conformation (β_{LT}) containing intramolecular Ser–Ser hydrogen bonds, two trans Ser–Pro peptide bonds, and ϕ angles as calculated from the $J_{N\alpha}$ of Table I and Karplus type equations,[8–11] corresponding to the sets of T resonances, is shown in Figure 5. It possesses Ser (ϕ, ψ) angles (Table II) appropriate to an (nondistorted) antiparallel L-residue β structure, trans′ Pro C_α–C═O bonds, and is formally analogous to the conformation designated β_L found[2] for *cyclo*(Pro-Ser-Gly-Pro-Ser-Gly). Note that interconversion to a *c*-$(PSG)_2$-type β_D conformer is precluded in the present case because of severe steric interaction of Ser side chains with neighboring Gly carbonyl and Pro δ-methylene groups. Quantitative support for the β_{LT} model proposed has been provided by approximate energy calculations.[12]

From the data above, particularly the marked upfield shift of all (C) NH resonances with increasing temperature, and the C_2-symmetry requirement, it is further concluded that the Ω_C conformation contains two cis Ser–Pro peptide bonds and no internal hydrogen bonds, a finding supported by the following additional observations. (1) The presence of double resonances for certain protons in the nmr spectrum of (the *c*-$(SPG)_2$ precursor) HBr-Ser-Pro-Gly-ON*p* indicate the occurrence of cis, as well as trans Ser–Pro peptide bonds, as has been reported for other linear oligomers containing proline.[13–15] (2) T and C resonances do not

(7) No experimental evidence for the presence of an asymmetric conformer was obtained from any nmr spectra examined for this cyclic peptide.

(8) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959); *ibid.*, **33**, 1842 (1960).

(9) M. Karplus, *J. Amer. Chem. Soc.*, **85**, 2870 (1963).

(10) M. Barfield and M. Karplus, *ibid.*, **91**, 1 (1969).

(11) V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov, and Yu. A. Ovchinnikov, *Tetrahedron*, **25**, 493 (1969).

(12) A. E. Tonelli, *J. Amer. Chem. Soc.*, **94**, 346 (1972).

(13) V. Madison and J A. Schellman, *Biopolymers*, **9**, 511 (1970).

(14) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, **92**, 6191 (1970).

coalesce at 80° in the spectrum of *c*-(SPG)$_2$, indicating that a high free energy barrier[16] separates the two conformers, such as would be provided by the ~20 kcal barrier to rotation about each Ser–Pro peptide bond. (3) From Figure 3a–e it is seen that a change in solvent can effect an almost complete conversion of the hexapeptide from β_{LT} to Ω_C. This solvent-induced conformational change is not novel in peptide chemistry; for example, isomerization of poly(L-proline) I (all *cis* Pro–Pro peptide bonds) to the all-trans form II is well documented.[17] Solvent effects on the equilibrium of cis and trans isomers have also been reported for linear proline amides[13] and oligomers.[14] Significantly, *c*-(SPG)$_2$ represents the first instance in which a change in population of peptide bond isomers *induced by solvent* is observed for a cyclic peptide. This molecule (dissolved in DMSO-d_6) appears also to be an example of a cyclic peptide in which a conformation containing the generally less-favorable cis X–Pro peptide bond isomers predominates.

A CPK model of *c*-(SPG)$_2$ conformation Ω_C is shown in Figure 5, having two cis Ser–Pro peptide bonds, no internal hydrogen bonds, and containing Ser and Gly ϕ angles in agreement with those predicted by Karplus type equations[3-11] and the (C) $J_{N\alpha}$ of Table I. A summary of the (ϕ, ψ) angles corresponding to both conformers[18] is provided in Table II. Although

Table II. Residue Rotation Angles[a] in Proposed Solution β_{LT} and Ω_C Conformations of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) in Water and Dimethyl Sulfoxide

Residue	$\phi(\beta_{LT})$, deg	$\psi(\beta_{LT})$, deg	$\omega(\beta_{LT})$, deg	$\phi(\Omega_C)$, deg	$\psi(\Omega_C)$, deg	$\omega(\Omega)_C$, deg
Ser	30	330	0	25	325	180
Pro	120	300	0	120	300	0
Gly	300	150	0	240	90	0

[a] Uncertainty ± 15°.

Figure 5 shows that β_{LT} and Ω_C have markedly different molecular shapes the $\beta_{LT} \rightleftharpoons \Omega_C$ transformation involves, in addition to rotations of 180° about the Ser–Pro peptide bonds, only rotations of *ca.* 60° of the Gly (ϕ, ψ) angles.

Despite the requirement of isomerization about two peptide bonds on changing solvent, models indicate *sequential rotations* about the two Ser–Pro peptide bonds can be accomplished without encountering severe steric interactions, suggesting that the $\beta_{LT} \rightleftharpoons \Omega_C$ transformation proceeds through an asymmetric conformation containing one cis and one trans Ser–Pro peptide bond. Such a sequential peptide bond isomerization is preferable to *simultaneous rotation* about both Ser–Pro peptide bonds, since the latter process would require surmounting a barrier of *ca.* 40 kcal (~20 per Ser–Pro bond). The reestablishment of equilibrium found on changing solvent (2–3 min) is consistent with the fact that a pathway of sequential isomerization is available to the system.

Conclusions

It is found in this and companion papers[2,12] that under certain conditions in solution, both *cyclo*(Pro-Ser-Gly-Pro-Ser-Gly) and *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) exist in all-trans β-type conformations, in which only residues preceding proline form intramolecular hydrogen bonds. The cyclic hexapeptide *c*-(PSG)$_2$ rapidly interconverts between conformations β_D and β_L (internally hydrogen-bonded Gly's) in which the Gly (ϕ, ψ) angles alternately correspond approximately to an L- and a D-residue β structure. In contrast, *c*-(SPG)$_2$ exists in solution in a β conformation (β_{LT}) having internally hydrogen-bonded Ser's, with Ser (ϕ, ψ) angles corresponding only to an L-residue β structure; severe steric interactions are present when an attempt is made to place an L-Ser residue into a conformation having Ser (ϕ, ψ) angles corresponding to a β structure with a D residue.

In addition to the all-trans structures of *c*-(PSG)$_2$, an asymmetric (and possibly interconverting) conformation (A) (having one cis and one trans Gly–Pro peptide bond, and one Gly–Gly internal H bond) is present in solution. In *c*-(SPG)$_2$, however, a second symmetric conformation, Ω_C, containing two *cis* Ser–Pro peptide bonds (and no intramolecular H bonds) is found to be in dynamic equilibrium with the all-trans *c*-(SPG)$_2$ conformation, β_{LT}. This latter equilibrium is quite sensitive to solvent and temperature.

Although our investigation, in conjunction with approximate energy calculations,[12] has yielded the detailed structural information summarized above, a number of intriguing questions remain. (1) Why is an asymmetric *c*-(PSG)$_2$ conformation energetically competitive with symmetric β-type conformations, while in *c*-(SPG)$_2$, a symmetric conformation is found in addition to the β-type structure? (2) Why are the relative populations of *c*-(SPG)$_2$ conformations, but not *c*-(PSG)$_2$ conformations, dependent upon solvent and temperature? (3) What is the distinct nature of the interactions responsible for stabilizing *c*-(SPG)$_2$ conformation β_{LT} (trans Ser–Pro bonds) in aqueous solution, but predominantly Ω_C (cis Ser–Pro bonds) in DMSO-d_6? An upper limit of $\leq$ 1 kcal is indicated in the free energy difference between the sets of conformers found for the cyclic hexapeptides in the present experiments;[19] such small energy differences serve to emphasize the subtleties of the interactions (intra- and intermolecular) which determine cyclic peptide conformation.

The facile solvent-induced conformational interconversions described herein add to the growing number of experimental observations[2,13-15,17] which have demonstrated the ability of prolyl residues to maintain cis and trans (X–Pro) peptide bonds in solution. Of the results discussed above, the discovery of solvent-induced changes in populations of trans β_{LT} and cis Ω_C conformers involving the Ser–Pro peptide bonds of

(15) V. J. Hruby, A. I. Brewster, and J. A. Glasel, *Proc. Nat. Acad. Sci. U. S.*, **68**, 450 (1971).

(16) A "high" free energy barrier means that a given rate of interconversion of conformers is sufficiently slow on the nmr time scale so that separate resonances for each conformer can be observed.

(17) F. A. Bovey and F. P. Hood, *Biopolymers*, **5**, 325 (1967), and references therein.

(18) It is possible that additional conformations having two cis Ser–Pro peptide bonds are also present in solution, since energy maps for peptide residues having cis peptide bonds are not available[12] and it remains to be verified that the (Ω_C) conformation proposed is the *lowest energy* cyclic structure consistent with the nmr data.

(19) This follows from the Boltzmann distribution and the relative areas of major and minor resonances obtained from the spectra of both cyclic hexapeptides. The number "1 kcal" should not be confused with much higher free energy barriers (~20 kcal) which must be surmounted for each peptide-bond isomerization.

cyclo(Ser-Pro-Gly-Ser-Pro-Gly) appears to be of particular significance, since it points to a possible bifunctional structural role for the proline residue. On the one hand, the presence of proline in a given peptide chain imparts to it conformational rigidity because of (a) the restriction to rotation about the Pro N–C_α bond (ϕ angle) due to the cyclic nature of the Pro side chain, and (b) the relatively small range of acceptable values of (ϕ,ψ) angles of an L residue preceding Pro.[20] On the other hand, the proline residue clearly allows two distinct, and in fact diametrically opposed, values of ω (0° and 180°) with respect to the preceding residue. Extension of these considerations to naturally occurring cyclic peptides containing proline suggests that their biological activity may be controlled by similar environment-induced changes in molecular conformation.

Experimental Section

Materials and Methods. The materials and methods employed herein were generally as described in the preceding paper.[2]

Nuclear Magnetic Resonance Spectra. Nmr spectra were obtained using the Varian HA-100 and HR-220 spectrometers at Bell Laboratories. Homonuclear spin decoupling was accomplished by Muirhead D-890-B and General Radio 1107-A audio oscillators. In some instances Varian and Fabri-Tek time-averaging computers (CATS) with 1024 channels were employed in conjunction with the HA-100 and HR-220 instruments to improve the signal-to-noise ratio of the spectra. In nonaqueous solvents, tetramethylsilane (TMS) was used as a reference and at 100 MHz as an internal lock. In aqueous solution, the *tert*-butyl resonance at τ 8.77 of *tert*-butyl alcohol-d_1 was used as internal reference and lock.

Scheme I.[a] Scheme of Synthesis of *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) (V) *via* the Cyclodimerization Reaction

tert-Boc-Pro-OH + HBr·H-Gly-ONp
↓ MA
tert-Boc-Pro-Gly-ONp (I)
↓ HBr–HOAc
HBr·H-Pro-Gly-ONp (II)
tert-Boc-Ser-OH ↓ MA
tert-Boc-Ser-Pro-Gly-ONp (III)
↓ HBr–HOAc
HBr·H-Ser-Pro-Gly-ONp (IV)
↓ pyridine–triethylamine
cyclo(Ser-Pro-Gly-Ser-Pro-Gly) (V)

[a] Abbreviations used are: *tert*-Boc = *tert*-butyloxycarbonyl; ONp = *p*-nitrophenyl ester; MA = mixed anhydride formed with isobutyl chloroformate and *N*-methylmorpholine; and HBr–HOAc = hydrobromic acid in acetic acid.

***tert*-Butyloxycarbonyl-L-prolylglycyl *p*-Nitrophenyl Ester (*tert*-Boc-Pro-Gly-ONp) (I).** *tert*-Boc-Pro-OH (2.15 g, 0.01 mole) was dissolved in 50 ml of dimethoxyethane (DME) and the solution was cooled to −15°. *N*-Methylmorpholine (1.12 ml, 0.01 mole) and isobutyl chloroformate (1.31 ml, 0.01 mole) were added and the solution was stirred for 1 hr at −15°. HBr·H-Gly-ONp (2.77 g, 0.01 mole) was added followed by an additional equivalent of *N*-methylmorpholine (1.12 ml); stirring for 1 hr at −15° and 20 hr at room temperature was carried out. A few drops of water were added, and solvents were removed under vacuum at room temperature. The residue was dissolved in chloroform, washed thoroughly with cold water, dried, and concentrated to provide a crude semisolid product. Upon solution of this product in a minimum of chloroform and precipitation with ether, a crystalline material was obtained in 81% yield (3.20 g): mp 153–154°; tlc, one spot (BuOH–AcOH–H_2O, 3:1:1), R_f 0.8.

L-Prolylglycyl *p*-Nitrophenyl Ester Hydrobromide (HBr·H-Pro-Gly-ONp) (II). *tert*-Boc-Pro-Gly-ONp (6.00 g) was dissolved in 40 ml of methylene chloride, and the solution cooled to 0° in an ice bath. A solution of HBr in acetic acid (5.50 *N*) was added slowly (4.50 ml). After 10 min, crystallization of the product began, and ether was added to complete the precipitation. Filtration and washing with ether gave a crude product, which was crystallized from methanol–ether in 80% yield (4.50 g): mp 195–196°; tlc, one spot (BuOH–AcOH–H_2O, 3:1:1), R_f 0.4.

***tert*-Butyloxycarbonyl-L-seryl-L-prolylglycyl *p*-Nitrophenyl Ester (*tert*-Boc-Ser-Pro-Gly-ONp) (III).** This preparation was accomplished by the mixed anhydride procedure as described for *tert*-Boc-Pro-Gly-ONp, with $CHCl_3$ as solvent. Thus *tert*-Boc-Ser-OH (0.68 g) was treated with *N*-methylmorpholine (0.37 ml) and isobutyl chloroformate (0.44 ml) at −15°, HBr·H-Pro-Gly-ONp (II) (1.25 g) and *N*-methylmorpholine (0.37 ml) were added, and the reaction mixture was stirred overnight. Work-up as described above for I yielded *tert*-Boc-Ser-Pro-Gly-ONp as a syrup which could not be crystallized, but which displayed appropriate ir spectrum and tlc, one spot (BuOH–AcOH–H_2O, 3:1:1), R_f 0.8.

L-Seryl-L-prolylglycyl-*p*-nitrophenyl Ester Hydrobromide (HBr·H-Ser-Pro-Gly-ONp) (IV). *tert*-Boc-Ser-Pro-Gly-ONp (5.64 g) was dissolved in 250 ml of methylene chloride at 0°, and treated with 8.50 ml of a 5.50 *N* solution of HBr–HOAc (a 4-molar excess of HBr). The reaction product soon began to separate, and after 20 min the solvents were removed and the residue treated under vacuum to eliminate excess HBr. After washing with ether, the product solidified upon scratching under acetonitrile. Crystallization was accomplished by dissolving the crude solid in a minimum of dimethylformamide and adding acetonitrile slowly to the cloud point: yield, 2.16 g (40%); mp 211–212°. *Anal.* Calcd for $C_{16}H_{21}N_4O_7Br$: C, 41.66; H, 4.59; N, 12.15. Found: C, 41.35; H, 4.59; N, 12.15.

***cyclo*(L-Seryl-L-prolylglycyl-L-seryl-L-prolylglycyl) (*cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) (V).** HBr·H-Ser-Pro-Gly-ONp (0.30 g) was dissolved in 5 ml of dimethylformamide (DMF) which already contained 0.3 ml of glacial acetic acid. This solution was added dropwise to 400 ml of spectroquality pyridine (preheated to 65°) containing 0.5 ml of triethylamine, over a period of 3 hr. After addition was complete, the mixture was stirred for 1 hr at 65°, and then at room temperature for 12 hr. The reaction mixture was concentrated under vacuum and methanol was added to aid the removal of residual DMF. The residue was treated with 10 ml of acetone, which was decanted, to remove *p*-nitrophenol. The residue was then taken up to 10 ml of methanol, upon which crystallization of the product commenced. Filtration provided *cyclo*(Ser-Pro-Gly-Ser-Pro-Gly) in 4% yield (11.2 mg). A further crystallization of a portion of this material from methanol gave an analytical sample: mp 315–319°; ir, absence of terminal COOH absorption above 1700 cm^{-1}; mass spectra, $M^+ - 2H_2O$ = 446 (theoretical value *m/e* 482); after trimethylsilylation, $M^+ + 4$ TMS = 770 (theoretical value *m/e* 770). *Anal.* Calcd for $C_{20}H_{30}N_6O_8 \cdot 0.5CH_3OH$: C, 48.19; H, 6.07; N, 16.86. Found: C, 48.47; H, 6.23; N, 16.54.

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