Carbon-13 NMR Spectroscopy of [20%-1,2-$^{13}C_2$-Gly^6]-Bradykinin. Role of Serine in Reducing Structural Heterogeneity

Robert E. London,*[1a] John M. Stewart,[1b] Robert Williams,[1b] John R. Cann,[1c] and N. A. Matwiyoff[1a]

Contribution from the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545, and the Department of Biochemistry and the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colorado 80262. *Received August 4, 1978*

Abstract: Substitution of a glycine residue for the serine residue at position six in the nonapeptide bradykinin leads to a dramatic increase in the cis/trans ratio about the sixth peptide bond (~38% cis). Decreases in the cis/trans ratio determined by NMR in 1 M $NaClO_4$ and in 60% $^{12}CH_3OH$ have been correlated with decreases in the 219-nm CD band. Chemical shifts of the 20% ^{13}C-labeled Gly^6 residue exhibit a very different pattern from that observed in the dipeptide Gly-L-Pro; the cis–trans shift difference is ~0 ppm for the glycine carbonyl resonances and ~1.1 ppm for the glycine methylene resonances. In contrast, the *cis*- and *trans*-glycine carbonyl resonances are resolved in 60% methanol and in 1 M $NaClO_4$. A series of model peptides has been examined to determine the structural factors leading to the observed shift pattern. These studies indicate the importance of Phe^8 both in determining the cis/trans ratio and in producing the observed chemical shifts. Glycine resonances in the tripeptide Gly-Pro-Phe are qualitatively similar to those observed in [Gly^6]-bradykinin. The most probable explanation of these data is a favorable hydrophobic/solvent-excluding Pro-Phe association in the cis peptide, which leads to a rotation about the Pro C_α–carbonyl bond, placing the carbonyl oxygen in close proximity to the Gly^6 methylene carbon. In the trans peptide, electrostatic repulsion between the Gly and Pro carbonyl oxygens opposes this effect. Significant differences in the cis/trans ratios between bradykinin and [Gly^6]-bradykinin have prompted careful bioassay studies, which show the potency of [Gly^6]-bradykinin to be only 50–70% that of bradykinin, although at saturating levels of peptide the responses are within experimental error. These data provide clues about the functional role of serine in bradykinin.

I. Introduction

Proline-containing peptides typically exhibit structural heterogeneity reflecting the presence of both cis and trans configurations of the X-Pro peptide bond. The biological significance of this heterogeneity is not presently clear. It has been suggested, for example, that on the basis of increased biological activity at high pH, the cis proline isomer of angiotensin II may be the more biologically active form of this peptide.[2] Unfortunately, the multiple perturbations caused by changing the pH, in particular deprotonation of the His^6 residue of the angiotensin, preclude conclusions regarding possible conformation–receptor affinity correlations. Another possibility is that conformational heterogeneity may be responsible for the different activities of peptide hormones in different tissues.[3] The high cis $\rightleftarrows$ trans interconversion barrier of ca. 20 kcal/mol[4-6] and resulting slow kinetics make this conformational feature an attractive target for probing the relation of conformation to receptor binding.

In the present study, we report on a bradykinin analogue, [Gly^6]-bradykinin, which in several previous bioassays has been found to have biological activity equivalent[7] or nearly equivalent[8] to that of bradykinin; our own bioassay results on isolated rat uterus and guinea pig ileum indicate a significant decrease in activity of [Gly^6]-bradykinin compared with bradykinin at nonsaturating levels of the peptide. These latter studies were prompted by ^{13}C NMR data, also reported here, which show that [Gly^6]-bradykinin has a considerably greater proportion of the cis peptide isomer about the sixth peptide bond than does bradykinin itself. The ^{13}C NMR data are of special interest because previous circular dichroism,[9] fluoresence,[10] and ESR[11] studies of bradykinin derivatives containing a glycine residue in the sixth position have all failed to indicate the high cis/trans ratio.

Although high cis/trans ratios have been observed in a number of cyclic peptides and in linear peptides containing a C-terminal proline residue,[12,13] the observation of a cis/trans ratio of 0.6 in a linear peptide with a nonterminal proline residue is unusual. Further, studies of [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin indicate unusual sensitivity of the glycine chemical shifts to the cis $\rightleftarrows$ trans equilibrium. In order to understand further the physical basis for the high cis/trans ratio and unusual glycine chemical shifts, ^{13}C NMR data for a variety of proline-containing peptides have also been collected. The model most consistent with the data is a hydrophobic interaction between the Pro^7 and Phe^8 side chains in the cis conformation, which in turn leads to a rotation of the Pro^7 carbonyl placing the carbonyl oxygen in close proximity with the glycine methylene group. An electric field contribution to the glycine chemical shifts is thus proposed as the basis for the observed unusual chemical shift behavior.

II. Materials and Methods

The peptides Gly-Gly-L-Pro, Gly-L-Pro-Gly-Gly, and L-Phe-Gly-L-Pro-L-Glu-L-Thr-L-Pro-NH_2 were obtained from Research Plus. Gly-L-Pro-Cha (Cha = cyclohexylalanine) was obtained from Bachem, Inc. These peptides were used without further purification and appeared to be free of carbon-containing contaminants as well as paramagnetic ions as judged by the ^{13}C NMR spectra. Gly-L-Pro-L-Phe, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), [Gly^6]-bradykinin, and [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin were synthesized using the solid-phase technique.[14] Arginine was used as the N^g-toluenesulfonyl derivative, and all Boc groups were removed with 25% trifluoroacetic acid in dichloromethane containing 1 mg/mL indole. Uniformly labeled 20% enriched [^{13}C]glycine was produced by the biosynthesis facility of Los Alamos Scientific Laboratory and was converted to the Boc derivative by treatment with *tert*-butyloxycarbonyl azide. After synthesis and cleavage from the resin by HF, the peptides were purified by countercurrent distribution (CCD).

Purification of [Gly^6]-bradykinin (both normal and ^{13}C enriched) was by successive CCD runs (*n*-BuOH:1% trifluoroacetic acid; 100 transfers; k = 1.22) followed by *n*-BuOH:HOAc:H_2O (4:1:5) (200 transfers; k = 0.205). The peptides were homogeneous by TLC on cellulose (R_f 0.28, *n*-BuOH:pyridine:HOAc:H_2O = 15:11:3:8), silica gel (R_f 0.37, EtOAc:pyridine:HOAc:H_2O = 5:5:1:3), and paper

0002-7863/79/1501-2455$01.00/0

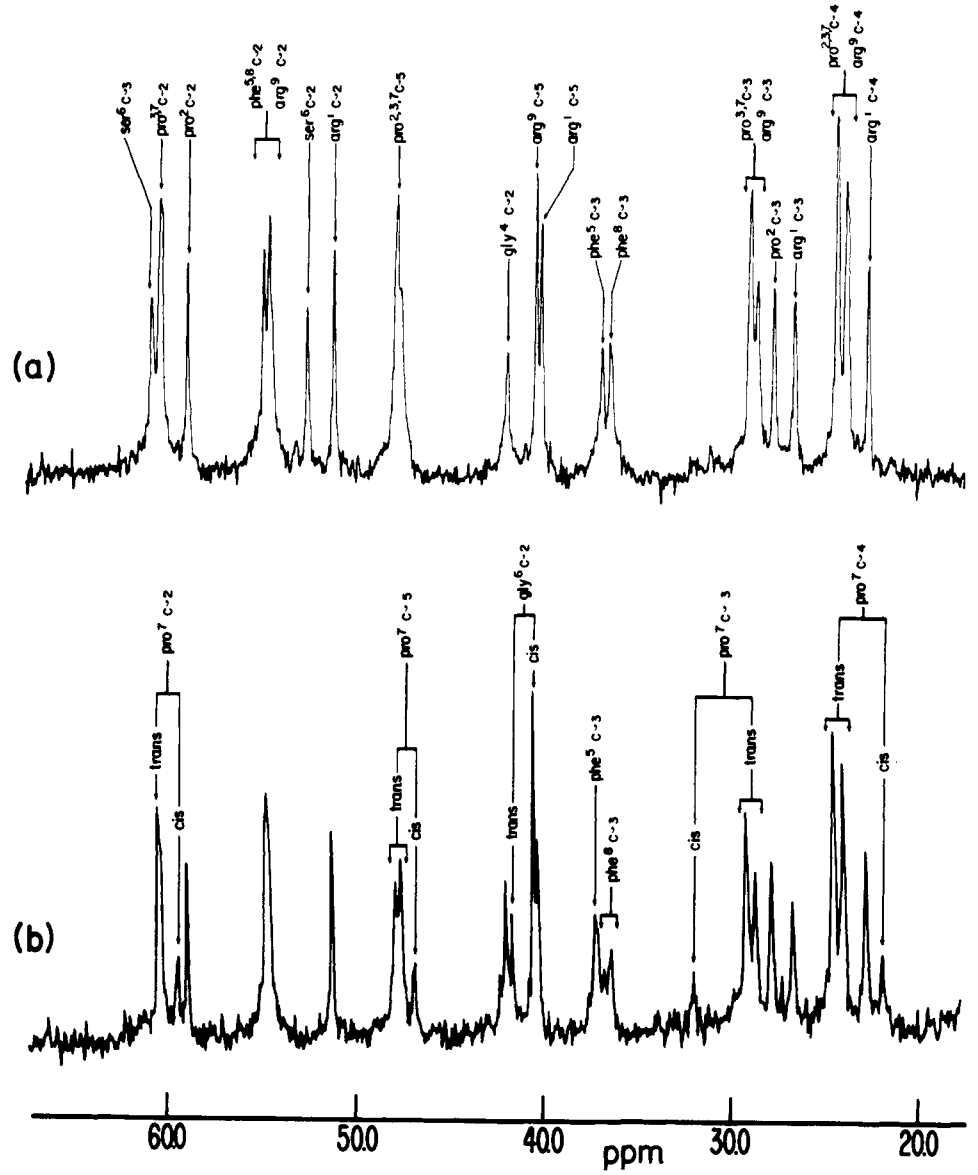


Figure 1. Upfield region of the ^{1}H-decoupled ^{13}C NMR spectra of (a) bradykinin and (b) natural abundance [Gly^6]-bradykinin. Spectra obtained in D_2O at 15 °C, pH (uncorrected meter reading) of 5.61 for a and 5.67 for b. Resonance assignments for bradykinin are those deduced previously.[21] In b, only assignments of selected resonances sensitive to the cis/trans isomerism are indicated. Other assignments are assumed identical with a. Note that intensities of resonances in b represent carbons indicated plus corresponding resonances in a. For example, the Gly^6 C-2 cis peak also includes a contribution from Arg^{1,9} C-5.

electrophoresis (E_{Lys} = 0.70, pH 5.0, pyridine-HOAc).

amino acid analysis:	Arg	Pro	Gly	Phe
[Gly^6]-bradykinin:	2.02	2.96	2.01	1.99
[^{13}C-Gly^6]-bradykinin:	2.02	2.98	1.98	2.01

Gly-Pro-Phe was homogeneous after CCD (*n*-BuOH:HOAc:H_2O = 4:1:5; 100 transfers; k = 0.59) as shown by paper electrophoresis (E_{Lys} = 0.45, pH 1.55, HOAc-HFor). Amino acid analysis: $Pro_{1.05}Gly_{0.97}Phe_{0.93}$.

Proton-decoupled ^{13}C NMR spectra were obtained at 25.2 MHz with a Varian XL-100-15 spectrometer interfaced to a Data General Nova 1210 computer for Fourier transform operation. Spectra were run at 15 °C in D_2O, the latter also serving for the deuterium lock. Measurements of spin-lattice relaxation times were carried out using the inversion recovery technique.[15]

CD spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 CD attachment. Spectra were recorded at 27 °C. Other details are as described previously.[16]

Bioassays of bradykinin and [Gly^6]-bradykinin were performed on isolated rat uterus and guinea pig ileum at 29 °C as previously described,[17] except that tissue baths were bubbled with 98% O_2–2% CO_2, to give a bath pH of 7.4. Rat blood pressure assays were done as previously described.[18]

III. Results and Discussion

Chemical-Shift Data. Proton-decoupled ^{13}C NMR spectra of the upfield regions of bradykinin as well as [Gly^6]-bradykinin are shown in Figure 1. Apart from the expected absence of the Ser^6 peaks and the appearance of a Gly^6 methylene resonance, the most significant difference between the spectra is the appearance of minor peaks in the [Gly^6]-bradykinin, which, on the basis of the chemical shifts,[19,20] can be assigned to cis proline resonances. Although there are some indications of cis proline resonances in the bradykinin spectra, these are difficult to observe and indicate that the amount of cis isomer for each of the three X-Pro bonds in bradykinin is $\lesssim$10%.[21] Since the structural difference between bradykinin and [Gly^6]-bradykinin involves residue 6, it is most probable that the observed cis resonances reflect isomerization about the Gly^6-Pro^7 bond, i.e., that the cis resonances arise from Pro^7 carbons rather than Pro^2 or Pro^3 carbons.

If the above assumption is correct, one would expect to see double peaks for the Gly^6 resonances as well. Such effects are difficult to observe in natural abundance Gly^6 bradykinin, but

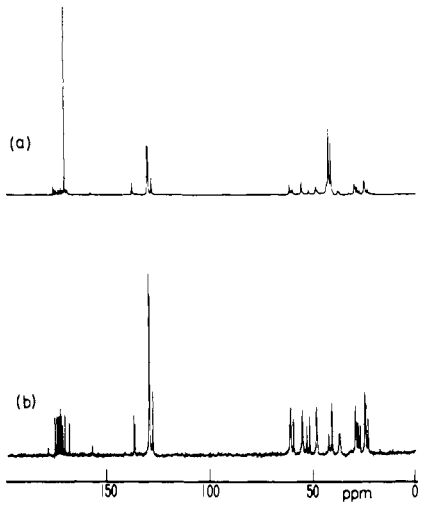


Figure 2. ^1H-decoupled ^{13}C NMR spectra of (a) [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin and (b) natural abundance bradykinin. Selective enrichment of peaks at δ 41.57, 42.71, and 169.83 ppm relative to external Me_4Si is apparent.

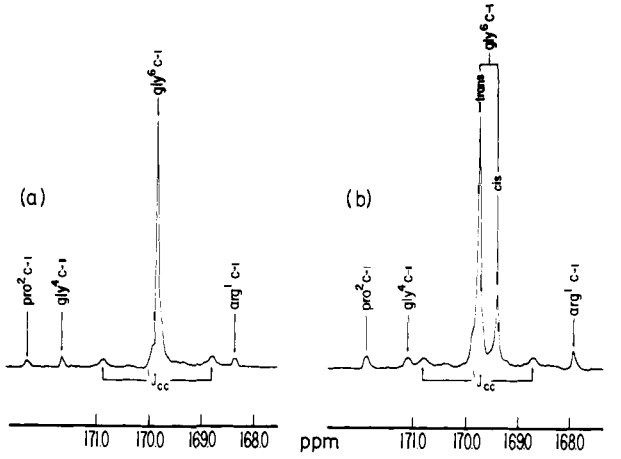


Figure 3. ^1H-decoupled ^{13}C NMR spectra corresponding to the portion of the carbonyl region containing the Gly^6 carbonyl resonance of [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin: (a) in D_2O; (b) in 40% D_2O-60% $^{12}CH_3OH$. Small satellites due to carbonyl-methylene $^1J_{CC}$ coupling in the 20% enriched glycine are indicated. In b, very weak satellites centered around the Gly^6 C-1 cis resonance can also be discerned. Other assignments are based on a comparison with previously assigned bradykinin resonances.[21]

should be readily observed if the Gly^6 carbons are enriched. The comparison of the ^{13}C spectra obtained for bradykinin and [20%-1,2-$^{13}C_2$-Gly^6]-bradykinin (Figure 2) indicates that there are clearly three enriched resonances in the ^{13}C-labeled molecule. Thus, a doubling of the Gly^6 methylene resonance is observed, but not of the Gly^6 carbonyl resonance. This pattern of chemical shifts differs dramatically from that observed in the dipeptide Gly-L-Pro, in which the δ (cis-trans) chemical-shift difference is larger for the Gly carbonyl than for the Gly methylene resonances (Table I). In order to test the hypothesis that the glycine carbonyl cis and trans resonances in [Gly^6]-bradykinin are indeed not resolved in aqueous solution, a perturbation leading to resolution of these resonances was sought. ^{13}C spectra corresponding to the glycine carbonyl region obtained in D_2O solvent and in 40% D_2O-60% $^{12}CH_3OH$ indicate resolution of the *cis*- and *trans*-glycine carbonyl resonances in the latter solvent (Figure 3).

In order to understand why the chemical-shift behavior of the glycine resonances in Gly-L-Pro differs dramatically from that of the glycine resonances in [Gly^6]-bradykinin, the δ (cis-trans) values for the glycine residues in a series of peptides containing the Gly-L-Pro linkage were obtained (Table I). Based on these data, several conclusions can be drawn: (1) The glycine δ (cis-trans) values for both the carbonyl and methylene resonances are relatively insensitive to whether the glycyl residue is at the N terminal position and, if it is, to whether or not the amino group is protonated. (2) In contrast, the δ (cis-trans) values are sensitive to whether or not the proline is at the carboxyl terminal position and, if it is, to whether or not the carboxyl group is protonated. (3) In the two cases examined in which two or more residues followed proline, viz., Gly-L-Pro-Gly-Gly and L-Phe-Gly-L-Pro-L-Glu-L-Thr-L-Pro-NH_2, the δ (cis-trans) value of the glycine carbonyl was ~0, indicating that for this parameter these peptides are reasonable models for [Gly^6]-bradykinin. (4) In the same two peptides considered above, the close agreement between the δ (cis-trans) values indicates that the presence of a Phe residue preceding the Gly does not significantly perturb this parameter. Thus, the Phe^5 residue in [Gly^6]-bradykinin is probably not the cause of the unusual δ (cis-trans) values. We note further that this observation is consistent with point 1 above. (5) In contrast to the above peptides, the tripeptide Gly-L-Pro-L-Phe gives δ (cis-trans) values for both glycine carbons that are qualitatively similar to those obtained in [Gly^6]-bradykinin. We also note that the cis/trans ratio obtained in this peptide

Table I. Cis/Trans Ratios and [^{13}C]Glycine Cis-Trans Chemical-Shift Differences in Peptides Containing the Gly-L-Pro Sequence

Peptide	pH[b]	δ(cis-trans)[a] carbonyl	δ(cis-trans)[a] methylene	cis/trans ratio
	1.0	0.25	0	0.18
Gly-Pro[c]	6.5	0.63	−0.25	0.61
	10.5	0.73	−0.22	0.79
	1.1	0.44	0	0.15
Gly-*Gly*-Pro	6.1	0.78	−0.29	0.73
	9.4	0.78	−0.33	0.71
Gly-Pro-Gly-Gly	5.4	0.12	0[d]	0.12
Gly-Pro-Ala	5.7	0.58	−0.18	0.17
Phe-*Gly*-Pro-Glu-Thr-Pro-NH_2	9.2	0.18	0[d]	0.1[e]
[*Gly*^{6}]-bradykinin (D_2O)	5.6	0	−1.14[g]	0.62[g]
60% CH_3OH-40% D_2O	1.8	−0.33	−1.09	0.28
	5.7	−0.24	−1.07	0.40
1 M $NaClO_4$	5	−0.28	−1.26	0.40
Gly-Pro-Phe	1.7	0	−0.68	0.30
	4.8[f]		−0.60	
	9.4		−0.43	0.41
Gly-Pro-Cha	1.0	0.44	−0.32	0.14

[a] δ(cis-trans) values positive for cis peak downfield of trans peak. [b] Uncorrected pH meter readings for peptides in D_2O or for [Gly^6]-bradykinin in solvents indicated. [c] Data from Fermandjian et al.[42] [d] Shoulder observed, but cis and trans resonances not resolved. [e] Cis/trans ratio approximate due to the presence of two prolines in this peptide. [f] Peptide insoluble at this pH, but maintained in solution for sufficient period to obtain data indicated. [g] Identical results obtained at pH 12, or in the presence of salt concentrations used in the bioassay.

is atypically high for peptides in which proline does not occupy the C-terminal position. Values of 0.30 and 0.41 for this ratio were obtained at pH 1.7 and 9.4, respectively.

In addition to the sensitivity of the glycine and proline chemical shifts to the conformation of the Gly-Pro bond, we have observed a significant dependence of the shifts corresponding to the Phe residue following the proline. This effect is clearly seen for the Phe^8 C-3 resonance in [Gly^6]-bradykinin (Figure 1b). In the tripeptide Gly-L-Pro-L-Phe at pH 7.43, the phenylalanine C-3, C-4, and ortho carbon shifts (where the ortho carbon resonances are assumed to be downfield of the meta carbon resonances[22]) are all sensitive to the conforma-

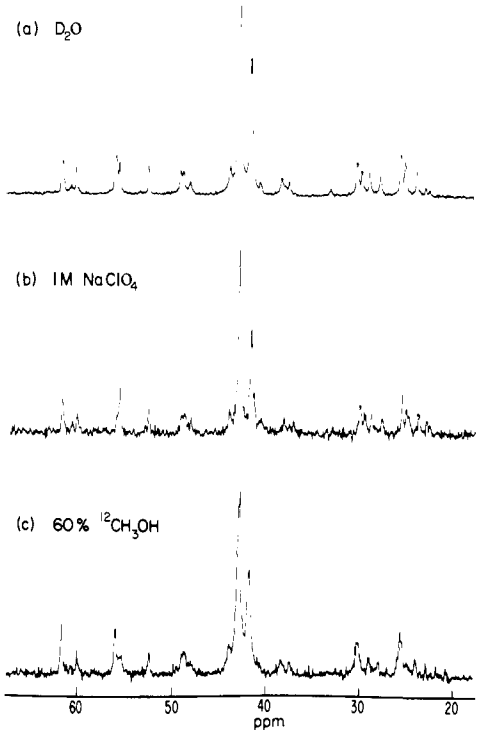


Figure 4. Upfield region of ^{13}C NMR spectrum of [Gly^6]-bradykinin in (a) D_2O; (b) 60% $^{12}CH_3OH$–40% D_2O; (c) 1 M $NaClO_4$.

tional state of the Gly-Pro bond. Values for δ (cis–trans) for each carbon are 0.24, 0.51, and −0.16 ppm, respectively. In contrast, at pH 1.7 the Phe C-2 but not C-3 resonances for cis and trans conformers are resolved.

Table I also contains data for the tripeptide Gly-Pro-Cha (Cha = cyclohexylalanine). Since, to our knowledge, this represents the first ^{13}C NMR study of a peptide containing the Cha residue, we note below several of the pertinent NMR parameters obtained for Gly-Pro-Cha. The cyclohexyl group gives rise to six resonances at 26.55, 26.75, 26.90, 32.42, 34.05, and 34.60 ppm, relative to external Me_4Si. In principle, two forms of the side chain corresponding to the equatorially and axially bonded cyclohexyl ring can exist. However, based on the axial/equatorial ratio observed in substituted cyclohexane[23] as well as on inspection of Corey–Pauling–Koltun (CPK) models, it appears unlikely that the axial species will be present at significant concentrations. Based on a comparison with the shifts observed in methylcyclohexane,[24] we can assign the three upfield peaks to the para and meta carbons and the three downfield peaks to the ortho and methine carbons. The data obtained in this case contrast with the pattern observed for the phenylalanine resonances in Gly-Pro-Phe, in which the ortho and meta carbons each give rise to only one resonance (neglecting the cis/trans dependence of the shifts). For cyclohexylalanine, the ortho and meta carbons are diastereotopic. Thus, the six cyclohexyl carbons are magnetically inequivalent. At low pH the cyclohexylalanyl C-3 peak was found to be sensitive to the cis/trans isomerism, with the cis and trans resonances at 38.13 and 38.73 ppm, respectively. The C-2 and C-1 resonance shifts were 51.63 and 177.14 ppm, respectively. Thus, the sensitivity of the Cha C-2 and C-3 resonances to the Gly-Pro cis/trans isomerism contrasts with that observed in Gly-Pro-Phe, in which C-2 but not C-3 yields resolved peaks reflecting cis and trans conformers at acid pH.

Solvent-Dependent NMR Studies of [Gly^6]-Bradykinin and Correlation with Other Techniques. Before considering the solvent dependence of the cis/trans ratio in [Gly^6]-bradykinin, we note that the cis/trans ratio of 1:2 obtained in D_2O is unusually high. In a series of di-, tri-, and tetrapeptides containing

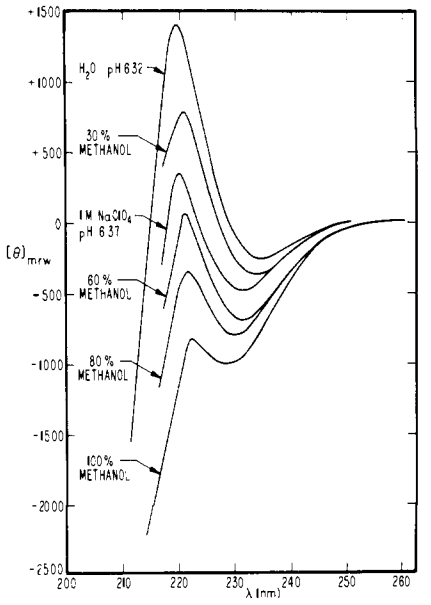


Figure 5. CD spectra of [Gly^6]-bradykinin in H_2O, 1 M $NaClO_4$, and various concentrations of methanol as indicated.

the Gly-Pro linkage, Grathwohl and Wüthrich[2] have observed 17 ± 3% cis (this applies to cases in which proline is not at the carboxyl terminal, or to cases in which the proline is at the carboxyl terminal but is protonated, i.e., conditions analogous to bradykinin). A possible explanation for the high ratio is that in [Gly^6]-bradykinin a favorable intramolecular hydrophobic/solvent-excluding interaction stabilizes the cis form of the peptide. Decreased cis/trans ratios observed in methanol and 1 M $NaClO_4$ (Figure 4; Table I) can then be explained by the conclusion that the loss of solvent structure favors the greater exposure of certain hydrophobic groups in the trans peptide conformation.

It is interesting that the solvent dependence of the upfield region of the ^{13}C NMR spectra of [Gly^6]-bradykinin (Figure 4) correlates with the solvent dependence of the peptide's CD in the spectral region, 260–215 nm (Figure 5). The CD spectrum in water (pH 6.3) shows two bands, a weak negative band with extremum at 234 nm and a much stronger positive band at 219 nm. Changing the solvent to 1 M $NaClO_4$ (pH 6.3) or to methanol (via progressive increase in the percentage of methanol in water–methanol solvent mixtures) has a pronounced effect on the spectrum. The difference spectrum in 100% methanol referred to water shows a single negative band with extremum at 218 nm, indicating that the change in solvent causes a decrease in the amplitude of the positive 219-nm band without significant effect on the negative 234-nm band. In view of the fact that this change is paralleled by a decrease in the cis/trans ratio of the peptide (Table I), it could be that there is a contribution to the 219-nm band by a chromophore sensitive to the cis/trans conformation about the Gly^6-Pro^7 bond, possibly one of the Phe residues.[25]

The results obtained for [Gly^6]-bradykinin have significant implications for the interpretation of CD data. Based on similarities between the CD spectra exhibited by bradykinin and [Gly^6]-bradykinin, Ivanov et al.[9] have concluded that the solution structures of the two peptides are similar. In subsequent studies based on fluroescence measurements[10] and ESR measurements of spin-labeled bradykinin analogues,[11] the Ser^6 and Gly^6 residues were interchanged freely. Marlborough et al.[26] have found that trifluoroethanol exerts a solvent effect on the CD of bradykinin similar to that reported here for methanol on [Gly^6]-bradykinin. In fact, the difference spectrum of bradykinin in trifluoroethanol referred to water, which

Table II. Glycine ^{13}C T_1 Values in [20%-1,2-$^{13}C_2$-Gly^6]-Bradykinin Measured in 60% $^{12}CH_3OH$–40% D_2O

temp, °C	carbonyl T_1, s		methylene T_1, ms	
	trans	cis	trans	cis
5			36	41
15	0.88	0.88	42	50
25	1.11	1.11	53	59
35			74	84

we constructed from the spectra presented in their Figure 1, shows a negative band at about 221 nm, indicating a decrease in the intensity of the positive 221-nm band without significant effect on the negative 234-nm band. They interpreted their findings primarily as a reflection of an increased probability of cis peptide bonds in trifluoroethanol, primarily involving the Arg^1-Pro^2 and Pro^2-Pro^3 bonds. In the present study, a comparison of the CD and ^{13}C NMR results obtained under several different solvent conditions indicates that a similar decrease in the intensity of the positive 219-nm band of [Gly^6]-bradykinin is correlated with a decreased cis/trans ratio about the Gly^6-Pro^7 bond. We note in this context that although the NMR and CD data were obtained at different peptide concentrations, recent CD measurements over a concentration range from 0.075 to 30 mg/mL obey Beers law within an experimental error of ±5–10%,[27] which indicates that interpretation of the NMR is not clouded by aggregation of the peptide. These results tend to underline the difficulties involved in the interpretation of CD data obtained for small peptides.

Relaxation Rates. Spin–lattice relaxation times for the glycine resonances of the specifically enriched bradykinin are summarized in Table II. Measurements were made in 60% methanol in order to obtain separate values for the carbonyl cis and trans resonances. Arrhenius plots of T_1 with inverse temperature are nonlinear, so that activation energies could not be obtained. This behavior may reflect differences in the activation energies associated with overall and segmental motions. A consistent difference between the cis and trans T_1 values for the glycine methylene carbon indicates greater mobility in the cis form. This difference is significant in light of the excellent signal-to-noise ratio for the enriched resonance leading to experimental errors $\lesssim$3%. This result could reflect a more ordered structure for the trans peptide. However, a similar effect is observed in the dipeptide Gly-L-Pro,[28] so that local cis–trans differences in steric interaction involving the glycine and proline residues and consequent variation in the rate of segmental motion are probably the dominant causes of the observed differences.

As noted in the previous section, both [Gly^6]-bradykinin and the tripeptide Gly-Pro-Phe exhibit an unusually large cis/trans ratio for peptides in which proline is not the C-terminal residue. In order to obtain further information on the intramolecular interactions responsible for this effect, we have studied the spin–lattice relaxation behavior of Gly-Pro-Phe; data were obtained at high pH (9.43) since cis and trans resonances for the phenylalanine ortho carbons are resolved under these conditions. Results for the protonated carbons are summarized in Table III. Differences in NT_1 values between cis and trans resonances for the carbons giving distinguishable peaks are in most cases very small and well below the estimated 10% error inherent in the measurements. The one exception to this conclusion is the glycine C-2 carbon for which, as in the case of [Gly^6]-bradykinin, the cis peak exhibits a significantly longer NT_1 value. NT_1 values for the phenylalanine and proline C-2 carbons indicate an overall isotropic rotational correlation time of 1.0×10^{-10} s for the tripeptide and significant internal motion about the glycine C-1–C-2 bond in both the cis and trans conformers (Table II). This behavior appears to be typical for terminal glycine residues.[29-31]

Table III. Calculated and Experimental Motional Parameters for Gly-Pro-Phe

residue	carbon	diffusion coeff,[a] s^{-1}		NT_1^{calcd}, ms	NT_1^{exp}, ms
Phe[b]	C-2	$D_0 = 1.6 \times 10^9$		449	440
	C-3	$D_{23} = 0.6 \times 10^9$		519	528 (trans)
					512 (cis)
	C_{ortho}	$D_{34} = 1.4 \times 10^9$		635	613 (trans)
					613 (cis)
	C_{meta}			635	650
	C_{para}			519	492
Gly[c]	C-2	$D_{12} = 1.2 \times 10^9$		583	584 (trans)
		$D_{12} = 2.0 \times 10^9$		660	660 (cis)
		β, deg	θ, deg		
Pro[d]	C-2	70.5	4	456	455 (trans)
		70.5	9	481	484 (cis)
	C-3	73	25	759	760 (trans)
		73	25	759	764 (cis)
	C-4	83	28	921	906 (trans)
		83	26	835	840 (cis)
	C-5	70.5	16	555	560 (trans)
		70.5	17	572	580 (cis)

[a] D_{ij} values correspond to diffusion about the C_i–C_j bond. [b] D_0 for overall isotropic motion derived from Phe C-2 NT_1 value. [c] Glycine NT_1 values assumed to reflect significant internal motion about the glycine C_1–C_2 bond relative to the peptide backbone. [d] Proline calculations based on the model of ref. 33 for $NT_1^{\gamma} > NT_1^{\beta} > NT_1^{\delta} > NT_1^{\alpha}$ and assuming $\tau_A = \beta_B = 10^{-12}$ s.

Theoretical NT_1 values for the Phe and Gly protonated carbons have been calculated using a free internal rotation model assuming uncorrelated rotations and employing the B matrix formalism.[32] This model is qualitatively consistent with the relaxation data. However, a small but significant difference, apparent from the partially relaxed Fourier transform spectra, between the NT_1 values for the benzyl ortho and meta carbons is not predicted using this model. This result may reflect greater contributions of nonbonded protons to the relaxation rate of the ortho carbons, as well as the possibility that the internal motion characterizing the phenylalanine residue is, in fact, somewhat restricted. In contrast, there appears to be no significant difference in the restriction of the phenylalanine motion between the cis and trans conformers. This result is somewhat surprising in view of the likelihood, discussed below, of a hydrophobic Phe-Pro interaction in the cis peptide conformer. It should be emphasized, however, that if the increase in the cis/trans ratio resulting from this interaction corresponds to the difference between the typical value of ~0.10 and the measured ratio of 0.41 at high pH, the stabilization due to this interaction amounts to only ~0.8 kcal/mol. This may be sufficiently small to have a negligible effect on the relaxation behavior.

Proline NT_1 values exhibit a typical pattern with $NT_1^{\gamma} > NT_1^{\beta} > NT_1^{\delta} > NT_1^{\alpha}$. This behavior can be explained using a ring puckering model such that the pyrrolidine ring jumps between C_β–C_γ half-chair forms with C_γ displaced further from the C_δ–N–C_α plane than C_β. A calculational procedure based on this model has been presented elsewhere.[33] We note that the small difference between cis and trans NT_1 C_α values translates to a relatively large difference in θ. This reflects the fact that, for small jumps, NT_1 changes very slowly as a function of θ. There is, therefore, a relatively large error (~5°) for the calculated θ value obtained for C_α.

Proposed Molecular Basis for the High Cis/Trans Ratio in [Gly^6]-Bradykinin. Molecular models have been examined in order to extract the information inherent in the unusual glycine

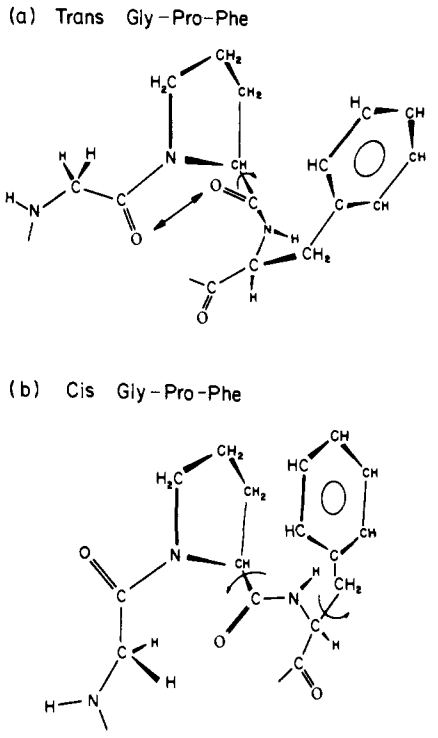


Figure 6. Model for the trans and cis structures of the Gly-Pro-Phe sequence. In the trans structure, a, glycine carbonyl–proline carbonyl repulsion prevents rotation about the proline C-1-C-2 bond required to bring the benzyl and pyrrolidine rings into close contact. In the cis conformation, b, rotation about the Pro C-1-C-2 bond leads to closer contact of these residues and more effective exclusion of solvent molecules. The Pro[2] carbonyl oxygen is also rotated closer to the Gly methylene carbon, thus explaining the upfield methylene shift in this conformer.

chemical shifts and the high cis/trans ratio exhibited by both [Gly[6]]-bradykinin and the tripeptide Gly-L-Pro-L-Phe. A viable model must be consistent with several facts, in particular: (1) the high cis/trans ratio of 0.62; (2) the unusual glycine chemical-shift differences between the cis and trans peptides; (3) the solvent dependence of the cis/trans ratio; (4) the results obtained with model peptides indicating the importance of the Phe residue following the proline in determining the measured NMR parameters. Two models have been considered to explain the ^{13}C NMR data:

(1) The glycine chemical-shift behavior is most readily explained by a model in which the benzyl ring of Phe[8] is in close proximity to the glycine methylene carbon in *cis*-[Gly[6]]-bradykinin. The unusual δ (cis–trans) value obtained is therefore explained on the basis of a ring current shift. This effect could readily account for the magnitude of the upfield methylene ^{13}C shift.[34] This model is also consistent with the model peptide studies, since the requirement for the phenylalanine residue following the proline is apparent. However, the magnitude and solvent dependence of the cis/trans ratio are less readily explained. One possibility is that in this proposed cis conformation of [Gly[6]]-bradykinin, the Phe[8] residue is sufficiently close to the Phe[5] ring to allow a significant hydrophobic interaction, thus stabilizing the cis conformation. This possibility is unlikely, however, in view of the fact that in the tripeptide Gly-Pro-Phe, in which no such interaction is possible, the cis/trans ratio is also high. Furthermore, the absence of significant ring current shifts of the phenylalanine benzyl carbons, as well as UV data,[35] indicates that any such interaction must be very weak. Another stabilizing contribution to this conformation could rise from a dipole-induced dipole interaction between an amide group and the aromatic phenylalanine ring, analogous to the interaction which occurs in diketopiperazines having an aryl methyl side chain[36] and in some linear peptides.[37] Since a high cis/trans ratio is also observed in Gly-Pro-Phe which lacks the Phe[5]-Gly[6] peptide bond of [Gly[6]]-bradykinin, the interactions would have to involve the Gly[6]-Pro[7] amide bond. This structure appears to be unfavorable based on an examination of molecular models. Furthermore, significant shifts of the cis glycine carbonyl peak would be expected, although the δ (cis–trans) value for the carbonyl resonances is similar to the values in some of the model peptides lacking an aromatic group. For these reasons, this model appears unlikely, although it cannot be completely ruled out.

(2) A second model consistent with the constraints enumerated above is based on the hypothesis of a favorable hydrophobic interaction between Pro[7] and Phe[8] which can occur in the cis peptide (Figure 6). Examination of CPK molecular models indicates that the electrostatic repulsion between the glycine and proline carbonyl groups in the trans peptide opposes rotation about the proline C_α–CO bond, which is required to bring the pyrrolidine ring of proline into close contact with the phenylalanine benzyl ring. When the Gly-Pro bond is cis, the reduction in this electrostatic interaction permits rotation about the proline C_α–CO bond, allowing the two hydrophobic groups to come much closer (Figure 6b). The upfield shift of the glycine methylene carbon in the cis peptide can then be explained on the basis of an electric field effect resulting from the proximity of the proline carbonyl oxygen. The theory of Batchelor for linear electric field shifts leads to the correct sign for this shift.[38]

In light of the two models proposed above, additional data were sought in order to substantiate further one or the other of these hypotheses. Perhaps the most clear-cut experiment in this series is the substitution of a residue for phenylalanine which would preserve the hydroprobic interactions, but eliminate the possibility of ring current shifts. We have, therefore, studied the tripeptide Gly-L-Pro-L-Cha. The glycine chemical-shift data (Table I) obtained for this tripeptide differ significantly from those obtained for [Gly[6]]-bradykinin and for the tripeptide Gly-Pro-Phe, but are closer to those obtained for the remaining model peptides. These data would tend to support model 1 above, since the aromatic contributions to the glycine shifts are removed. However, this tripeptide also exhibits a significantly smaller cis/trans ratio than either Gly-Pro-Phe or [Gly[6]]-bradykinin, so that it must be concluded that the nature of the hydrophobic interactions has also been significantly changed. CPK models of Gly-Pro-Cha suggest that the bulkier cyclohexyl group cannot get quite as close to the proline ring, so that less volume is "saved" by this interaction. It thus appears that this experiment does not permit an unequivocal conclusion regarding the two models considered above.

Another implication of the second model proposed above is a chemical-shift contribution to the proline resonances in the cis peptide resulting from the interaction with the phenylalanine ring. Since this effect will be observed only for the cis peptide, it should be reflected in the δ (cis–trans) values observed for proline in Gly-Pro-Phe compared with those for the other model peptides. It must be noted, however, that the perturbation in question involves *both* an aromatic contribution to the proline shift arising from the Phe residue, as well as a change in the electrostatic contribution due to rotation of the proline carbonyl. This combined effect is difficult to predict. Comparison of these values for several of the model peptides indicates differences <0.5 ppm which are most pronounced for the proline C_α and C_β carbons. The lack of a suitable reference compound precludes a definite conclusion, however.

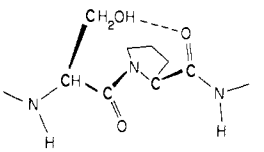
Despite the absence of significant proline ^{13}C chemical shifts reflecting an interaction with the phenylalanine ring, as well as the lack of significantly greater motional restriction of the Phe side chain in the cis peptide as indicated by T_1 measure-

ments, there are some data in the literature supporting the importance of a hydrophobic phenylalanine–proline interaction. In particular, studies of Grathwohl and Wüthrich on the cis/trans ratios observed in a series of X-Pro dipeptides[12] indicate unusually high values in L-Phe-L-Pro: 0.41 if Pro is protonated, 3.17 if Pro is deprotonated. These data can be explained by postulating a hydrophobic/solvent-excluding interaction in the cis peptide which stabilizes this conformation. Studies with CPK molecular models support this possibility. Furthermore, theoretical calculations of Galaktionov et al.[39] indicate the importance of this type of interaction. A stereospecific collision complex between benzene and the proline H_α of *cyclo*-(L-Pro)$_3$ has also been observed[40] and attributed to an attraction between the electron-rich aromatic ring and the positively charged peptide nitrogen atom or α protons. In the present case, however, the observed solvent dependence appears to indicate a hydrophobic interaction stabilizing the cis conformation.

Biological Activity of Peptides. Our comparison of the potency of bradykinin and [Gly^6]-bradykinin on the contraction of the isolated rat uterus and guinea pig ileum (Figure 7) shows that at half-saturating levels of the peptides, [Gly^6]-bradykinin exhibits approximately 50–70% of the activity of bradykinin on both tissues. Within experimental error, the [Gly^6] analogue of bradykinin has full intrinsic activity at saturating levels of the peptide (caption, Figure 7), although there *may* be a marginally longer lag time before the beginning of the contraction when [Gly^6]-bradykinin is applied. These data may be analyzed in a number of ways, but the simplest interpretation suggests that either the [Gly^6]-bradykinin cis peptide does not bind to the receptor or, if it does bind, it has a lower affinity and/or a lower intrinsic potency than the dominant *trans*-[Ser^6,Pro^7]-bradykinin natural hormone. In this context it is of interest to note that there is no significant difference between the two peptides in rat blood pressure response, with respect either to potency or lag time to onset of the depressor response when the peptides are injected intraarterially.

The Role of Serine in Bradykinin. Prior to the present study, the role of Ser^6 in bradykinin has been an enigma, since substitution with a glycine residue was reported to give full activity in several bioassays.[7,8] The present study indicates that the presence of the Ser^6 residue significantly reduces the cis/trans ratio of the sixth peptide bond relative to [Gly^6]-bradykinin. Since a specific receptor interaction involving the serine hydroxyl side chain is apparently not required for activity, it appears that only a "spacer" residue is required; the least costly such residue in terms of cellular materials is glycine. The present study indicates, however, that the Ser residue of bradykinin may have evolved in part to reduce the naturally occurring heterogeneity resulting from the significant percentages of both cis and trans conformers of the sixth peptide bond of bradykinin. Based on this hypothesis, it seems likely that where there is a possibility of structural heterogeneity due to the presence of an imino acid, additional factors may act to reduce this heterogeneity. In this context, we note that the cis/trans ratio is in general largest if there is a carboxyl terminal imino acid.[12,13] Several biologically active peptides, e.g., thyrotropin releasing hormone and thyrocalcitonin, contain a C-terminal prolinamide rather than a proline. One apparent function of the terminal amide group is therefore to reduce the heterogeneity which would characterize the corresponding peptide in a free acid form.

There are several possible explanations for the effect of Ser^6 on the cis/trans ratio: (1) The steric interaction resulting from the additional CH_2OH group in bradykinin could prevent the type of rotation illustrated in Figure 6b, thus blocking the favorable hydrophobic interaction proposed to stabilize the cis conformation. (2) Even in the absence of the above effect, the additional steric factor resulting from the proximity of the serine side chain (or the side chain of any L-amino acid) and the proline carbonyl will destabilize the cis conformation. (3) Molecular models indicate that a serine hydroxyl–proline carbonyl hydrogen bond may be a significant factor in the stabilization of the trans conformation of bradykinin:



In this context, we note again that, on the basis of the difference in the cis/trans ratios observed between bradykinin and [Gly^6]-bradykinin, an interaction supplying only 1 kcal/mol is required to explain the effect.

Differences in the activities of bradykinin and [Gly^6]-bradykinin presented here may reflect an enhancement in the association constant with the receptor resulting from a specific interaction with the Ser hydroxyl, and/or an effect associated with the altered cis/trans ratio. In connection with the latter, we note that ^{13}C NMR studies of bradykinin itself give evidence for the presence of a cis peptide isomer.[21] Although the corresponding peptide bond could not be assigned with certainty, studies of the tripeptides Arg-Pro-Pro and Ser-Pro-Pro[41] indicate that if the terminal carboxyl is not charged (analogous to the situation in bradykinin), the cis probabilities are very small so that the observed cis proline resonances in bradykinin are most probably associated with Pro^7. Furthermore, examination of the ^1H NMR spectrum of Ser-Pro-Phe-Arg indicated a 13% cis probability for the Ser-Pro bond.[21] Estimating a 10% cis probability for the Ser^6-Pro^7 bond of bradykinin, the difference between the percent cis of bradykinin and [Gly^6]-bradykinin would lead to a 28% reduction in activity if the cis peptide does not bind to the receptor. However, possible formation of an inert complex with the cis peptide, as well as the possibility of cis $\rightleftarrows$ trans isomerism of the bound cis peptide, precludes definite conclusions at present. Marginal differences between the time lag for onset of activity suggest the possibility of relating cis $\rightleftarrows$ trans isomerism to peptide activity, but additional data are required before a definite conclusion can be reached.

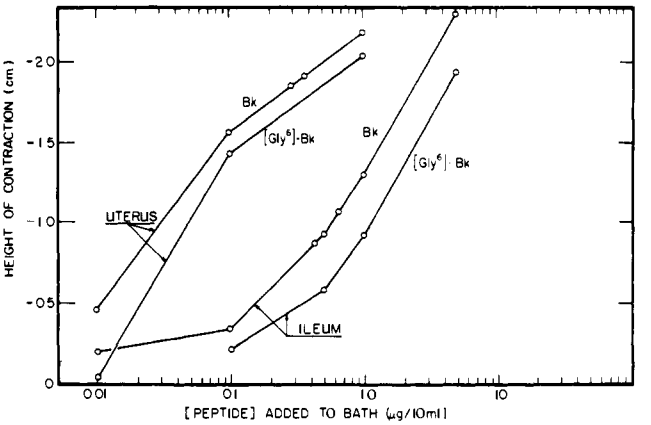


Figure 7. Contraction of rat uterus and guinea pig ileum as a function of added bradykinin (Bk) or [Gly^6]-bradykinin ([Gly^6]-Bk). Maximum contractions were equal, within experimental error.

Acknowledgment. This work was performed under the auspices of the U.S. Department of Energy and was supported in part by Research Grant No. RR-00962-02 from the Division of Research Resources, National Institutes of Health, Department of Health, Education and Welfare (N.A.M.), and in part by Research Grant No. HL13909-27 from the National Heart, Lung and Blood Institute, NIH (J.R.C.). This publi-

cation is No. 723 from the Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colo.

References and Notes

(1) (a) Los Alamos Scientific Laboratory, University of California; (b) Department of Biochemistry, University of Colorado Medical Center; (c) Department of Biophysics and Genetics, University of Colorado Medical Center.
(2) (a) Galardy, R. E.; Bleich, H. E.; Ziegler, P.; Craig, L. C. *Biochemistry* **1976**, *15*, 2303–2309. (b) Bleich, H. E.; Freer, R. J.; Stafford, S. S.; Galardy, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3630.
(3) Deslauriers, R.; Komoroski, R. A.; Levy, G. C.; Paiva, A. C. M.; Smith, I. C. P. *FEBS Lett.* **1976**, *62*, 50.
(4) Maia, H. L.; Orrell, K. G.; Rydon, H. N. *J. Chem. Soc. Perkin Trans. 2*, **1976**, 761–763.
(5) Cheng, H. N.; Bovey, F. A. *Biopolymers*, **1977**, *16*, 1465–1472.
(6) Brandts, J. F.; Halvorson, H. R.; Brennan, M. *Biochemistry* **1975**, *14*, 4953–4963.
(7) (a) Bodanszky, M.; Sheehan, J. T.; Ondetti, M. A.; Lande, S. *J. Am. Chem. Soc.*, **1963**, *85*, 991–997. (b) Bodanszky, M.; Ondetti, M. A.; Sheehan, J. T.; Lande, S. *Ann. N.Y. Acad. Sci.* **1963**, *104*, 24–34.
(8) Schröder, E.; *Justus Liebigs Ann. Chem.* **1964**, *673*, 186–195.
(9) Ivanov, V. T.; Filatova, M. P.; Reissmann, Z.; Reutova, T. O.; Chekhlyaeva, N. M. *Bioorg. Khim.* **1977**, *3*, 1157–1168.
(10) Efremov, E. S.; Filatova, M. P.; Reutova, T. O.; Stepanova, L. N.; Reissmann, Z.; Ivanov, V. T. *Bioorg. Khim.* **1977**, *3*, 1169–1180.
(11) Filatova, M. P.; Reissmann, Z.; Reutova, T. O.; Ivanov, V. T.; Grigoryan, G. L.; Shapiro, A. M.; Rozantsev, E. G. *Bioorg. Khim.* **1977**, *3*, 1181–1189.
(12) Grathwohl, C.; Wüthrich, K. *Biopolymers*, **1976**, *15*, 2025–2041, 2043–2057.
(13) Evans, C. A.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1974**, *96*, 7312.
(14) Stewart, J. M.; Young, J. D. "Solid-Phase Peptide Synthesis"; W. H. Freeman: San Francisco, 1969.
(15) Vold, R. L.; Waugh, J. S.; Klein, M. P.; Phelps, D. E. *J. Chem. Phys.* **1968**, *48*, 3831.
(16) Cann, J. R.; Stewart, J. M.; London, R. E.; Matwiyoff, N. A. *Biochemistry* **1976**, *15*, 498–504.
(17) Freer, R. J.; Stewart, J. M. *J. Med. Chem.* **1972**, *15*, 1–5.
(18) Freer, R. J.; Stewart, J. M. *Arch. Int. Pharmacodyn.* **1975**, *217*, 97–109.
(19) Thomas, W. A.; Williams, M. K. *J. Chem. Soc., Chem. Commun.* **1972**, 994.
(20) Dorman, D. E.; Bovey, F. A. *J. Org. Chem.* **1973**, *38*, 2379–2383.
(21) London, R. E.; Stewart, J. M.; Cann, J. R.; Matwiyoff, N. A. *Biochemistry* **1978**, *17*, 2270–2277.
(22) Howarth, O. W.; Lilley, D. M. *Progr. NMR Spectrosc.* **1978**, *12*, 1–40.
(23) Thomas, W. A. *Annu. Rep. NMR Spectrosc.* **1970**, *3*, 91–147.
(24) Pehk, T.; Lippmaa, E. *Eesti NSV Tead. Akad. Toim. Keem. Geol.* **1968**, *17*, 291.
(25) Cann, J. R.; Stewart, J. M.; Matsueda, G. *Biochemistry* **1973**, *12*, 3780–3788.
(26) Marlborough, D. I.; Ryan, J. W.; Felix, A. M. *Arch. Biochem. Biophys.* **1976**, *176*, 582–590.
(27) Cann, J. R.; London, R. E.; Stewart, J. M.; Matwiyoff, N. A., *Int. J. Pept. Protein Res.*, in press.
(28) Fossel, E. T.; Easwaran, K. R. K.; Blout, E. R. *Biopolymers* **1975**, *14*, 927–935.
(29) Deslauriers, R.; Smith, I. C. P.; Walter, R. *J. Am. Chem. Soc.* **1974**, *96*, 2289–2291.
(30) Deslauriers, R.; Somorjai, R. L. *J. Am. Chem. Soc.* **1976**, *98*, 1931–1939.
(31) Cutnell, J. D.; Glasel, J. A.; Hruby, V. J. *Org. Magn. Reson.* **1975**, *7*, 256–261.
(32) London, R. E.; Avitabile, J. *J. Chem. Phys.* **1976**, 2443–2450; *ibid.* **1977**, *66*, 4254.
(33) London, R. E. *J. Am. Chem. Soc.* **1978**, *100*, 2678–2685.
(34) Duvernet, R.; Boekelheide, V. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2961–2964.
(35) Brady, A. H.; Ryan, J. W.; Stewart, J. M. *Biochem. J.* **1971**, *121*, 179.
(36) (a) Kopple, K. D.; Marr, D. H. *J. Am. Chem. Soc.* **1967**, *89*, 6193–6200. (b) Kopple, K. D.; Ohnishi, M. *J. Am. Chem. Soc.* **1969**, *91*, 962–970. (c) Ziauddin; Kopple, K. D. *J. Org. Chem.* **1970**, *35*, 253.
(37) (a) Bovey, F. A.; Tiers, G. V. D. *J. Am. Chem. Soc.* **1959**, *81*, 2870–2878. (b) Halpern, B.; Nitcki, D. E.; Weinstein, B. *Tetrahedron Lett.* **1967**, 3075.
(38) Batchelor, J. G. *J. Am. Chem. Soc.* **1975**, *97*, 3410–3415.
(39) Galaktionov, S. G.; Sherman, S. A.; Shenderovich, M. D.; Nikiforovich, G. V.; Leonova, V. I. *Bioorg. Khim.* **1977**, *3*, 1157–1168.
(40) Torchia, D. A.; Deber, C. M. *Biopolymers* **1972**, *11*, 653–659.
(41) London, R. E.; Stewart, J. M.; Cann, J. R.; Matwiyoff, N. A. *Biochemistry* **1978**, *17*, 2277.
(42) Fermandjian, S.; Tran-Dinh, S.; Savrda, J.; Sala, E.; Mermet-Bouvier, R.; Bricas, E.; Fromageot, P. *Biochim. Biophys. Acta* **1975**, *399*, 313–338.

Crystal and Molecular Structure of Oxythiamin Chloride Hydrochloride Monohydrate. A Thiamin Antagonist with a Conformation That Differs from Thiamin

Whanchul Shin,[1] James Pletcher,* Martin Sax,* and Gary Blank

Contribution from the Biocrystallography Lab, VA Medical Center, Pittsburgh, Pennsylvania 15240, and Crystallography Department, University of Pittsburgh, Pittsburgh, Pennsylvania 15260.

Received August 17, 1978

Abstract: The hydrochloride salt of oxythiamin assumes a conformation with respect to its C(3,5′) methylene bridge that differs substantially from the characteristic conformations of thiamin or its C(2) substituted derivatives. Its intermolecular bonding patterns are very similar to those observed in thiamin structures, although N(3′) is now a hydrogen bond donor and the 4′-oxo group is a hydrogen bond acceptor. There is also a close contact between a chloride ion and the oxopyrimidine ring normal to the plane of the ring. The crystal structure was determined using diffractometer data obtained by the θ:2θ scan technique with Cu radiation from a crystal having $P2_1$ space group symmetry and unit cell parameters a = 13.072 (4), b = 8.977 (3), c = 15.097 (4) Å, and β = 110.17 (2)°. The structure was solved by direct methods and refined by least squares to an R = 0.104 for all 3039 independent reflections and an R = 0.041 for the 1788 observed reflections.

Oxythiamin is a broadly active and potent antagonist of thiamin[2] (vitamin B_1), which differs from thiamin only in that an oxygen atom replaces the 4′-amino group. Oxythiamin can be readily converted to its pyrophosphate ester, since it is a suitable substrate for thiamin kinase, the enzyme that catalyzes the phosphorylation of thiamin into the active coenzyme, thiamin pyrophosphate. Furthermore, it has been found that oxythiamin pyrophosphate, OTPP, is as acceptable as thiamin pyrophosphate at the binding site of pyruvate decarboxylase, and that pyruvate reacts with the OTPP holoenzyme to form the C(2) adduct intermediate, but the reaction does not proceed to release the acetaldehyde product.[3] The inhibitory properties of oxythiamin have been attributed to its inability to form the intramolecular N···O hydrogen bond between the 2α-hydroxyl group and the 4′ substituent. This intramolecular hydrogen bond has been proposed to both stabilize the adduct and assist in proton removal. In order to achieve this interaction, the C(2) adduct of thiamin is depicted as assuming a **V** conformation. (See ref 4 for a description of several conformations.) However, this is not supported by the crystal-

0002-7863/79/1501-2462$01.00/0