On the Solution Conformation of Bradykinin and Certain Fragments†

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ABSTRACT: A circular dichroism (CD) study of [D-Pro^2]- and [D-Pro^3]-bradykinin, selected peptide fragments, and the model compound, *N*-acetyl-L-phenylalaninamide, support our previous conclusion (*Biochemistry 12*, 3780, 1973) that the positive 221-nm CD band of bradykinin is a composite of bands due to two chromophores, the 217-nm band characteristic of the Phe residues overlying the 223-nm band of the N-terminal sequence, Arg-Pro-Pro. The results also indicate that the 223-nm band of Arg-Pro-Pro is associated with the configuration of the Pro-Pro sequence, Arg-D-Pro-Pro and Arg-Pro-D-Pro virtually being diastereoisomers. Accordingly, the conformation of Arg-Pro-Pro was probed in further detail. Upon increasing the temperature from about 27 to 65 °C, Arg-Pro-Pro undergoes a conformational transition characterized by large positive values of ΔH° and ΔS°, which is interpreted to mean that the structure of water and, thus, solute–solvent interactions play a dominant role in determining the conformation of the peptide. ^{13}C nuclear magnetic resonance spectroscopy indicates that the effect of lowering the pH on the CD of Arg-Pro-Pro is explicable in terms of hydrogen-bond formation between the carboxyl group and Pro^2 carbonyl oxygen at acid pH with concomitant cis to trans isomerization.

The circular dichroism (CD) spectrum of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) shows a weak negative band centered at 234 nm and a weak positive band at 221 nm (Figure 1). In a previous study (Cann et al., 1973) of the CD behavior of bradykinin, several of its analogues and peptide fragments and model compounds, the 234-nm band was assigned to a hydrogen-bonded configuration in which a 3 → 1 type hydrogen bond bridges the Pro^7 residue. Evidence was presented that the 221-nm band is a composite of bands due to two chromophores: the 217-nm band characteristic of the Phe residues overlying the 223-nm band associated with the N-terminal sequence, Arg-Pro-Pro.

Our assignment of the 234-nm band to a hydrogen-bonded configuration finds support in a preliminary communication of Filatova et al. (1973). These investigators conclude from the comparative CD behavior of bradykinin and 8-phenyllactic acid-bradykinin, an analogue in which the amide group between amino acid residues 7 and 8 is replaced by an ester bond, that the NH group of Phe^8 in bradykinin participates in a hydrogen bond. From the temperature dependence of the NH chemical shifts in the 100-MHz proton nuclear magnetic resonance (NMR) spectrum of bradykinin, they further conclude that the NH proton of one amino acid residue participates in an intramolecular hydrogen bond.

We have now examined the CD of two D-Pro containing analogues[1] of bradykinin and selected peptide fragments and the CD and ^{13}C NMR of Arg-Pro-Pro. The results presented below focus attention on the conformational origin of the 221-nm CD band associated with the N-terminal sequence of bradykinin.

Materials and Methods

Bradykinin and its D-Pro analogues and peptide fragments used were synthesized by the solid-phase method as described previously (Cann et al., 1973), except for Arg-Pro-Pro and Arg-Gly-Gly, which were purchased from Cyclo Chemical Co. The peptides and their physical properties are given in Table I. *N*-Acetyl-L-phenylalaninamide was obtained from Cyclo Chemical Co.

The peptide trifluoroacetates when dissolved in water to a concentration of about 0.15 mg/ml gave about pH 4; the peptide acetates, about pH 5. The pH was adjusted with either HCl or NaOH.

CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment fitted with a thermostatable cell holder. The temperature of the solution was checked with a thermistor probe. Slits were programmed to yield a 15-Å bandwidth at each wavelength. Concentrations and path lengths were dictated by the absorbancy of the solution. Since measurements were extended down to 188 nm it was necessary to eliminate the possibility of artifactual CD signals on passing through intense absorption bands. Such error signals are caused by mechanical vibrations in the instrument. Our instrument was tested in accordance with the manufacturer's recommendations and found to be free of this effect. In any case, our experimental conditions were not those required to produce the effect; and the CD spectrum of *N*-acetyl-L-phenylalaninamide was the same, within experimental error, for absolute absorbances at 190 nm of 0.82 and 1.63. Molar

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[1] Abbreviations used are: [D-Pro^2]-bradykinin, Arg-D-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; [D-Pro^3]-bradykinin, Arg-Pro-D-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

Table I: Properties of Synthetic Peptides.

Compound[a]	Partition Coefficient[b]	Electrophoretic Mobility[c]	Amino Acid Composition[d]					
			Arg	Ser	Pro	Gly	Phe	Ile
[D-Pro^2]-Bradykinin	1.30 (T)	0.60	2.00	1.00	2.94	1.12	1.98	
[D-Pro^3]-Bradykinin	1.29 (T)	0.62	2.25	1.10	2.92	0.91	2.00	
Arg-D-Pro-L-Pro	0.12 (T)	0.67	1.00		1.92			
Arg-L-Pro-D-Pro	0.15 (T)	0.66	1.00		1.95			
Ile-L-Pro-L-Pro	*e*	0.50			2.26			1.00
Ser-L-Pro-L-Pro	0.210 (A)	0.51		1.00	2.22			

[a] For Arg-L-Pro-L-Pro, Arg-L-Pro-L-Pro-Gly, and Ser-Pro-Phe-Arg, see Table I in Cann et al. (1973). [b] Determined after countercurrent distribution in 1-butanol–acetic acid–water (4:1:5)(A) or 1-butanol–1% trifluoroacetic acid (T). [c] Relative to lysine on paper electrophoresis in 0.1 *M* pyridine–acetic acid buffer (pH 5.0). [d] Ratios determined after hydrolysis in 6 *N* HCl at 110 °C for 22 h. [e] Purified by continuous flow electrophoresis in 0.25 *M* acetic acid.

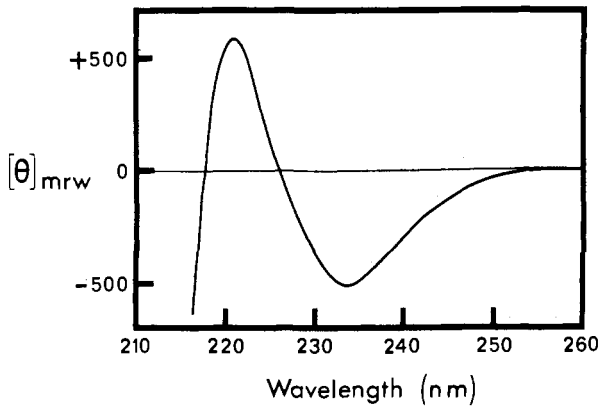


FIGURE 1: The CD spectrum of bradykinin in water at 27 °C. The average values of the ellipticities at the extrema as determined over a 4-year period with four different preparations of the peptide are as follows: 234-nm band, $[\theta]_{mrw} = -540 \pm 35°$ with a range of −470 to −611°; 221-nm band, 670 ± 83° ranging from 540 to 800°. Within experimental error the spectrum is independent of pH from 7.2 to 1.7.

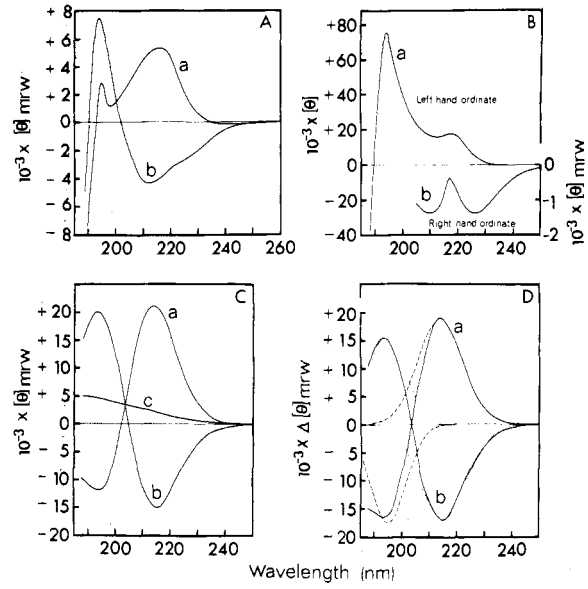


FIGURE 2: CD spectra of D-Pro containing analogues of bradykinin and selected peptide fragments in water at pH 4 and 27 °C: (A) curve a, CD spectrum of [D-Pro^3]-bradykinin; curve b, [D-Pro^2]-bradykinin; (B) curve a, CD spectrum of acetyl-L-phenylalaninamide; curve b, Ser-Pro-Phe-Arg; (C) curve a, CD spectrum of Arg-Pro-D-Pro; curve b, Arg-D-Pro-Pro; curve c, Arg-Gly-Gly; (D) curve a, CD difference spectrum of Arg-Pro-D-Pro referred to the CD spectrum of Arg-Gly-Gly as baseline; curve b, Arg-D-Pro-Pro referred to Arg-Gly-Gly; broken line curves (- - -) resolve the difference spectrum of Arg-Pro-D-Pro into two Gaussian bands, $\Delta[\theta]_1 = 1.90 \times 10^4 \exp[-(\lambda - 214)^2/(11.5)^2]$ and $\Delta[\theta]_2 = -1.75 \times 10^4 \exp[-(\lambda - 195)^2/(9.25)^2]$ with rotational strengths, $R_1 = 12.6 \times 10^{-40}$ and $R_2 = -10.2 \times 10^{-40}$ erg cm^2 rad, respectively; dotted curves (· · ·) resolve the difference spectrum of Arg-D-Pro-Pro into two Gaussian bands $\Delta[\theta]_1 = -1.71 \times 10^4 \exp[-(\lambda - 215)^2/(12.0)^2]$ and $\Delta[\theta]_2 = 1.61 \times 10^4 \exp[-(\lambda - 194.5)^2/(9.30)^2]$ with $R_1 = -11.8 \times 10^{-40}$ and $R_2 = 9.52 \times 10^{-40}$.

ellipticities, $[\theta]$, and mean residue ellipticities, $[\theta]_{mrw}$, (deg cm^2)/dmol, were calculated in the usual fashion. Each spectrum is the average of at least two determinations.

Natural abundance, proton decoupled ^{13}C NMR Fourier transform spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Supernova using 4K time domain data points. The Arg-Pro-Pro diacetate was dissolved in H_2O to a concentration of about 35 mg/ml, and a D_2O capillary was used for the lock. All spectra were obtained at 27 °C.

Results

CD of D-Pro Analogues of Bradykinin and Their Peptide Fragments. The CD spectra of [D-Pro^2]- and [D-Pro^3]-bradykinin are compared in Figure 2A. The most prominent feature of the spectrum of [D-Pro^3]-bradykinin (curve a) is the strong, asymmetrical positive band with maximum ellipticity at 217 nm flanked by a shallow negative band at 238 nm and a relatively weak positive band centered at 195 nm. The spectrum of [D-Pro^2]-bradykinin (curve b) shows a strong negative band characterized by an extremum at 212 nm and a prominent shoulder at about 225 nm and a strong positive band at 194 nm. The contrast between the two spectra is so striking that we were led to examine the CD of the N-terminal peptide fragments, Arg-Pro-D-Pro and Arg-D-Pro-Pro. Their spectra are displayed in Figure 2C along with the spectrum of Arg-Gly-Gly. The spectrum of Arg-Pro-D-Pro (curve a) shows a very strong positive band centered at 214 nm and a weaker negative band at 194 nm, while the spectrum of Arg-D-Pro-Pro (curve b) is almost the mirror image. In fact, as shown in Figure 2D, the two spectra are virtually mirror images when reflected through the rather flat spectrum of Arg-Gly-Gly and are then readily resolvable into two Gaussian bands centered at 215 and 195 nm, presumably corresponding to amide n–π^* and π–π^* Cotton effects, respectively (Madison and Schellman, 1970). The conclusion reached is that the CD spectra of the tripeptides are dominated by the sterically constrained and thus conformationally restricted D-Pro-Pro and Pro-D-Pro sequences, which also dominate aspects of the CD of [D-Pro^2]- and [D-Pro^3]-bradykinin.

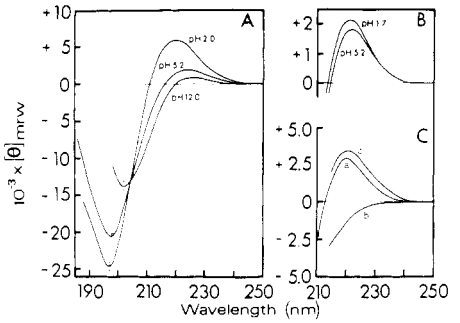


FIGURE 3: The CD spectra of Arg-Pro-Pro and its analogues. (A) Effect of pH on the CD of Arg-Pro-Pro; within experimental error the CD is independent of pH between 4.0 and 10.5; the spectrum at pH 2.0 was resolved into Gaussian bands, $[\theta]_1 = 7.00 \times 10^3 \exp[-(\lambda - 218)^2/(12.0)^2]$ and $[\theta]_2 = -2.50 \times 10^4 \exp[-(\lambda - 197)^2/(10.5)^2]$ with rotational strengths, $R_1 = 4.75 \times 10^{-40}$ and $R_2 = -16.4 \times 10^{-40}$ erg cm^2 rad. (B) CD of Arg-Pro-Pro-Gly. (C) Curve a, Ile-Pro-Pro at pH 4.0; curve b, Ser-Pro-Pro at pH 4.0; curve c, Ser-Pro-Pro at pH 1.0. Error bars in Figure 3A indicate mean deviation; the mean deviations of the maximum ellipticity of the positive bands are also small, e.g., at pH 5.2 the average value of the ellipticity at 223 nm from six determination on two stock solutions is 1950 ± 50° with a range of 1880 to 2110°

The CD spectra of the bradykinin analogues can be understood in terms of contributions from at least three chromophores: (1) the N-terminal sequence (Figure 2C); (2) the Phe residue(s) characterized by two positive bands located at 217 and 195 nm as shown by the model compound, acetyl-L-phenylalaninamide (curve a in Figure 2B); and (3) the C-terminal sequence, Ser-Pro-Phe-Arg, which shows two negative bands (curve b in Figure 2B), the one at 227 nm having previously been assigned to a configuration in which a 3 → 1 hydrogen band bridges the Pro residue (Cann et al., 1973). In the case of [D-Pro^3]-bradykinin (curve a in Figure 2A) the major contributions to the 217-nm band are the positive 214-nm band of Arg-Pro-D-Pro and the 217-nm band of one Phe residue; together they obscure the spectrum of Ser-Pro-Phe-Arg except for the long wavelength tail of its 227-nm band which gives rise to the shallow negative band at 238 nm. The relatively weak positive 195-nm band of the analogue is the resultant of the negative 194-nm band of Arg-Pro-D-Pro and the positive 195-nm band of the two Phe residues. Turning to [D-Pro^2]-bradykinin (curve b in Figure 2A) we see that the negative 215-nm band of Arg-D-Pro-Pro together with the negative bands of Ser-Pro-Phe-Arg outweigh the positive 217-nm Phe band to give a strong negative band at 212 nm with a shoulder ascribable to the 227-nm band of Ser-Pro-Phe-Arg, while the positive 193-nm band of Arg-D-Pro-Pro reinforces the 195-nm band of the Phe residues to give a strongly positive band centered at 194 nm. These assignments are evidently unambiguous, since simulated spectra of the analogues constructed by the weighted addition of the spectra of the peptide fragments and model compound give the correct sign and location and the qualitative shape of the bands. In passing we note that, as previously found for bradykinin (Cann et al., 1973), the intensities of the spectra of the peptide fragments and model compound do not sum quantitatively to the absolute band intensities because of the different asymmetrical environments and the residue interactions in the intact analogues.

Since these findings bear directly upon the CD of bradykinin itself, particularly with respect to assignment of its positive 221-nm band, the conformation of Arg-Pro-Pro was probed in further detail.

Effect of pH on the CD of Arg-Pro-Pro. As illustrated in Figure 3A the CD spectrum of Arg-Pro-Pro at pH 5.2 shows a weak positive band with maximum ellipticity at 223 nm and a strong negative band centered at 198 nm. Within experimental error the intensity and position of the 223-nm band are independent of pH over the range 4.0–10.5, but the spectrum changes when the pH is adjusted to values outside of this range. Thus, lowering the pH to 2.0 causes a rapidly reversible threefold intensification of the positive band with an accompanying blue shift to 220 nm, and a 25% intensification of the negative band with a blue shift to 197 nm. In contrast, raising the pH to 12.0 causes a 50% decrease in intensity of the positive band which is red shifted to 225 nm and a 34% diminution of the negative band with a red shift to 202 nm. The effect of pH 12 is 90% reversed when the pH is readjusted to 5.3 and the solution aged for 6 h at room temperature before recording the spectrum, with about 65% reversal when the spectrum is recorded immediately after readjustment of pH.

The spectra exhibit an isoellipticity point (204.5 nm) which permits explanation in terms of two forms of the peptide. The possibility of a pH-dependent equilibrium between aggregated and nonaggregated structures appears to be eliminated by experiments which showed that the spectra obey Beers law over the 40-fold range of concentration (0.021–0.85 mg/ml) examined at pH 5.2; eight-fold range (0.021–0.17 mg/ml) at pH 2.0; and 20-fold range (0.043–0.85 mg/ml) at pH 12.0. Since it is well established that the cis–trans isomerization of the model proline compounds *N*-acetylproline, Gly-Pro, and Pro-Pro is pH dependent with acid pH favoring the trans isomer (Madison and Schellman, 1970; Bedford and Sadler, 1974), we entertained this possibility for Arg-Pro-Pro. As described below ^{13}C NMR spectroscopy has established that cis–trans isomerization does occur upon lowering the pH from 5 to 1, although as we shall see this alone does not account for the CD changes.

In contrast to Arg-Pro-Pro, but like bradykinin (legend to Figure 1), Arg-Pro-Pro-Gly shows an essentially negligible effect of pH on its CD (Figure 3B).

As seen from Figure 3C, N-terminal amino acid residues other than Arg in X-Pro-Pro can give these effects. Thus, the CD spectrum of Ile-Pro-Pro at pH 4.0 (curve a) shows a positive band at 220 nm; and while Ser-Pro-Pro at pH 4.0 does not exhibit the band (curve b), it does show it at pH 1.0 (curve c).

Effect of Temperature on the CD of Arg-Pro-Pro. The intensity, but not the position, of the 223-nm band of Arg-Pro-Pro is highly sensitive to temperature. The temperature dependence of the ellipticity at pH 4 is shown in Figure 4. At least two effects of temperature are discernible since the ellipticity–temperature curve shows two inflection points: one at about 27 °C and another at about 48 °C. Between 10 and 27 °C the ellipticity decreases about 25%, and the curve is concave downwards; between about 27 and 65 °C the ellipticity decreases reversibly by about 75% in a manner indicative of a conformational transition. It is reasonable to interpret the latter observation in terms of two interconvertible conformers, A and B. The inherent temperature dependence of the ellipticity of the high-temperature conformer, B, is taken to be the linear extension of the high-temperature values of the ellipticity to lower temperature (broken line a). The inherent temperature dependence of the low-temperature conformer, A, was constructed by extending the low-temperature values of the ellipticity to higher temperatures (broken curve b) such that the limiting

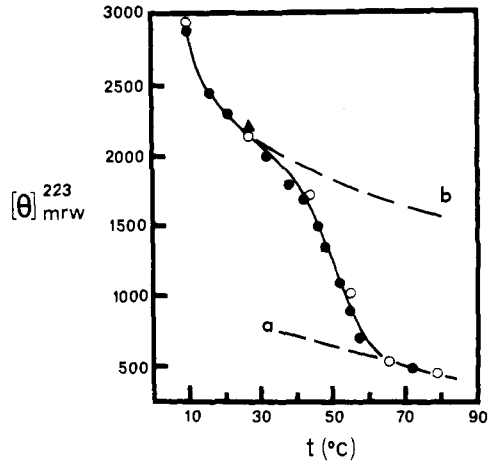


FIGURE 4: Effect of temperature, t, on the ellipticity, $[\theta]^{223}_{mrw}$, of the 223-nm band of Arg-Pro-Pro: (○ and ●) two different stock solutions of the peptide, aliquots of which were adjusted to pH 4.0 just prior to recording the CD spectra; (▲) sample held at 65.5 °C for 1 h and then cooled over a period of 0.5 h to 27 °C thereby demonstrating the reversibility of the effect of high temperature; curve a, extension of high temperature values of ellipticity to lower temperature; curve b, extension of low temperature values of ellipticity to higher temperatures. All but three of the points are averages of two or three determinations.

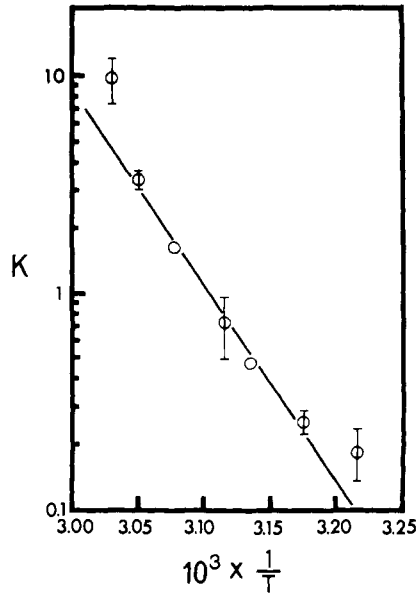


FIGURE 5: van't Hoff plot of logarithm of the equilibrium constant, K, against reciprocal of the absolute temperature, $1/T$, for Arg-Pro-Pro using data from the closed circles of Figure 4. Error bars indicate mean deviations.

slope is the same as the slope of broken line a.[2] The equilibrium constant for the assumed two-state transition, A ⇌ B, is then given by

$$K = ([\theta] - [\theta]_A)/([\theta]_B - [\theta]) \quad (1)$$

where $[\theta]$ is the observed ellipticity at a specified temperature between 38 and 57 °C inclusive, and the subscripts A and B designate the ellipticities of the pure conformers at the same temperature as given by the corresponding extensions to the ellipticity–temperature curve.

[2] This is the most logical way to extend the low-temperature values of ellipticity. Other constructions, such as linear extension of a line through the values at 21 and 27 °C, were tried and found to yield the same qualitative conclusions.

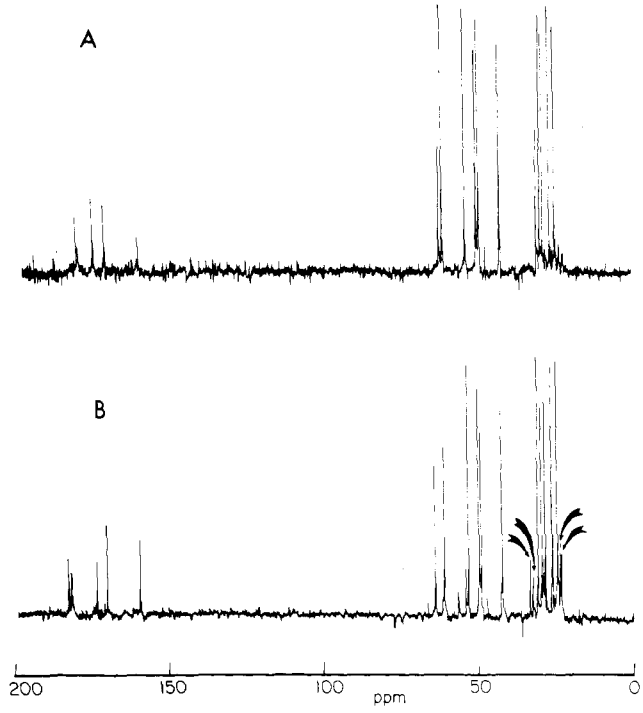


FIGURE 6: ^{13}C NMR spectra of Arg-Pro-Pro in H_2O at 27 °C measured at (A) pH 1.88 and (B) pH 4.67. The spectra represent 5532 and 10 458 scans, respectively. Arrows indicate peaks assigned to the cis resonances of the proline C-3 and C-4 carbons. Spectra obtained at higher resolution revealed an upfield shoulder on the Pro[3] C-2 resonance and a low-field shoulder on the Pro[2] C-2 resonance. The cis peaks increased in intensity with the deprotonation of the terminal carboxyl and remained roughly constant in intensity with further increases in pH. In addition to the tripeptide resonances, relatively small acetate peaks are also present in the spectrum. Although the acetate/tripeptide ratio was 2:1 the relative intensities of the acetate peaks are reduced due to the relatively long T_1 values since the pulse spacing (0.9 sec) was insufficient for these peaks to relax.

The van't Hoff plot of log K against $1/T$ is presented in Figure 5. The plot is approximately linear between 42 and 55 °C and yields $\Delta H° = 40$ kcal mol^{-1} and $\Delta S° = 120$ eu at 48 °C. The magnitude of the thermodynamic functions is interpreted to mean that the structure of water is involved and, thus, solute–solvent interactions are major factors in determining the conformation of Arg-Pro-Pro.

Since the data points designated by the closed circles in Figure 4 are internally consistent with respect to peptide concentration, apparent standard enthalpy changes, $\Delta H'(T)$, can be calculated from successive points over the temperature range, 38 to 57 °C. Although this treatment amplifies experimental error, it is clear that $\Delta H'(T)$ does not go through a maximum as is borne out by inspection of Figure 5. This observation satisfies one of the two criteria for a two-state transition (Lumry et al., 1966).

^{13}C NMR of Arg-Pro-Pro. In addition to the CD measurements, ^{13}C NMR spectra were obtained as a function of pH for Arg-Pro-Pro (residues are designated Arg[1], Pro[2], and Pro[3]). Spectra taken at pH 1.88 and 4.67 are shown in Figure 6 and the complete titration curves of the major peaks assigned to the trans–trans isomer are shown in Figure 7. Peak assignments were made on the basis of comparison with free amino acids (Stothers, 1972) and model oligopeptides (Christl and Roberts, 1972) as well as the pH titration data. The most striking aspect of the NMR spectrum is the appearance of additional peaks in the spectrum whose intensity roughly follows the carboxyl deprotonation.

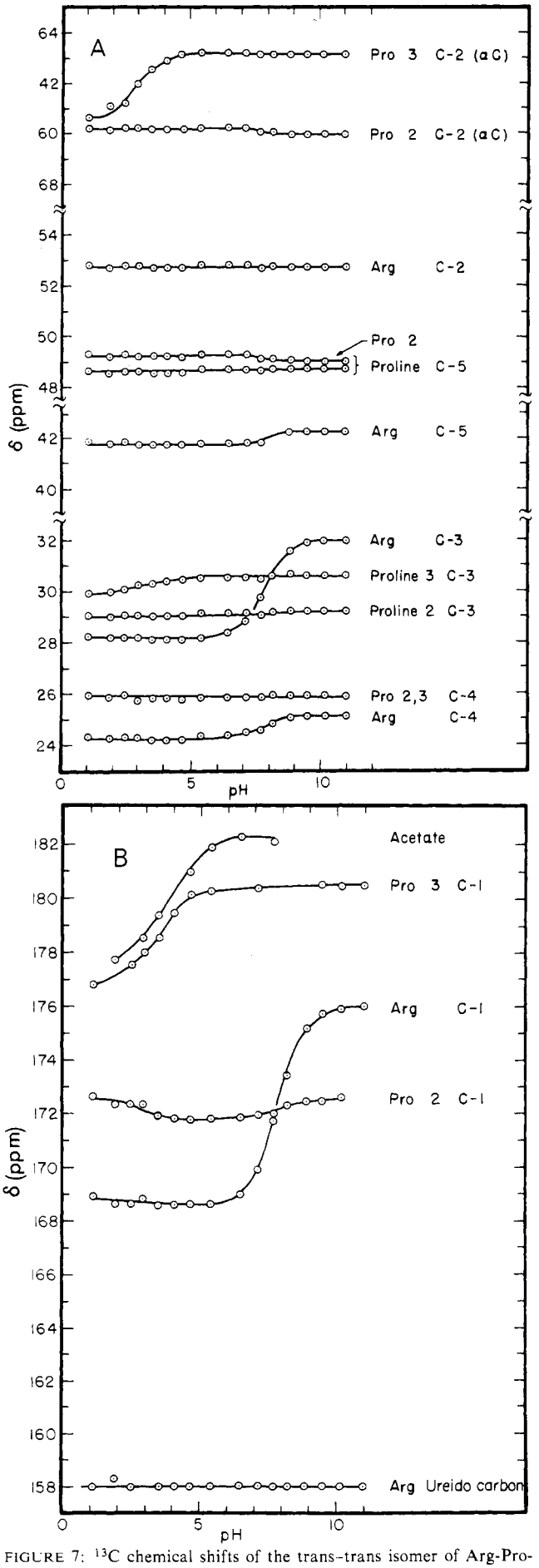


FIGURE 7: ^{13}C chemical shifts of the trans-trans isomer of Arg-Pro-Pro as a function of pH relative to external Me_4Si: (A) upfield region of the spectrum; (B) downfield region. Spectra were taken at 27 °C.

Spectra of proline-containing peptides often are characterized by multiple peaks corresponding to each carbon (Christl and Roberts, 1972; Thomas and Williams, 1972; Evans and Rabenstein, 1974; Dorman et al., 1973). These resonances arise because of the hindered rotation about the peptide bond leading to slow exchange (on the NMR time scale) of the cis and trans rotational isomers. On the basis of the chemical shifts of these additional peaks, we assign the major ones to the all-trans configuration of the tripeptide and the minor ones to a configuration in which one or both of the peptide bonds is in the cis configuration. Thus, deprotonation of the terminal carboxyl (pK of 2.9) appears to be the driving force which stabilizes the cis conformation or destabilizes the trans conformation or both. The relative peak areas of the cis and trans bands indicate that after the carboxyl has been deprotonated approximately 20% of one or both peptide linkages is in the cis conformation. This is discussed in greater detail later.

The pH titration data can be divided into intra- and inter-residue effects. The intra-residue effects were used to distinguish between the Pro^2 and Pro^3 resonances. Thus, the Pro^3 carboxyl (δ 180.4 ppm at pH 7.0), C-2 (δ 63.2 ppm at pH 7.0), and C-3 (δ 30.6 ppm at pH 7.0) were assigned on the basis of sensitivity to the carboxyl titration. Titration of the terminal amino group (pK of 7.9) produces shifts of the Arg^1 C-1, C-3, C-4, and C-5 peaks, but no shift in the C-2 resonance position. In previous studies it has been observed that amino group titrations result in an anomalously small effect on the directly bonded carbons (Christl and Roberts, 1972). In addition to the intra-residue effects of the amino and carboxyl titrations, several inter-residue effects can be observed. The Pro^2 carbonyl carbon is sensitive to both titrations, and the Pro^2 C-2 and C-5 carbons are also sensitive to the amino group titration. The Pro^2 C-5 resonance has been assigned on this basis.

While many of the peaks corresponding to the cis conformation can be seen only as shoulders of the trans proline peaks (see legend to Figure 6), carbons C-3 and C-4 give rise to well defined cis resonances 2 to 3 ppm downfield and upfield, respectively, of the trans C-3 and C-4 peaks. Similarly, the C-3 and C-4 peaks of cis-polyproline are the most readily resolved (Dorman et al., 1973). The C-4 cis resonances both appear to be sensitive to the carboxyl titration. In addition, the cis resonance of the Pro^3 C-2 shifts downfield into the trans peak as the terminal amino group is deprotonated. This is the only interaction between Pro^3 and Arg^1 which has been clearly observed. Although precise titration of the Pro^3 C-2 cis band must await experiments on ^{13}C-enriched material, it can be concluded at this time that the amino group of the cis conformer has p$K > 8.2$.

The cis–trans equilibrium not only affects the proline resonances, but the arginine resonances as well, and shoulders can be seen on several of the arginine peaks. Unfortunately, due to the relatively low intensities of the cis peaks, particularly as the pH is lowered, reliable titration data could not be obtained.

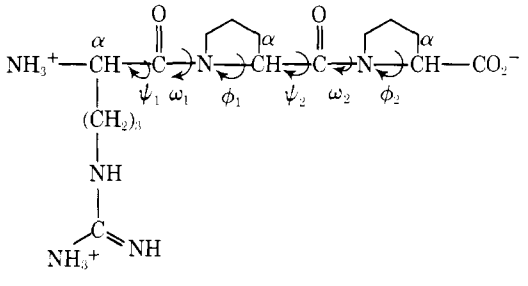
Discussion

Our previous conclusion that the positive 221-nm CD band of bradykinin is a composite of bands with contributions from the N-terminal sequence, Arg-Pro-Pro, as well as the Phe residues was reached by comparing the spectra of bradykinin and its N-terminal peptide fragments. Whereas both Arg-Pro and Arg-Pro-NH_2 show strongly negative CD from 255 nm down to at least 210 nm, Arg-

Pro-Pro and Arg-Pro-Pro-Gly are characterized by a positive band at 223 nm; and Arg-Pro-Pro-Gly-Phe shows the positive 218-nm band of Phe with a strong shoulder at 223 nm due to the Arg-Pro-Pro moiety. In the intact bradykinin molecule the resultant of the overlying Phe and Arg-Pro-Pro bands, when superimposed on the tail of the strongly negative band centered at about 200 nm, is the positive 221-nm band in question. The results of the present investigation constitute convincing supporting evidence for this assignment. Moreover, the evidence is compelling that the 223-nm band of Arg-Pro-Pro is associated with the configuration of the Pro-Pro sequence, Arg-D-Pro-Pro and Arg-Pro-D-Pro virtually being diastereoisomers.

The fact that at pH 4 the positive band shown by Arg-Pro-Pro is of maximum intensity at 223 nm, while the corresponding bands of its D-Pro analogues are centered at 214–215 nm, invites comment. Most likely this difference is in large part attributable to the enantiomorphism of Pro and D-Pro. On the other hand, it does appear that in the case of Arg-Pro-Pro (Figure 3A) the overlap of strongly negative and weakly positive bands distorts the apparent contribution of the positive band and gives a maximum shifted into the red from its true center. Although analysis of the Arg-Pro-Pro spectra at pH 4–12 in terms of two Gaussian bands is precluded by the observation that two forms of the peptide (cis and trans isomers) contribute to the CD, such analysis seems justified at pH 2 where ^{13}C NMR indicates that the peptide is almost exclusively in the trans configuration. The spectrum at pH 2 can be satisfactorily resolved into two Gaussian bands, a positive band centered at 218 nm and a negative one centered at 197 nm (see legend to Figure 3A). These bands are presumed to correspond to amide $n-\pi^*$ and $\pi-\pi^*$ Cotton effects, respectively (Madison and Schellman, 1970).

The positive sign of the 223-nm band can be understood in terms of the distinctive molecular geometry of Arg-Pro-Pro conferred by the Pro-Pro sequence.

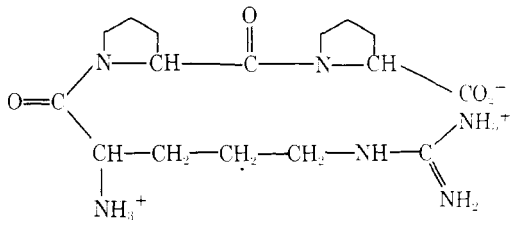


where ψ_i, ϕ_i, and ω_i are the dihedral angles which measure rotation about the corresponding bonds. Not only is the Pro-Pro moiety sterically constrained by the pyrrolidine rings which prevent rotation about the two N-C^α bonds (ϕ_i fixed), but rotation about the C^α—C=O bond (ψ_2) is highly hindered (Steinberg et al., 1960). Consequently, Arg-Pro-Pro is restricted to a relatively small set of conformations as compared, for example, with Arg-Val-Val. Another special feature of Arg-Pro-Pro is that both peptide bonds may be able to adopt either the cis or trans conformation, ω_i = 180 or 0° (Deber et al., 1970; Torchia, 1972; Dorman and Bovey, 1973). Madison and Schellman (1970) have found theoretically that both the cis and trans isomers of the dipeptides, Gly-Pro and Pro-Pro, are restricted to small regions of ψ_1–ψ_2 conformational space; and Bayley et al. (1969) have presented maps over ψ–ϕ conformational space of predicted contributions of individual electronic transitions to the optical activity of molecules containing two peptide groups. These maps show that the sign of the rotational strength is quite sensitive to conformation. Thus, the magnitude of the optical activity of a proline-containing molecule such as Arg-Pro-Pro will depend on the exact region of conformational space to which it is restricted. Since the optical activity is the weighted sum over accessible conformations, the rotational strength for a given transition (e.g., $n-\pi^*$) may be either positive or negative or even zero. These considerations provide the formal explanation for the fact that, whereas Arg-Pro-Pro at pH 2-12 and Ile-Pro-Pro at pH 4 show a positive 223-nm CD band, Ser-Pro-Pro does not show the band at pH 4 but does so at pH 1 (Figure 3).

Similar considerations apply to the temperature dependence of the intensity of the 223-nm band of Arg-Pro-Pro. According to Kauzmann and Eyring (1941) the major effects of raising the temperature on the optical activity of a solution are to change the distribution of solute molecules among accessible conformations by increasing the freedom of rotation about bonds, and to decrease solute–solvent interactions which influence intramolecular solute interactions and, perhaps, also solute conformation. Both effects are discernible in the ellipticity–temperature profile of Arg-Pro-Pro (Figure 4), which we interpret in terms of two interconvertible conformers. The low-temperature conformer is conceptualized as a set of conformations restricted to a small region of conformational space. Raising the temperature from 10 to 27 °C increases the relative population of molecules in higher energy conformations in accordance with the Boltzmann distribution law. Accordingly, the magnitude of the CD, which is a weighted sum over accessible conformations, changes. A priori, the effect of temperature on the optical activity of ring compounds is difficult to predict (Kauzmann and Eyring, 1941), but the observed decrease in ellipticity is consistent with increased rotation about the two C^α—C=O bonds (ψ_1, ψ_2) and the bonds of the Arg side chain in the Arg-Pro-Pro molecule. At the same time as the conformational states are being repopulated, solute–solvent interactions are being lessened as water structure changes. Eventually, disruption of solute–solvent interactions results in a conformational transition (between about 27 and 65 °C) to a high-temperature conformer. The dominant role of water structure in this process is indicated by the magnitude of the thermodynamic functions. The high-temperature conformer is conceptualized as a new set of conformations restricted to a different region of conformational space than the low-temperature conformer, and its CD is also intrinsically temperature dependent due to repopulation of states.

It is not clear what intramolecular reorientations occur during the transition from low- to high-temperature conformer, but they would not seem merely to involve trans $\rightleftharpoons$ cis isomerization. The values of ΔH° and ΔS° are 1–2 orders of magnitude greater than for the trans $\rightleftharpoons$ cis interconversion of *N*-acylproline esters in deuteriochloroform (Maia et al., 1971) and Gly-Pro peptide bonds in aqueous solutions of poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) (Torchia, 1972). Also, ΔH° is two orders of magnitude greater than for isomerization of the Pro-Pro peptide bonds in poly(L-proline) (Steinberg et al., 1960). It is possible that the conformational change involved is a change in the spatial disposition of the side-chain group of Arg[1]. Inspection of molecular models indicates that the side chain can either extend out into the solvent or fold around to give a closely packed structure in which the guanidium group is close

enough to the carboxylate group to form an ionic bond, diagrammatically:



This structure is sterically permitted in all four cis–trans isomers of Arg-Pro-Pro. Such compact structures with charged groups intramolecularly compensated would be favored when a change in water structure disrupts solute–solvent interactions. Previous studies of the effect of temperature on the CD of Ser-Pro-Phe-Arg and bradykinin (Cann et al., 1973) led to a similar conclusion; namely, that disruption of water structure favors the conformation in which a 3 → 1 hydrogen bond bridges Pro^2 in the tetrapeptide and Pro^7 in bradykinin.

The ^{13}C NMR spectra provide information on the conformation of the all-trans Arg-Pro-Pro as well as on the cis–trans conformational transition. We note first that the relatively low pK of 7.9 found for the amino group suggests that no significant intramolecular hydrogen bonding exists in the all-trans peptide. Furthermore, this pK is close to that observed for the single tripeptide studied by Evans and Rabenstein (1974) but below that for all of the dipeptides. This suggests that a salt bridge which can form in the dipeptide cannot form in the all-trans tripeptide. The fact that the Pro^3 resonances appear to be completely insensitive to the amino titration is similarly consistent with the lack of an interaction between the amino and carboxyl groups. In contrast, the amino group titration does lead to shifts for the Pro^2 C-1, C-2, and C-5 carbons. These shifts might reflect a hydrogen bonding interaction or, more probably, an electrostatic effect. The sensitivity of the Pro^2 C-1 to the carboxyl titration suggests an intramolecular hydrogen bond between the protonated carboxyl and the Pro^2 carbonyl oxygen. Similar conclusions have been reached for peptides with proline as the carboxyl terminal residue by Evans and Rabenstein (1974). If this is the case, the appearance of the cis peaks could result in part from a destabilization of the Pro-Pro trans isomer due to electrostatic repulsion of the carboxylate and Pro^2 carbonyl groups. This conclusion is consistent with the absence of any significant CD effects accompanying deprotonation of the terminal carboxyl group in Arg-Pro-Pro-Gly. If this interaction is responsible for the shift of the cis–trans equilibrium,[3] at least one pair of the cis peaks observed corresponds to Pro^3. It is clear from the spectrum presented in Figure 6B that two cis peaks are present for the proline C-3 and two for the C-4 carbon. This could reflect an indirect effect in which the Pro–Pro peptide linkage affects the shifts of Pro^2 as well as Pro^3. Alternatively, both peptide bonds may tend to adopt cis linkages so that the minor conformation of the peptide is made up predominantly of the cis–cis conformation. Examination of molecular models indicates that if the Arg-Pro linkage also adopts a cis conformation, the terminal amino group will be in closer proximity to the deprotonated carboxyl. The fact that the shoulder of the Pro^3 C-2 resonance, which we assign to the all-cis isomer, is shifted by the amino titration is consistent with this interaction. This model also predicts a higher pK (>8.2 as observed) for the amino titration of the cis–cis conformation.

In light of these findings, the cis–trans isomerization alone cannot account for the large change in CD of Arg-Pro-Pro on going from pH 4 to 2 because only about 20% of the peptide linkages are in the cis conformation at the higher pH. The intensification and blue shift of the 223-nm band at the low pH (Figure 3A) can best be understood in terms of formation of a hydrogen bond between the protonated carboxyl group and the Pro^2 carbonyl oxygen in the all-trans isomer.

Detailed assessment of the contributions of cis and trans isomers of the Arg-Pro-Pro moiety to the conformation of bradykinin must await complete assignment of the resonances in the ^{13}C NMR spectrum of bradykinin. Preliminary experiments indicate that complete assignment of resonances will require synthesis of bradykinins in which particular residues are enriched in ^{13}C. Such studies have been initiated.

[3] Madison and Schellman (1970) explain the shift of the cis–trans equilibrium toward the trans isomer upon titration of the carboxylate group of *N*-acetyl-L-proline, Gly-Pro, and Pro-Pro in terms of the relative electrostatic energies of the two isomers. Their calculations indicate that for the carboxylate forms of these three compounds the intramolecular electrostatic energy of interaction is lower in the cis isomer, while for the carboxyl forms it is lower in the trans isomer.

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