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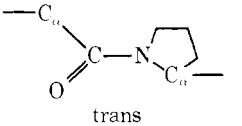
^{13}C Nuclear Magnetic Resonance Study of the Cis–Trans Isomerism in X-Pro-Pro Tripeptides†

Robert E. London,* Nicholas A. Matwiyoff, John M. Stewart, and John R. Cann

ABSTRACT: ^{13}C nuclear magnetic resonance has been used to characterize quantitatively the cis–trans isomerism about both peptide bonds in the tripeptides Ser-Pro-Pro and Arg-Pro-Pro. Detailed pH titration data indicate that the configuration about both peptide bonds is closely linked to titration of the terminal carboxyl group and, to a lesser extent, to titration of the terminal amino group. The Pro^2 C-3 resonance has been found particularly useful for interpretation due to its sensitivity to the isomerization about both peptide bonds. Analysis of the probabilities of the trans–trans, cis–cis, cis–trans, and trans–cis isomers in aqueous solution indicates a stability decrease in the order given. Similarities in the isomerization behavior of the two peptides indicate that side chain interactions involving the first residue have very little effect on the observed cis/trans ratios. The sensitivity of the cis/trans ratio to titration of the terminal amino group is most readily explained on the basis of an indirect effect on carbonyl–carbonyl repulsion.

The interdependence of the configurations about various bonds in model peptides is a conformational question of great importance to understanding the structure and function of proteins and peptide hormones. The oligopeptides containing proline are particularly useful models because, on the one hand, the rotational constraints introduced by the pyrrolidine ring simplify structural analyses considerably and yet, on the other hand, the presence of an X-Pro linkage can introduce structural heterogeneity corresponding to two possible conformations about the X-Pro bond.

Although peptide bonds preceding α-amino acids show an overwhelming preference for the trans configuration, both cis and trans isomers have been observed for peptide bonds preceding the imino acids sarcosine, proline, and hydroxyproline: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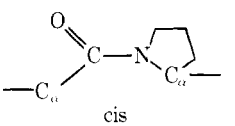
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† From the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545 (R.E.L., N.A.M.), and the Departments of Biochemistry (J.M.S.) and Biophysics and Genetics (J.R.C.), University of Colorado Medical Center, Denver, Colorado 80262. *Received July 18, 1977.* Supported in part by Research Grant RR-00962-01 from the Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, and supported in part by Research Grant HL13909-25 from the National Heart and Lung Institute, National Institutes of Health. This publication is No. 672 from the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado 80262, and supported in part by the U.S. Department of Energy.

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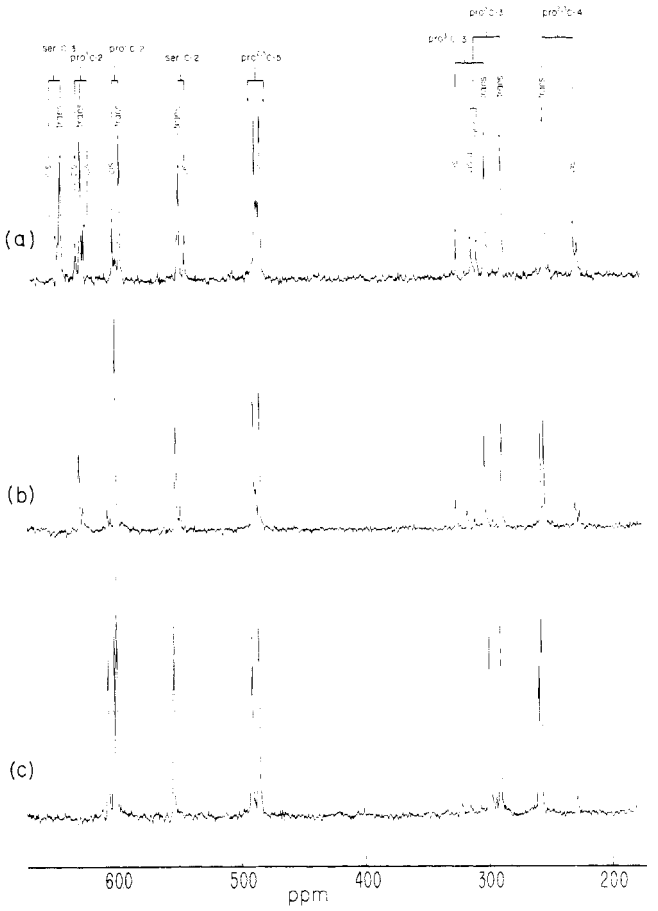


FIGURE 1: Proton noise decoupled ^{13}C NMR spectra of the upfield region of Ser-Pro-Pro at (a) pH 9.7; (b) pH 6.2; (c) pH 1.6.

Observations of *cis*-proline ^{1}H and ^{13}C resonances have been made in both synthetic (Deber et al., 1970; Madison & Schellman, 1970; Torchia et al., 1972a,b; Deber et al., 1972; Thomas & Williams, 1972; Dorman & Bovey, 1973; Dorman et al., 1973; Voelter et al., 1973, 1974; Evans & Rabenstein, 1974; Wu et al., 1975; and references therein) as well as biologically active peptides (Hruby et al., 1971; Patel, 1973; Wurthrich et al., 1972; Deslauriers et al., 1972, 1973a,b; Haar et al., 1975; Galardy et al., 1976). These studies have shown that the cis/trans equilibrium involving X-Pro peptide bonds depends on many factors, including the solvent (Grathwohl & Wurthrich, 1976b; Higashijima et al., 1977), the protonation state of the C-terminal proline carboxyl group (Evans & Rabenstein, 1974; Grathwohl & Wurthrich, 1976a,b), and the identity of the X residue (Grathwohl & Wurthrich, 1976a). Furthermore, it has been found that there are only small stability differences between the cis and trans isomers, suggesting that more than one configuration of X-Pro peptides may be biologically important.

The X-Pro-Pro sequence also occurs frequently in proteins and peptides, e.g., human β lipotropin (Li & Chung, 1976), pancreatic trypsin inhibitor (Kassell & Laskowski, 1965), as well as human hemoglobin β chain, immunoglobin G1 heavy chain, bovine catalase, human carbonic anhydrase B, bovine luteinizing hormone β chain, and soybean protease inhibitor (Dayhoff, 1972). However, there have been few systematic studies of the four cis/trans isomers (trans–trans, trans–cis, cis–trans, and cis–cis) possible in model peptides containing this linkage. In proline oligopeptides like *tert*-butyloxycarbonyl(L-proline)$_n$ benzyl esters, the population of cis linkages depends strongly on the number of proline residues present, random distributions of cis and trans peptide bonds occurring for $n = 2$–4 and an all-trans configuration being dominant for $n = 5, 6$ (Deber et al., 1970). A quantitative analysis of the data for the former peptides ($n = 2$–4) (Tonelli, 1970) indicates that the bond isomerizations are roughly independent, i.e., the probability of a cis–cis isomer is roughly proportional to the product of the probabilities of the occurrence of the cis isomer for each bond.

In the present ^{13}C NMR study, we have analyzed the cis/trans equilibria of the tripeptide Ser-Pro-Pro in detail and report also more limited data for Arg-Pro-Pro. The detailed study was possible because of the inequivalence of the Pro^2 and Pro^3 resonances, the large dependence of chemical shift of Pro C-3 on the bond configuration (Thomas & Williams, 1972) and, in particular, because the Pro^2 C-3 resonance is sensitive to *both* bond izomerizations. In contrast to the study of the *tert*-butyloxycarbonyl(L-proline)$_n$ benzyl esters, the ^{13}C NMR spectra of the free peptide Ser-Pro-Pro has allowed a determination of the effect of the titrations of the C-terminal carboxyl and the N-terminal amino groups on both peptide bonds. A particularly interesting finding is that the isomer with the cis–cis configuration exhibits a significantly higher population than those with the trans–cis and cis–trans when the C-terminal carboxyl group is deprotonated, showing that in the Ser-Pro-Pro and Arg-Pro-Pro tripeptides the peptide bond isomerizations are *not* independent.

Materials and Methods

^{13}C NMR Studies of Peptides. Proton noise decoupled ^{13}C NMR spectra were obtained on a Varian XL-100-15 NMR spectrometer interfaced to a Nova 1210 Computer for operation in the pulse Fourier transform mode. A concentric D_2O capillary was used for the lock. A sweep width of 1250 Hz was used for the upfield peptide region with 1024 data points for an accumulation time of 0.82 s. All spectra were obtained at 27 ± 1 °C. The peptides (~50 mg) were dissolved either in H_2O or in 50% $^{12}CH_3OH$–50% H_2O (v/v) and pH was adjusted by addition of 6 M HCl and 6 M NaOH standard solutions. The $^{12}CH_3OH$ carbon-13 depleted methanol was a gift of Vernon Kerr at the Los Alamos Scientific Laboratory. Peak intensities were determined using a program developed by W. E. Wageman and T. E. Needham for use on the Nova computers which integrates peak areas. The results were generally in excellent agreement with measurements of peak height. In several cases where there was substantial overlap, a cut and weigh method was used.

Peptides. Arg-Pro-Pro was purchased from Cyclo Chemical Co. and Ser-Pro-Pro synthesized by the solid phase method as described previously (Cann et al., 1973, 1976). The peptides and their physical properties are given in Table I of Cann et al. (1973, 1976).

Results

A. Peak Assignments. The ^{13}C NMR spectra in the high-field region for solutions of Ser-Pro-Pro at several pH values are reproduced in Figure 1. As observed previously for Arg-Pro-Pro (Cann et al., 1976), several minor resonances disappear at pH values below the pK (~3.4) of the terminal carboxyl group. The major resonances have been assigned on the basis of comparison with small peptides (Christl & Roberts, 1972; Keim et al., 1974; Cann et al., 1976). The minor resonances have been assigned to species having a cis configuration about one or two of the peptide bonds on the basis of chemical shift (Thomas & Williams, 1972; Evans & Rabenstein, 1974; Dorman et al., 1973). Proline resonances were assigned to either Pro^2 or Pro^3 on the basis of sensitivity to pH titration

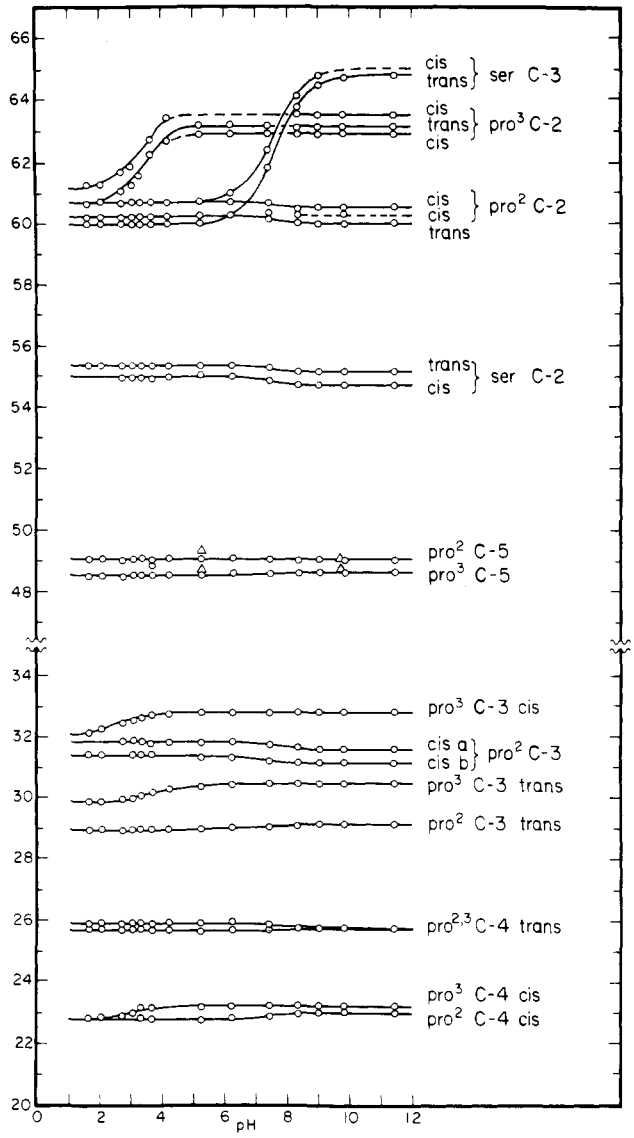


FIGURE 2: ^{13}C chemical shifts of the upfield peaks of Ser-Pro-Pro as a function of pH (○). In some cases, resonance positions could not be obtained due to low intensity of the cis peaks. This applies to most of the cis resonances at low pH and to the Pro³ C-2 downfield cis peak in the region pH 5–8. Overlapping of the cis and trans resonances at some pH values also resulted in an abrupt disappearance of the cis peak at some pH values. This applies to one of the Pro² C-2 cis peaks and to the Ser C-3 cis peak at high pH. Approximate titration behavior is indicated by a dotted line. Chemical shifts of the Pro² and Pro³ C-5 peaks obtained in Arg-Pro-Pro are also indicated in the figure (△).

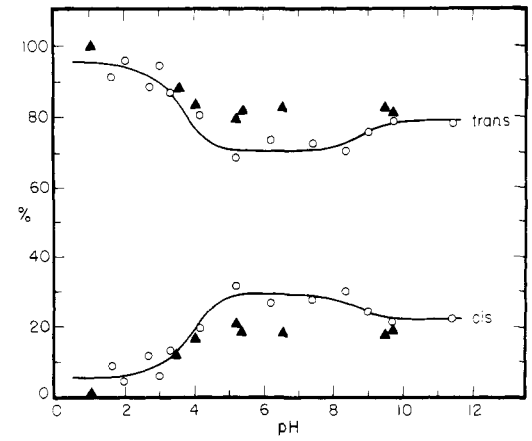


FIGURE 3: Percentages of cis and trans conformers deduced for Ser-Pro-Pro from Pro³ C-3 resonances. Corresponding data obtained for Arg-Pro-Pro are also indicated by the solid symbols.

(Figure 2). The behavior observed appears to be typical for linear peptides in which titration of a terminal amino or carboxyl group produces relatively large chemical shifts for the resonances of the terminal amino acid and smaller but measurable shifts of the adjacent residue (Zimmer et al., 1972; Cann et al., 1976; Christl & Roberts, 1972; Deslauriers et al., 1974). Thus, Pro³ resonances should show a strong sensitivity to the carboxyl titration and the Pro² resonances a weaker sensitivity to both the carboxyl and amino titration; the Pro C-3 cis resonance furthest downfield is assigned to Pro³ and the two cis resonances labeled cis a and cis b (Figures 1 and 2) were assigned to Pro². These assignments give values of δ(cis–trans) of 2.3 ppm for Pro³ and 2.8 ppm and 2.3 ppm for the cis a and cis b resonances of proline 2 at pH 6.2. Typical values of the shift difference δ(cis–trans) for proline C-3 resonances include 2.05 ppm for Gly-Pro, 2.15 ppm for Ala-Pro, 2.48 ppm for Val-Pro (Thomas & Williams, 1972), 2.18 ppm for Gly-Pro-Gly (Fermandjian et al., 1975), 1.97 ppm (pH 9) and 2.25 ppm (pH 1.6) for thyrotropin-releasing hormone (Haar et al., 1975), 2.2 ppm for poly(Pro-Gly) and 2.6 ppm for poly(Gly-Gly-Pro-Gly) (Torchia & Lyerla, 1974). The observation of only two resonances corresponding to *cis*- and *trans*-Pro³ C-3 indicates that the Pro³ C-3 resonance is sensitive only to the isomerism about the Pro-Pro bond. On the other hand, the observation of more than two resonances of Pro² C-3 indicates a sensitivity to both bond configurations. The detailed assignments are discussed in the following section. Minor resonances can be noted for a number of other carbons as well. In particular, Pro³ C-2 exhibits both upfield and downfield satellites, indicating sensitivity of that resonance to both bond configurations also. A fairly well resolved cis resonance can also be seen for Ser C-2 (Figure 1).

B. *Peak Intensities.* The intensities of the Pro³ C-3 cis and trans resonances as a function of pH are summarized in Figure 3. These intensities show a strong pH sensitivity in the pH range corresponding to the carboxyl titration and a somewhat smaller sensitivity in the pH range corresponding to the amino titration. Some representative data for Arg-Pro-Pro are also included in Figure 3, the only apparent difference being a slightly greater fraction of trans isomers. Intensity data for several Ser-Pro-Pro resonances as a function of pH are summarized in Table I. It must be noted in the case of Pro³ C-2 that the abrupt drop in the intensity of the upfield cis resonance (and increase in the trans intensity) as the pH is decreased below 5.2 corresponds to the overlap of the upfield cis and trans peaks. Due to the poorer resolution attainable with these resonances, intensity data are considerably less reliable than those obtained from the proline C-3 resonances. For this reason, quantitation of the four peptide isomers discussed in the following section is based exclusively on the Pro C-3 data. The longer T_1 values and lower nuclear Overhauser enhancement for the carbonyl resonances render these unsuitable for quantitative evaluation.

Although reproducibility of the intensity measurements using different methods (digital integration of peak areas or cut and weight) was always excellent ($\lesssim$5% in most cases, higher for the smaller cis peaks and poorly resolved Pro³ C-2 cis peaks), significant differences were obtained between spectra run at similar pH values before and after extensive pH titrations. In all cases, the latter spectra exhibited ~5% lower cis peak intensities. This effect is most probably caused by the increasing salt concentration in the sample as a result of the

TABLE I: pH Dependence of Fractional Resonance Intensities.

pH	Pro^2 C-3			Pro^3 C-3		Ser C-2[c]		Pro^3 C-2[a]		
	Cis a	Cis b	Trans	Cis	Trans	Cis	Trans	Cis downfield	Cis upfield	Trans
1.6		0.06	0.94	0.09	0.91		1.00	0.05		0.95
1.9[b]		0.03	0.97	0.04	0.96		1.00	0.05		0.95
3.0[b]		0.06	0.93	0.06	0.94	0.05	0.95	0.03		0.97
3.3[b]	0.06	0.04	0.90	0.13	0.87	0.09	0.91			
4.2[b]	0.13		0.87	0.20	0.80	0.12	0.88	0.01		0.99
5.2	0.20	0.07	0.73	0.31	0.69	0.21	0.79		0.28	0.68
6.2	0.16	0.08	0.73	0.27	0.73	0.19	0.81		0.30	0.70
7.4	0.18	0.12	0.70	0.28	0.72	0.20	0.80		0.30	0.70
9.0	0.19	0.16	0.66	0.24	0.76	0.16	0.84	0.06	0.25	0.68
9.7	0.18	0.15	0.67	0.21	0.79	0.18	0.82	0.16	0.22	0.63
11.4	0.18	0.14	0.68	0.22	0.78	0.21	0.79	0.08	0.22	0.70

[a] As indicated in Figure 2, the Pro^3 C-2 upfield cis resonance is resolved only above pH 5. The apparent abrupt changes in intensity above this pH correspond to an abrupt change in resolution rather than in isomer composition. The Pro^3 C-2 downfield cis peak is similarly not well resolved at intermediate pH values. [b] Spectra obtained near the end of the experiment representing samples with greater salt concentrations due to the previous titrations. Cis peak intensities were consistently low compared with spectra obtained at similar pH values earlier in the experiment. [c] The assumption that the Ser C-2 cis resonance corresponds to the two configurations with the Ser-Pro bond cis gives somewhat different numerical results than those summarized in Table II. Comparison with Pro^2 C-3 data indicates that the Ser cis resonance probably corresponds to only the "cis a", i.e., the cis-cis configuration.

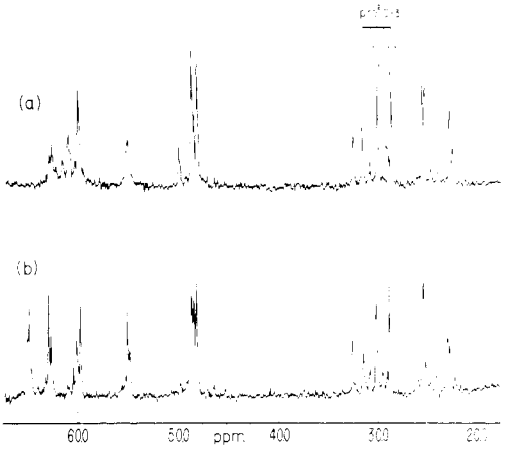


FIGURE 4: Proton noise decoupled ^{13}C NMR spectra of Ser-Pro-Pro in 50% $^{12}CH_3OH$–50% H_2O at (a) pH 7.2; (b) pH 11.9. Resonance assignments for Pro^2 C-3 indicated in a are discussed in the Discussion section.

addition of NaOH and HCl in the pH titrations. Similar decreases in the cis/trans ratio for Ala-Pro as a function of increasing $NaClO_4$ concentration have been noted by Grathwohl & Wurthrich (1976b). The mechanism for this effect is presumably a reduction of the electrostatic repulsions which are important in determining the relative cis/trans stabilities under conditions of increasing ionic strength.

Interpretation of the intensity measurements in terms of isomer concentrations is based on the assumption of equal nuclear Overhauser enhancements and equal spin–lattice relaxation times for cis and trans resonances. Deslauriers et al. (1974), Fermandjian et al. (1975), and Fossel et al. (1975) all report minor differences in the proline carbon spin–lattice relaxation of several cis and trans peptides. In addition, based on the molecular weight of Ser-Pro-Pro, the motion should be sufficiently rapid to ensure a full nuclear Overhauser enhancement for all proton-bearing carbons. It thus appears that the proportionality of peak intensity to isomer concentration should be valid.

Experiments in 50% $^{12}CH_3OH$–50% H_2O. The observation of three separate resonances for Pro^2 C-3 presents a problem of interpretation. If the chemical shift of this resonance is sensitive to both peptide bond isomerizations, four peaks should be discernible. In order to resolve this problem, we have obtained several spectra in a different solvent system: 50% $^{12}CH_2OH$–50% H_2O. (The $^{12}CH_3OH$ ^{13}C depleted methanol, which was used in order to minimize the size of the solvent resonance, showed only a small peak at 49.9 ppm corresponding to the residual $^{13}CH_3OH$.) ^{13}C spectra obtained in this system are nearly identical with those obtained in H_2O with several exceptions: (1) The intensities of the *cis*-Pro C-3 resonances are somewhat higher than those observed in H_2O. (2) The C-2 resonances appear somewhat broader. This probably reflects the increased intensities of the closely spaced cis peaks observed in the H_2O spectra which are no longer resolvable. (3) An additional peak slightly downfield of the *trans*-Pro^2 C-3 resonance is clearly resolved (Figure 4). We believe that this resonance corresponds to the fourth cis/trans isomer which is not resolved in pure H_2O. Resonance assignments of Figure 5 are discussed in the following section.

Comparison of Ser-Pro-Pro and Arg-Pro-Pro Assignments. Despite the overwhelming similarities in the isomerization behavior of Arg-Pro-Pro and Ser-Pro-Pro there are several interesting differences. First, the Ser C-2 cis resonance is ~0.5 ppm upfield of the trans peak, whereas in Arg-Pro-Pro the Arg C-2 cis resonance is ~0.5 ppm downfield of the trans peak (see Figure 6 of Cann et al., 1976). This surprising difference underlines the difficulty of interpretation of the Ser C-2 cis/trans intensity ratio. A second difference is observed in the pH behavior of the two (trans) Pro C-5 resonances. As discussed by Cann et al. (1976), the assignment of these peaks in Arg-Pro-Pro is based on the greater sensitivity of the downfield peak to the amino titration. The corresponding resonances in Ser-Pro-Pro exhibit less sensitivity to this titration and have been assigned by comparison with Arg-Pro-Pro.

Discussion

Assignment of Minor Resonances. As is noted in the previous section, the minor resonances observed in the Ser-Pro-Pro and Arg-Pro-Pro spectra are assigned to X-Pro cis–trans isomers, an assignment based principally on the chemical shifts

of the minor proline C-3 and C-4 resonances. Other slowly exchanging conformations such as the cis′ and trans′ states which are related by 180° rotation about ψ have also been identified in proline containing peptides (Deber et al., 1974), e.g., *cyclo*-(L-Pro-Gly)$_2$, in which the C-2 resonances were found to undergo greater shifts than the proline C-3 and C-4 peaks. In addition, it is uncertain whether the steric barrier to the cis′–trans′ equilibrium in Ser-Pro-Pro is sufficient to result in slow exchange at 27 °C. Even in the highly constrained *cyclo*-(L-Pro-Gly)$_2$ system the Pro C-2 cis′ and trans′ peaks are not well resolved at 32 °C (Deber et al., 1974). We note further that the cis′ $\rightleftarrows$ trans′ barrier for a Pro-Pro peptide has recently been estimated at 10 kcal/mol (Madison, 1977); however, the 15–16 kcal/mol barrier for the urethane bond is insufficient to produce well-resolved cis and trans ^{13}C resonances for Pro C-3 and C-4 (Young & Deber, 1975).

In the following discussion, the Ser-Pro bond is denoted by P and the Pro-Pro bond by P′. On the basis of the observed chemical shifts we have assigned the Pro^3 cis resonances to isomers in which the P′ bond is cis, i.e., to the trans-cis + cis-cis isomers. The observed "trans" peak thus represents the sum of the trans-trans and cis-trans resonances. Similarly, *both* the cis a and cis b resonances corresponding to Pro^2 C-3 are assigned to isomers in which the P bond is cis, i.e., to the cis-cis and cis-trans isomers. Although the Pro^2 C-3 resonance is sensitive to both bond isomerizations, its chemical shift should be perturbed considerably more by isomerization about the P bond. This conclusion is based primarily on the ^{13}C shifts observed for the C-3 resonance of amino acid X in several X-Pro systems. Thus, δ(cis-trans) for Ala C-3 in Ala-Pro is 0.22 ppm, for Val C-3 in Val-Pro δ(cis-trans) = −0.54 ppm (Thomas & Williams, 1972). and for the Ser C-3 resonance in Ser-Pro-Pro δ(cis-trans) varies with pH and is ca. +0.25 ppm at pH 9.0.

Although isomerization of the P′ bond probably cannot produce a 2 ppm shift of the Pro^2 C-3 resonance, it apparently is sufficient to produce the 0.5 ppm shift difference between the cis a and cis b resonance. It must be noted at this point that the effects of the cis/trans isomerizations about bonds P and P′ on the Pro^2 C-3 resonances are clearly *not* additive. If they were, then on the basis of shift difference between the cis a and cis b resonances, the isomerization of bond P′ should lead to a 0.5-ppm shift of the resonance. Thus, a peak corresponding to the trans-cis isomer should be either 0.5 ppm upfield or downfield of the trans-trans resonance. We believe that this peak has been resolved in the 50% methanol solution in which a clear peak 0.26 ppm downfield of the larger trans-trans peak is observed (Figure 4). Thus, nonadditive effects of the P and P′ bond isomerizations on the chemical shift of Pro^2 C-3 emphasize the existence of long-range interactions which may, for example, change the average dihedral angles about bonds which are free to rotate.

Additional confirmation of the above assignments comes from a consideration of the chemical shifts of the cis a and cis b resonances as a function of pH. Upon deprotonation of the terminal amino group both the cis a and cis b resonances exhibit a 0.3-ppm upfield chemical shift, whereas the "trans" peak undergoes a smaller 0.1-ppm downfield shift. Based on CPK models of Ser-Pro-Pro in which the most probable conformation has been chosen to minimize steric effects, the terminal amino group comes closer to Pro^2 C-3 and to the Pro^2 carbonyl in conformations in which the P bond is cis (Figure 5c,d). The effect on the Pro^2 C-3 chemical shift could be via a through-space electrostatic effect or it could arise from an amino-carbonyl hydrogen-bonding interaction involving the Pro^2 carbonyl. Thus, the sensitivity of the Pro^2 C-3 resonances

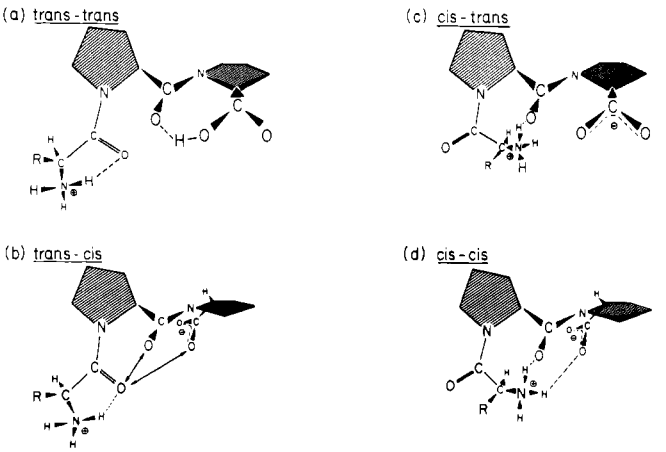


FIGURE 5: Representations of the four possible structural isomers of Ser-Pro-Pro at varying pH values. (a) Trans-trans. The Pro^3 carboxyl-Pro^2 carbonyl hydrogen bond formed at low pH is illustrated as well as the proposed hydrogen bond between the terminal amino and Ser^1 carbonyl. (b) Trans-cis isomer at neutral pH. The repulsive carbonyl-carbonyl and carbonyl-carboxyl interactions which destabilize this state are indicated with double headed arrows. (c) Cis-trans isomer. The possible amino-carbonyl hydrogen bond is indicated. (d) Cis-cis isomers. The possible amino-Pro^2 carbonyl hydrogen bond and possible amino-carboxyl salt bridge are indicated.

to the terminal amino titration appears to be consistent with the assignment of the cis a and cis b resonances to the cis-cis and cis-trans isomers.

The fact that an additional Pro^2 C-3 peak is observed in 50% methanol solutions provides a basis for distinguishing between the cis-cis and cis-trans Pro^2 C-3 resonance. Based on the above discussion, this resonance, which is 0.26 ppm downfield of the trans peak, is assigned to the trans-cis conformer. It then becomes possible to compare the intensity of the Pro^3 trans-cis + cis-cis peak with the intensity of the Pro^2 trans-cis + cis a or trans-cis + cis b peaks. The former comparison is considerably better, particularly at high pH. For example, at pH 8.5 we measure 37% for the Pro^3 trans-cis + cis-cis peak compared with 23% for the Pro^2 trans-cis + cis b resonance and 36% for the Pro^2 trans-cis + cis a resonance. We, therefore, conclude that the cis a resonance represents the cis-cis peptide and the cis b resonance the cis-trans peptide. This assignment is also reasonable because it predicts that the effect of the cis P′ bond on the Pro^2 C-3 resonance will be a downfield shift regardless of whether P is cis or trans. Although the effects of the two bond isomerizations are clearly not quantitatively additive, this assignment seems preferable to the reverse one which would require the cis P′ bond to induce an upfield shift if P is cis and a downfield shift if P is trans. Finally, the interpretation given above appears to be more readily understandable on the basis of molecular models, as discussed below.

Populations of the Four Peptide Isomers. The populations of the four peptide isomers calculated for a variety of solvent conditions are summarized in Table II. For the H_2O solutions, values for the trans-cis isomers could not be obtained directly and were calculated by subtracting the intensity of the Pro^2 cis-cis peak from that of the Pro^3 [cis-cis + trans-cis] peak. For the 50% methanol solutions the values were obtained by measurement of the intensities of the four resolvable Pro^2 C-3 resonances. The intensities thus obtained for the cis-cis + trans-cis isomers were then compared with the intensity of the Pro^3 C-3 cis-cis + trans-cis peak, and the agreement was generally within 5%.

The most surprising feature of these results is the fact that

TABLE II: Relative Populations (%) of Peptide Isomers.

Isomer P-P′	H_2O			50% H_2O–50% CH_3OH	
	pH 1	pH 7	pH 11	pH 7	pH 11
Trans-trans	91	65	61	46	49
Cis-trans	3	6	16	14	17
Trans-cis	6	9	3	15	8
Cis-cis	~0	20	20	25	25

except at very low pH values the cis-cis isomer is the second most stable peptide conformation. If the two bond isomerizations were assumed to be independent so that the probability of a cis P conformation were P_1, and the probability of a cis P′ conformation were P_2, the cis-cis probability would be $P_1P_2 \ll 0.2$. Clearly this hypothesis is incorrect, and the strongest evidence for this is the dependence of both the P and P′ bond isomerization on the titration of the terminal carboxyl group (pH 1 and 7 data in Table II).

As is evident from Table II, there is a significant decrease in the probability of the trans-trans isomer in methanol-water relative to water. Each of the isomers containing one or more cis bonds shows a small increase so that the relative stabilities of these conformations do not appear to be significantly altered. These observations are consistent with the trend observed in a series of oligoproline peptides studied by Rothe & Rott (1976) such that solvents which form stronger hydrogen-bonding interactions with peptide CO groups stabilize the trans configuration about the peptide bonds. Such solvent effects appear to be maximal for relatively short peptides which should be most analogous to the system studied here.

Comparison of Isomer Probabilities with Molecular Models. The probabilities obtained for the four peptide isomers differ significantly from those obtained previously for *tert*-butyloxycarbonyl(L-proline)$_n$ benzyl esters (Deber et al., 1970; Tonelli, 1970). Whereas in the di- and triproline derivatives no cis-cis or cis-cis-cis isomers were detected, in Ser-Pro-Pro the cis-cis isomer appears to be more probable than either the trans-cis or the cis-trans isomer. In order to interpret these results, molecular models corresponding to the four peptide isomers were examined (Figure 5).

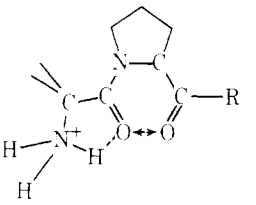
In the trans-trans conformation a hydrogen bond between the Pro^3 carboxyl and the Pro^2 carbonyl which can form at low pH may be a factor stabilizing the trans configuration of the Pro-Pro bond as has been postulated previously for X-Pro and X-Sar peptides (Evans & Rabenstein, 1974; Gerig, 1971). We note in addition that in Ser-Pro-Pro acceptance of a hydrogen bond by the Pro^2 carbonyl will reduce the carbonyl-carbonyl electrostatic repulsion between the Ser carbonyl and the Pro^2 carbonyl explaining in part why, at low pH, the all-trans conformation is so strongly predominant (Figure 5a and Table II). This interaction may be one basis for the linkage of isomerization about both peptide bonds to deprotonation of the terminal carboxyl.

The relatively low stability of the trans-cis isomer may reflect the fact that this structure appears to have maximal proximity of all carbonyl and carboxyl groups (Figure 5b). This conclusion is consistent with recent studies of Grathwohl & Wurthrich (1976) indicating the carboxyl-carbonyl repulsion may be the dominant factor causing an increase in the cis/trans ratio upon deprotonation of the terminal carboxyl group in X-Pro peptides. Further, Grathwohl & Wurthrich observe considerably less cis isomer in the X-Pro bond of Ala-Ala-Pro and Ala-Ala-Ala-Pro compared with Ala-Pro, consistent with greater repulsion in the trans-cis and trans-trans-cis peptide configurations. Finally, the repulsive interactions in Ser-Pro-Pro will be greater than in *tert*-butyloxycarbonyl-Pro-Pro benzyl ester, in which there are no charged groups, consistent with the fact that in the present case the cis-cis isomer is more probable than the trans-cis isomer.

A final factor which is evident from the models is the possibility of hydrogen bonding between the terminal amino group and the Pro^2 carbonyl in the cis-trans and cis-cis isomers (Figures 5c,d). A salt bridge between the terminal carboxyl and amino groups in the cis-cis isomer may also be significant, although there is no specific evidence for such an interaction.

Effect of Amino Group Deprotonation on Cis-Trans Isomerism. It is interesting that the cis-cis + cis-trans total probability increases from 26% at neutral pH to 36% at high pH in H_2O and behaves similarly in CH_3OH-H_2O (Table I). We note that an analogous phenomenon appears to occur in the dipeptides Gly-Pro, Ala-Pro, and Leu-Pro studied by Grathwohl & Wurthrich (1976a) and Fermandjian et al. (1975); in each case a significant increase in the cis probability occurs upon deprotonation of the terminal amino group: 7% for Gly-Pro, 14% for Ala-Pro, and 15% for Leu-Pro. The possibility that these changes reflect an amino-carboxyl interaction between the two terminal groups is inconsistent with the fact that such an interaction occurs in the cis isomer. Thus, the observed increase in probability would imply that the NH_2-^-O_2C interaction is stronger than the NH_3^+-^-O_2C interaction.

One explanation for this behavior may be an indirect effect of the terminal amino group in modulating the carbonyl repulsions. Specifically, the formation of a hydrogen bond between the amino and carbonyl group of the N-terminal residue leads to a relatively stable five-membered ring configuration, thereby reducing the effective field produced by the carbonyl oxygen of the N-terminal amino acid and, in turn, reducing the carbonyl-carboxyl repulsions. An inductive interaction may also contribute to this effect.



This indirect effect should be more pronounced if there is a net positive charge for the amino group to share with the carbonyl. Thus, at high pH reduction of the indirect effect should lead to increased carbonyl-carbonyl repulsion and consequently a destabilization of the trans isomers.

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