left in the aqueous state. Hemocyanin was hydrolyzed in 6 M HCl (constant boiling) at 110 °C for 22 h in an evacuated sealed tube. The hydrolyzates were prepared in duplicates. Cyst(e)ine content was determined as cysteic acid, following oxidation with performic acid.

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References and Notes

(1) (a) Department of Botany; (b) Department of Chemistry.
(2) (a) E. I. Solomon, P. J. Clendening, H. B. Gray, and F. J. Grunthaner, *J. Am. Chem. Soc.*, **97**, 3878 (1975); (b) P. R. Milne, J. R. E. Wells, and R. P. Ambler, *Biochem. J.*, **143**, 691 (1974).
(3) M. M. Millard, *Adv. Exp. Med. Biol.*, **48**, 589 (1974).
(4) L. N. Kramer, Ph.D. Thesis, University of California, Berkeley, Calif., 1971.
(5) F. J. Grunthaner, Ph.D. Thesis, California Institute of Technology, Pasadena, Calif., 1974.
(6) J. Peeling, D. T. Clark, I. M. Evans, and D. Boulter, *J. Sci. Food Agric.*, **27**, 331 (1976).
(7) K. Siegbahn, C. Nordling, A. Fahlman, R. Nordbert, K. Hamrin, J. Hedman, G. Johansson, T. Bergmark, S. E. Karlson, I. Lindgren, and B. Linberg, *Nova Acta Regiae Soc. Sci. Ups.*, **20** (1967).
(8) B. J. Lindberg, K. Hamrin, G. Johansson, U. Gelius, A. Fahlman, C. Nordling, and K. Siegbahn, UUIP-638, March 1970.
(9) J. A. M. Ramshaw, M. D. Scawen, C. J. Bailey, and D. Boulter, *Biochem. J.*, **139**, 583 (1974).
(10) M. D. Scawen and D. Boulter, *Biochem. J.*, **143**, 257 (1974).
(11) J. A. M. Ramshaw, R. H. Brown, M. D. Scawen, and D. Boulter, *Biochim. Biophys. Acta*, **303**, 269 (1973).
(12) "Cysteine" and "cystine" are used sensu original publications.

^{13}C NMR Relaxation Studies of Complexes between *cyclo*(L-Pro-Gly)$_3$ and Amino Acids. Conformational Aspects of Stepwise Binding†

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Abstract: ^{13}C NMR relaxation and chemical shift measurements have been used to study the complexes of proline and valine amino acid salts (HCl·Pro-OBz and HCl·Val-OMe) with the synthetic ion-binding cyclic hexapeptide, *cyclo*(L-Pro-Gly)$_3$. While HCl·Pro-OBz was found to form a discrete 1:1 complex with the cyclic peptide, HCl·Val-OMe forms a 2:1 complex, an "amino acid sandwich", as well as a 1:1 complex. Evidence from appropriate changes in NT_1 values and ^{13}C chemical shifts confirmed the existence of these various complexes; for example, the NT_1 values found for α-carbon atoms of the free amino acid, HCl·Pro-OBz, and the free peptide decreased in the complexes to values which were identical within experimental error. By observation of selective broadening of ^{13}C resonances upon binding the paramagnetic Mn^{2+} cation, the primary binding site of *cyclo*(L-Pro-Gly)$_3$ is inferred to be the Gly carbonyl groups.

Complexes between "larger" and "smaller" molecules, such as the binding of an enzyme to its substrate, are widespread in chemistry and biology.[2] In the larger moiety, binding is often accompanied by induced structural alterations which produce cooperative and/or allosteric phenomena.[3-5] Direct observation of the conformational adjustments of an enzyme, induced upon binding a substrate or cofactor, has been limited by the low molar concentrations obtainable and the complexities of proteins. Consequently, it is often difficult to probe the restrictions to motion, the detailed stoichiometry, and mutual conformational adjustments which occur in both components upon binding. Since aspects of molecular motion may be examined through ^{13}C spin-lattice relaxation measurements (T_1's),[6-11] while changes in molecular conformation of interacting species can be correlated with variations in chemical shifts,[12-14] ^{13}C nuclear magnetic resonance (NMR) spectroscopy is particularly well-suited for investigation of binding interactions.[15] These circumstances led us to apply ^{13}C NMR spectroscopic methods to a system in which aspects of conformation and molecular motion could be elucidated for both the larger and smaller components of a molecular complex.

In this communication we report the use of ^{13}C NMR to monitor the conformational changes which take place in the formation of complexes between a synthetic ion-binding peptide and certain amino acid salts. The stabilizing interactions involved in binding include, primarily, delocalization of the net positive charge of the amino acid ammonium group over the peptide carbonyl oxygens, as well as possible hydrogen bonding and hydrophobic interactions similar to those expected to stabilize enzyme:substrate or hormone:receptor complexes.

Impetus for the present investigation was provided by the discovery that amino acid ester hydrochlorides (which may be viewed as alkylammonium salts; e.g., $RNH_3^+Cl^-$) bind specifically to the cyclic hexapeptide, *cyclo*(L-Pro-Gly)$_3$[13] (designated hereafter as $c(PG)_3$)[16]. The cation binding capabilities of this peptide have already been described.[17] (We propose the trivial name "aminophore" to describe the class of compounds, including the present ion-binding cyclic peptide, capable of interacting specifically with and/or transporting compounds containing protonated amino groups.)

In contrast to metal ion: ionophore complexes, both components of amino acid:cyclic peptide complexes contain carbon atoms (and are of suitable molecular size) such that detailed structural features in both species can be studied spectroscopically by ^{13}C NMR. If a discrete amino acid:$c(PG)_3$ complex is formed (see Figure 1), its existence would be confirmed by the decrease in the average relaxation times, T_1, of the carbon atoms of both the peptide and the amino acid (relative to the T_1 values of the free components). A similar decrease of T_1's of the protons of acetylcholine was reported recently upon complex formation between ATP and acetylcholine,[18] and in a related approach, binding of ^{13}C-enriched ox-

† This paper is dedicated to Professor R. B. Woodward on the occasion of his sixtieth birthday. E.R.B. is pleased to acknowledge over thirty years of stimulating scientific and personal association.

ytocin to neurophysin II was examined.[19] A preliminary account of the present work has appeared.[20]

It has already been demonstrated, at least for metal cations such as magnesium, that the conformational change induced in $c(PG)_3$ upon binding one mole of salt leads to the formation of a second binding site on the opposite face by concomitant reorientation of appropriate carbonyl groups.[17] Thus, since there are two *potential* binding sites on $c(PG)_3$ located on opposite faces of this disk-shaped molecule (consisting, respectively, of three Gly and three Pro carbonyl groups), the binding of more than one molecule of amino acid to each cyclic peptide is possible.

In the present report the conformational details of Pro-OBz:$c(PG)_3$ and Val-OMe:$c(PG)_3$ complexes are examined through the application of ^{13}C NMR measurements of their T_1 and chemical shift changes. The existence of an "amino acid sandwich", Val-OMe:$c(PG)_3$:Val-OMe, is demonstrated, and its formation is discussed in terms of stepwise conformational adjustments upon binding.

Materials and Methods

cyclo(Pro-Gly)$_3$ was prepared as described previously[21] and was used as the dimethylformamide adduct. Proline benzyl ester hydrochloride (HCl·Pro-OBz) was prepared according to the procedure of Newman and Smith.[22] Valine methyl ester hydrochloride (HCl·Val-OMe) was purchased from Ajimoto Co., Inc., Japan. Deuteriochloroform (99.8% *d*) was purchased from Merck Sharp and Dohme Isotopic Products, Teterboro, N.J.

Sample Preparation. A weighed quantity of amino acid hydrochloride was dissolved in 1.0 ml of $CDCl_3$ (to give a ca. 0.2 M solution) and transferred to an 8 or 10 mm (o.d.) NMR sample tube. After T_1 data were obtained on this sample, 0.25 equiv of cyclic peptide was added, and the T_1 data were taken again. T_1 data were generally collected for mole ratios of salt:peptide of 4:1, 2:1, and 1:1. Duplicate experiments with gassed and degassed samples yielded comparable results within experimental error. Degassing was accomplished by bubbling argon gas through the solvent for 10 min prior to sample preparation. Most T_1 values were determined on undegassed samples.

T_1 Measurements. All spectra were recorded on a Varian CFT-20 ^{13}C NMR spectrometer. The field strength of this instrument is 18.682 kG. The ambient temperature of the probe is ca. 30 °C. The required pulse widths of 180 and 90° were determined from a standard *t*-BuOH/DMSO-d_6 sample. Two pulse sequences were used to determine T_1 values: the inversion recovery method which utilizes a $-(180°\text{-}t\text{-}90°\text{-}5T_1)_n-$ sequence, and the progressive saturation method which uses a $-(90°\text{-}t\text{-}90°)_n-$ sequence.[23] The latter method was used with the "random-wait" parameter as described by Freeman and Hill.[24]

T_1 values were determined from a non-linear least-squares fitting procedure for the following equations:

$$A = A_0(1 - 2e^{-t/T_1}) \qquad -(180°\text{-}t\text{-}90°\text{-}5T_1)_n-$$

$$A = A_0(1 - e^{-t/T_1}) \qquad -(90°\text{-}t\text{-}90°)_n-$$

Here, A and A_0 are peak intensities at any time, t, and at $t = \infty$, respectively. The standard error of reported T_1 values is 10%. Nuclear Overhauser enhancements for both the salts and the cyclic peptide were found to be 3.0, within experimental error for aliphatic protonated carbons. This result indicates that dipolar coupling is the dominant relaxation mechanism.[6-8] The relaxation times of the aromatic and nonprotonated carbons were not determined.

Analysis of T_1 Data. For the molecules and complexes reported herein, the ^{13}C-^1H dipolar interaction dominates the ^{13}C relaxation of protonated carbons. Furthermore, at our magnetic field strength (20 MHz) the ^{13}C-^1H dipolar relaxation satisfies the extreme narrowing condition; i.e., $(\omega_H + \omega_C)^2\tau_c^2 \ll 1$. Thus, the T_1 values of the protonated carbons are related in a straightforward manner to the rotational correlation time, τ_c, of the ^{13}C-^1H pair, as described in eq 1.

$$\frac{1}{NT_1} = \frac{\gamma_C^2\gamma_H^2\hbar^2}{r^6}\tau_c \qquad (1)$$

Here, N is the number of directly bonded protons; γ_C and γ_H are the gyromagnetic ratios of carbon and hydrogen, respectively; $\hbar$ is Planck's constant divided by 2π; r is the C-H bond distance; and τ_c is the rotational correlation time of the C-H pair. For a C-H bond length of 1.09 Å, eq 1 becomes

$$1/NT_1 = 2.15 \times 10^{10}\tau_c \qquad (2)$$

where τ_c may represent the rotational correlation time of a molecule, provided that the C-H pair within the molecule has no internal degrees of freedom.

If the exchange rate between the free and complexed state is fast on the NMR time scale, only a single resonance will be observed for each (nonequivalent) carbon of the salt and peptide. The T_1 value of a given salt or peptide carbon atom will then be a function of the relative populations of free and bound salt. This is quantified in eq 3:

$$\frac{1}{T_1^{obsd}} = \frac{n_B}{T_1^B} + \frac{n_F}{T_1^F} \qquad (3)$$

where n_B and n_F are the mole fraction of bound and free amino acid, respectively, and T_1^B and T_1^F are the relaxation times of the bound and free amino acid, respectively. Evidence for the existence of fast exchange for these complexes in chloroform solution has been presented in a previous communication[13] and is further verified here by the observation of single averaged resonances at all salt to peptide ratios. In that report the ammonium (or "immonium" for Pro) moiety of the amino acid hydrochloride was proposed as the site at which the cyclic peptide complexes the amino acid, as shown in Figure 1.

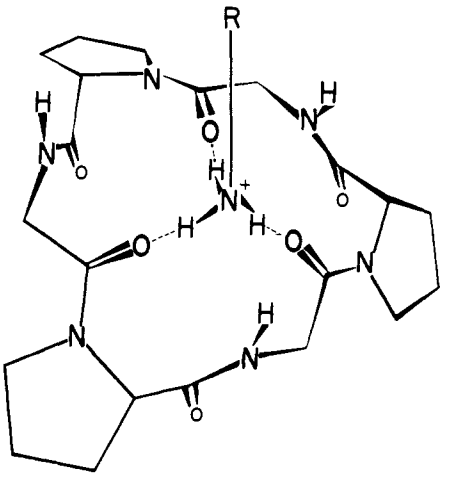

Figure 1. The binding scheme of an amino acid salt to $c(PG)_3$. An amino acid salt, e.g., Val-OMe, is represented by RNH_3^+; Pro-OBz would be $RR'NH_2^+$. The N-C_α bond of the salt has been elongated for clarity. Binding is shown to the trio of Gly carbonyls. All six peptide bonds in the cyclic peptide are trans.

Table I. NT_1 Values (s) of HCl·Pro-OBz and *cyclo*(Pro-Gly)$_3$

Mole ratio salt/peptide	Salt	Peptide	
	Pro_α	Pro_α	Gly_α
Free	0.59	0.44	0.47
4:1	0.50		
2:1	0.40	0.32	0.30
1:1	0.34	0.31	0.32

Results

Complexes with HCl·Pro-OBz. The NT_1 value of the HCl·Pro-OBz α-carbon decreases monotonically as the equivalents of $c(PG)_3$ are increased (see Table I). Simultaneously, NT_1 values for the cyclic peptide α-carbons also become shorter in the presence of salt. These results are consistent with the fact that an amino acid salt of molecular weight 252, complexed with a peptide of molecular weight 462, should have NT_1 values characteristic of a molecule of molecular weight 714. The amino acid experiences the larger percentage gain

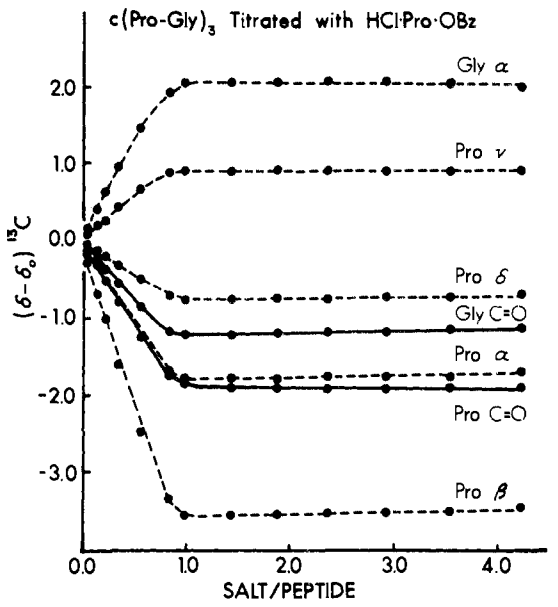

Figure 2. Variations of ^{13}C chemical shifts of $c(PG)_3$ when titrated with Pro-OBz; solvent, $CDCl_3$; concentration of $c(PG)_3$, 0.1 M. ($\delta - \delta_0$) represents the difference in chemical shift between that of the free peptide, δ_0, and the peptide in the presence of a given concentration of amino acid, δ, in ppm.

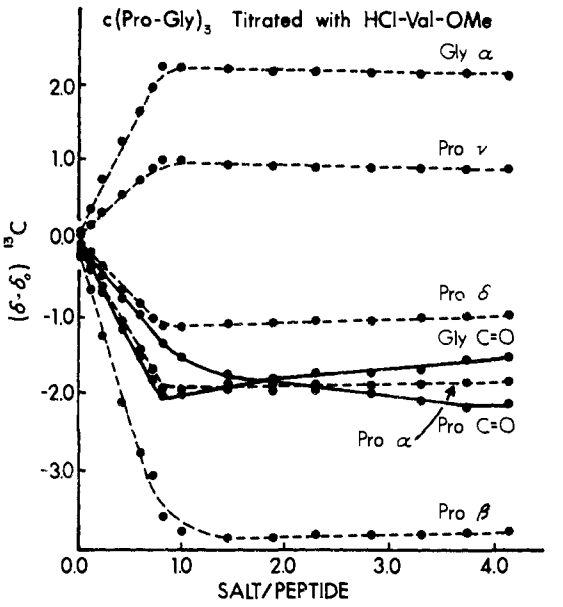

Figure 3. Variation of ^{13}C chemical shifts of $c(PG)_3$ when titrated with Val-OMe; solvent, $CDCl_3$; concentration of $c(PG)_3$, 0.1 M. ($\delta - \delta_0$) represents the difference in chemical shift between that of the free peptide, δ_0, and the peptide in the presence of a given concentration of amino acid, δ, in ppm.

in molecular weight and, hence, displays the larger percentage drop in T_1's. While these molecular weights include the chloride anion of the amino acid salt, the data provide no information as to its location with respect to the complex or whether it "tumbles along with the complex".

Since the cyclic peptide Pro C_α and Gly C_α carbons comprise a portion of the relatively rigid peptide backbone, it is assumed that the corresponding C_α–H pairs have no internal degrees of freedom (other than possible puckering around the sterically unhindered Gly C_α, particularly in free $c(PG)_3$). This assumption is supported by the observation that NT_1 values of the Pro C_α and Gly C_α of $c(PG)_3$ are nearly identical in both the free and complexed state. At a mole ratio of 1:1 Pro-OBz:$c(PG)_3$, the NT_1 of the amino acid α-carbon (0.34 s) is equal, within experimental error, to the NT_1's of the cyclic peptide α-carbons (Pro, 0.31s; Gly, 0.32 s). Assuming that the Pro-OBz:$c(PG)_3$ complex tumbles isotropically, this result provides confirmatory evidence for the existence of a discrete complex.

Other carbon atoms in both components display qualitatively similar decreases in NT_1 values. Interestingly, the NT_1 of the benzyl methylene carbon in the ester portion of Pro-OBz (OCH_2Ph) drops from 1.8 s (free amino acid) to 0.49 s (1:1 complex). The fact that both of these NT_1 values are greater than the corresponding α-carbon values (0.59 s free, 0.34 s bound) indicates the occurrence of internal motion in this portion of the amino acid salt, as might be anticipated for atoms in Pro-OBz other than those contained in the rigid pyrrolidine ring.

Chemical shift changes in both species were also monitored during these titrations, and the results for $c(PG)_3$ are shown graphically in Figure 2. The chemical shifts of $c(PG)_3$ have apparently reached their limiting values at a 1:1 mol ratio of amino acid:peptide. This finding supports the inference from T_1 measurements of the formation of a 1:1 amino acid:cyclic peptide complex. Chemical shift changes in the salt (Pro-OBz) were of lesser magnitude but also reached their limiting values at 1:1 molar ratios.

Preliminary studies with the *N*-methylated amino acid, HCl·Sar-OMe, indicate that it binds $c(PG)_3$ similarly.

Table II. NT_1 Values (s) of HCl·Val-OMe and *cyclo*(Pro-Gly)$_3$

Mole ratio, salt/peptide	Salt	Peptide	
	Val_α	Pro_α	Gly_α
Free	0.62	0.44	0.47
4:1	0.41	0.27	0.28
2:1	0.34	0.28	0.28
1:1	0.48	0.29	0.32

Complexes with HCl·Val-OMe. As with the Pro-OBz salt, the α-carbon NT_1 value for valine salt decreases with increasing cyclic peptide concentration (Table II). In the Val-OMe case, however, α-carbon NT_1 values *do not decrease monotonically;* at a mole ratio of 2:1 Val-OMe:$c(PG)_3$ the Val-OMe C_α NT_1 goes through an apparent minimum, and as the concentration of peptide increases, NT_1 increases but does not return to its value in the free Val-OMe salt. Additional unusual aspects of the Val-OMe:$c(PG)_3$ system are apparent from inspection of the plots of chemical shift changes of $c(PG)_3$ titrated with HCl·Val-OMe (Figure 3). Up to a 1:1 mol ratio the trends in the titration curves of the $c(PG)_3$ carbonyl carbon chemical shifts are similar, using either Pro-OBz or Val-OMe as the titrant (compare Figures 2 and 3). Beyond the 1:1 mol ratio, however, in the Val-OMe case the $c(PG)_3$ carbonyl chemical shifts continue to change as more Val-OMe is added (although the $c(PG)_3$ side chains and α-carbon resonances have essentially leveled off in this region).

Determination of the "Primary" Binding Face of $c(PG)_3$ Using a Paramagnetic Cation, Mn^{2+}. In an effort to determine precisely which trio of carbonyl groups (i.e., the three Pro carbonyls or the three Gly carbonyls), if either, comprises the *primary* binding site for cations with $c(PG)_3$, the ^{13}C spectrum of $c(PG)_3$ was monitored as small amounts of manganese *tert*-butyloxycarbonylalanate ($Mn(Boc\text{-}Ala)_2$) were added to the NMR sample.[25] Since Mn^{2+} is a paramagnetic cation, it can effectively relax nearby nuclei[15] with the result that the resonances due to carbons closest to the cation will experience significant broadening. As shown in Figure 4, the Gly carbonyl

Table III. Correlation Times, τ_c, of HCl·Val-OMe, HCl·Pro-OBz, *cyclo*(Pro-Gly)$_3$, and Their Complexes

Species	$\tau_c \times 10^{-10}$, s
HCl·Pro-OBz, free	0.75[a]
HCl·Val-OMe, free	0.75[a]
cyclo(Pro-Gly)$_3$, free	1.1[b]
HCl·Pro-OBz/c(PG)$_3$ complex, 1:1	1.5[b]
HCl·Val-OMe/c(PG)$_3$ complex, 1:1	1.5[b]

[a] τ_c determined from NT_1 values of amino acid α-carbons (using eq 2). [b] τ_c determined from NT_1 values of c(PG)$_3$ α-carbons.

resonance has broadened significantly relative to the Pro carbonyl resonance. This result suggests that the primary binding site for Mn^{2+} is on the c(PG)$_3$ face containing the Gly carbonyls, although one cannot rule out the possibility that some primary binding also occurs at the Pro carbonyls. Preliminary circular dichroism studies indicate that added Mn^{2+} ion produces parallel conformational changes in c(PG)$_3$ to those of Mg^{2+} ion.[26] While the various c(PG)$_3$ cation complexes studied thus far have varying stoichiometries (e.g., Mg^{2+}, Mn^{2+}, Val-OMe$^+$ form ion sandwiches, although Li^+, Na^+, Ca^{2+}, Ba^{2+}, and Pro-OBz$^+$ do not[17]), the present results may be tentatively extrapolated to indicate that the Gly carbonyls are also the primary binding site for other cations and amino acid salts.

Discussion

Since the molecular weight of any complex is larger than the individual molecular weights of its components, the rotational correlation time of the complex should be longer (i.e., slower motion) than the corresponding values for its individual components. Thus, an increase observed in the correlation time, τ_c, as manifested by a decrease in experimental NT_1 values, provides good evidence for the formation of a discrete complex in solution. Indeed, as indicated in the Results section, the T_1 data reported herein confirm the existence of Pro-OBz:c(PG)$_3$ and Val-OMe:c(PG)$_3$ complexes; both amino acid and cyclic peptide display NT_1 values which have decreased significantly relative to the corresponding NT_1 values for the free species and have, for the Pro-OBz case, converged to a common value. The data, therefore, show that the Pro-OBz:c(PG)$_3$ and Val-OMe:c(PG)$_3$ species are specific complexes, in which the components tumble as an entity, in contrast to nonspecific complexes (e.g., peptide:solvent) in which the components do interact but behave essentially as independent species.

The correlation times for the various components and complexes, calculated from the measured NT_1 values of the appropriate α-carbons using eq 2, are given in Table III. If there are no internal degrees of rotational freedom, the NT_1 values of both species in a 1:1 complex should be equal. However, the NT_1 value of Val-OMe C_α has not decreased to the value of the c(PG)$_3$ α-carbons at a 1:1 mol ratio (as has the Pro-OBz C_α, see Tables I and II). Since essentially all Val-OMe molecules are expected to be bound under these conditions, this result may be attributed to some free rotation about the Val-OMe N-C_α bond in the complex. By comparison, the corresponding N-C_α bond in Pro-OBz is incorporated into the pyrrolidine side chain and would not have such rotational freedom. If the C_α carbon of Val-OMe is considered to be a rotating methine carbon attached to an isotropically rotating body, the internal rotational correlation time about the Val-OMe N-C_α bond can be determined.[7,8] Using the result (Table III) that the Val-OMe:c(PG)$_3$ complex tumbles with a correlation time of 1.5×10^{-10} s, the rotational correlation time about the Val-OMe N-C_α bond is computed to be 0.83×10^{-10} s. This finding indicates that motion about the Val-OMe N-C_α bond is more rapid than the overall rotation of the complex.

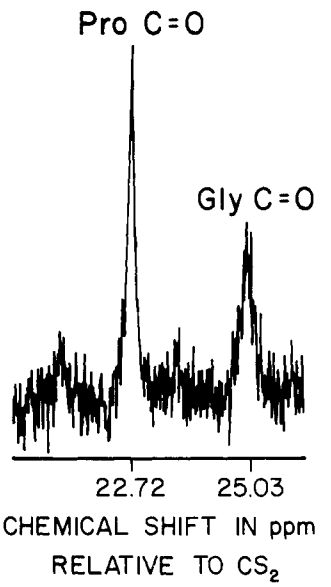

Figure 4. ^{13}C spectrum of the carbonyl resonances of c(PG)$_3$ in the presence of Mn(Boc-Ala)$_2$. The molar ratio of salt to peptide is 3.6×10^{-5}. Carbonyl resonances were assigned through ^{13}C enrichment at the Gly carbonyl (in another c(PG)$_3$ sample[17a]).

Table IV. ^{13}C Chemical Shift Changes of *cyclo*(Pro-Gly)$_3$ Carbonyl Carbons

Salt (HCl)	Equiv salt/ equiv c(PG)$_3$	$(\delta - \delta_0)$,[a] ppm	
		Pro C=O	Gly C=O
Pro-OBz	1/1	−1.85	−1.24
Pro-OBz	4/1	−1.89	−1.13
Val-OMe	1/1	−1.50	−2.00
Val-OMe	4/1	−2.31	−1.51

[a] $(\delta - \delta_0)$ = change in chemical shift vs. free *cyclo*(Pro-Gly)$_3$ in chloroform solutions.

"Amino Acid Sandwich" Complexes of Val-OMe·HCl and *cyclo*(Pro-Gly)$_3$. Evidence for the presence of higher order complexes of Val-OMe with c(PG)$_3$ emerged from several aspects of the experimental data. It has been noted in the Results section (and may be seen in Table II) that, upon titration with c(PG)$_3$, NT_1 values for Val-OMe go through a minimum at ca. a 2:1 ratio of Val-OMe:c(PG)$_3$. This finding suggests the formation of a "higher order" species (which reverts to the 1:1 complex when sufficient c(PG)$_3$ has been added). Furthermore, c(PG)$_3$ chemical shifts continue to change as excess Val-OMe (but not Pro-OBz) is added, specifically at carbonyl positions where (additional) binding would be occurring. Table IV compares the behavior of the c(PG)$_3$ carbonyls upon addition of 1 and 4 equiv respectively of both Pro-OBz and Val-OMe. Although the change in c(PG)$_3$ carbonyls is negligible on going from 1:1 to 4:1 ratios of Pro-OBz:peptide, the addition of excess Val-OMe moves the c(PG)$_3$ Pro carbonyl further downfield by 0.81 ppm and the Gly back upfield 0.49 ppm. These results indicate that the cyclic peptide carbonyls are interacting to a further extent with excess Val-OMe.

c(PG)$_3$ is known to form both "peptide sandwiches" (two peptides:one cation) and "cation sandwiches" (two cations:one peptide), the latter particularly with magnesium ion.[17] Presumably, the two binding sites in the cation sandwich are the three Gly and three Pro carbonyl oxygen atoms on opposite faces of the peptide. Metal cations are "spherical" and can

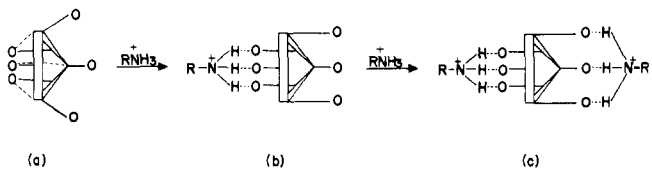

Figure 5. Schematic of the stepwise binding of Val-OMe to $c(PG)_3$. (a) Free $c(PG)_3$ in γ-turn conformation, - - - indicates hydrogen bonds to Gly NH's; (b) 1:1 complex (salt presumably bound to Gly carbonyls); (c) 2:1 complex (for Val-OMe, R = $CH(CH_3)_2CO_2CH_3$).

reside between two cyclic peptide molecules. However, one does not expect the formation of 2:1 cyclic peptide:amino acid complexes in the present experiments, since alkylammonium cations are bulky, dissymmetric species with positively charged heads oriented toward the peptide face and hydrophobic tails which prohibit the approach of another cyclic peptide molecule. However, in the presence of excess RNH_3^+ species, the formation of a 2:1 amino acid:cyclic peptide complex (e.g., Val-OMe:$c(PG)_3$:Val-OMe) is a strong possibility. Indeed, considering the body of data presented above, we believe that evidence for the existence of the "valine sandwich" complex is compelling.

Stepwise Binding of Val-OMe to *cyclo*(Pro-Gly)$_3$. Given that the primary binding site on the cyclic peptide is composed of the three Gly carbonyls, as deduced from the Mn^{2+} experiments, the following model for the stepwise binding of Val-OMe molecules to $c(PG)_3$ may be proposed. The free peptide in chloroform is known to have its three Gly carbonyls involved in intramolecular hydrogen bonds ("γ turns")[17] and its three Pro carbonyls on the opposite face arrayed away from the central peptide cavity (Figure 5a). When the first molecule of Val-OMe is introduced, rotations about backbone single bonds occur,[17] causing the Gly carbonyls to pucker slightly outward to receive the incoming cation, while also causing the three Pro carbonyls to rotate inward to form a second binding pocket (Figure 5b). In effect, this transformation converts the three Pro carbonyls from a *potential binding site* to an *actual binding site*, and the conformation of the cyclic peptide is now such that it is "set up" to receive a second Val-OMe molecule at the three Pro carbonyls. This latter binding step (depicted in Figure 5c), which produces a ternary complex, would not be nearly as favorable (vide infra) had not formation of the binary Val-OMe:$c(PG)_3$ complex produced the necessary conformational change in the cyclic peptide.

That this second binding event occurs with little or no *further* conformational change in the cyclic peptide is supported by the ^{13}C chemical shift data. The relatively large (some greater than 3 ppm) chemical shift changes in resonances due to atoms *remote from binding sites* upon addition of the first mole of Val-OMe (Figure 3) must reflect almost exclusively *conformational* changes in both components of the incipient complex (such as the relief of the Pro-C_β-Pro-C=O eclipsing interaction present in free $c(PG)_3$). Perhaps a significant portion of the change, even in carbonyl chemical shifts, is also due to conformational change. However, since the binding of the second Val-OMe molecule to the cyclic peptide produces significant changes *only* in $c(PG)_3$ carbonyl chemical shifts, it may be concluded that $c(PG)_3$ maintains a relatively "constant conformation" throughout the second binding step.

Because of the relative simplicity of the amino acid:$c(PG)_3$ complexes, we have been able to monitor the conformational accommodations to binding displayed by the larger component of the complexes. In this context, one is struck by certain analogous features of the present systems to examples of protein interactions with small molecules, since, at least conceptually, both allosterism and cooperativity are phenomena whereby subsequent events are facilitated only after preliminary events have occurred.[2-5] In the present systems the formation of the second binding pocket upon complexing of the first Val-OMe molecule to $c(PG)_3$ facilitates the binding of the second molecule of Val-OMe. Thus, in the sense that $c(PG)_3$ has a site specific for a substrate (Val-OMe), adjusts its conformation, and produces a site specific for a further substrate molecule (another Val-OMe), it may be concluded that an allosteric event has occurred. It would seem that the amino acid:cyclic peptide systems could be modified to prepare mixed sandwich complexes containing different amino acids on opposing faces, or one amino acid:one metal cation, in accord with the more usual examples of allosterism.

However, before describing the binding of two Val-OMe molecules to $c(PG)_3$ as "cooperative", one must recognize the qualitative aspects of the existing data vs. the quantitative requirements for "true" or "positive" cooperativity. Thus, if the Val-OMe:$c(PG)_3$:Val-OMe system were cooperative, as in the stepwise binding of oxygen molecules to hemoglobin, then each time a Val-OMe:$c(PG)_3$ 1:1 complex formed, a second Val-OMe molecule would preferably bind to this species and form the Val-OMe:$c(PG)_3$:Val-OMe species before many additional 1:1 complexes formed. In such an idealized situation a solution containing c-$(PG)_3$ in excess over Val-OMe would consist largely of Val-OMe:$c(PG)_3$:Val-OMe and free $c(PG)_3$ species. In reality, the binding constant for the second Val-OMe molecule is unlikely to be greater than that for the first molecule, since (a) the collision of three molecules (of approximately the same order of magnitude in size) to form a ternary complex is not an entropically favorable process and (b) the direct approach of two net positive charges (of alkylammonium groups) toward each other, as required in the formation of Val-OMe:$c(PG)_3$:Val-OMe, is not an electrostatically favorable process. In fact, experimental observations have established that the change from an ammonium binding moiety to the "immonium" group of Pro-OBz is apparently sufficient to preclude the second binding step with Pro-OBz. It remains to be determined in the Val-OMe:$c(PG)_3$ system what the concentration is, if any, of Val-OMe:$c(PG)_3$:Val-OMe species in solutions containing ca. 1:1 molar ratio of Val-OMe and cyclic peptide. Despite these caveats, proton NMR experiments with the magnesium perchlorate:$c(PG)_3$ system revealed[17] that at 1:1 molar ratios of Mg^{2+} to $c(PG)_3$ the solution already contained ca. 30% of Mg^{2+}:$c(PG)_3$:Mg^{2+} species.

We believe the continued use of ^{13}C NMR relaxation methods will allow studies in similar detail of more complicated synthetic and natural binding systems in which complexes of biochemical significance are found.

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References and Notes

(1) This is Cyclic Peptides. 16; for the preceding paper in this series, see P. E. Young, V. Madison and E. R. Blout, *J. Am. Chem. Soc.*, **98**, 5365 (1976).
(2) I. M. Klotz, *Acc. Chem. Res.*, **7**, 162 (1975).
(3) J. Wyman, *Adv. Protein Chem.*, **19**, 223 (1964).
(4) J. Monod, J. Wyman, and J.-P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).
(5) D. E. Koshland, Jr., G. Nemethy, and D. Filmer, *Biochemistry*, **5**, 365 (1966).
(6) A. Allerhand, D. Doddrell, V. Glushko, D. W. Cochran, E. Wenkert, P. J. Lawson, and F. R. N. Gurd, *J. Am. Chem. Soc.*, **93**, 544 (1971).
(7) A. Allerhand, D. Doddrell, and R. Komoroski, *J. Chem. Phys.*, **55**, 189 (1971).
(8) D. Doddrell, V. Glushko, and A. Allerhand, *J. Chem. Phys.*, **56**, 3683 (1972).
(9) A. Allerhand and R. Komoroski, *J. Am. Chem. Soc.*, **95**, 8228 (1973).
(10) D. A. Torchia and J. R. Lyerla, Jr., *Biopolymers*, **13**, 97 (1974).
(11) R. Deslauriers, I. C. P. Smith, and R. Walter, *J. Biol. Chem.*, **249**, 7006 (1974).
(12) E. T. Fossel, W. Veatch, Yu, A. Ovchinnikov, and E. R. Blout, *Biochemistry*,

13, 5264 (1974).
(13) C. M. Deber and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 7566 (1974).
(14) D. A. Torchia, J. R. Lyerla, Jr., and C. M. Deber, *J. Am. Chem. Soc.*, **96**, 5009 (1974).
(15) R. A. Dwek, "Nuclear Magnetic Resonance in Biochemistry; Applications to Enzyme Systems", Clarendon Press, Oxford, 1973.
(16) Abbreviations: *c*(PG)$_3$, *cyclo*(L-Pro-Gly)$_3$; Pro-OBz, proline benzyl ester hydrochloride; Val-OMe, valine methyl ester hydrochloride.
(17) (a) V. Madison, M. Atreyi, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **96**, 6725 (1974); (b) C. M. Deber, V. Madison, and E. R. Blout, *Acc. Chem. Res.*, **9**, 106 (1976).
(18) E. R. Richards and R. R. Sharp, *Biochem. Biophys. Res. Commun.*, **64**, 851 (1975).
(19) I. M. Chaiken, R. E. Randolph, and H. C. Taylor, *Ann. N.Y. Acad. Sci.*, **248**, 442 (1975).
(20) C. M. Deber, B. Bartman, and E. R. Blout, "Peptides: Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Ed., Ann Arbor Science, Ann Arbor, 1975, pp 203–207.
(21) C. M. Deber and E. R. Blout, *Isr. J. Chem.*, **12**, 15 (1974).
(22) R. E. Newman and E. L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).
(23) T. C. Farrar and E. D. Becker, "Pulse and Fourier Transform NMR", Academic Press, New York, N.Y., 1971, Chapter 2.
(24) R. Freeman and H. D. W. Hill, *J. Magn. Reson.*, **4**, 366 (1971).
(25) The use of this manganese salt was dictated by our desire to overcome a recurrent experimental problem; namely, that complexes of *c*(PG)$_3$ with salts having "harder" (more inorganic) anions tend to precipitate from chloroform solutions. Salts of N-protected amino acid carboxylic acids, such as *t*-Boc-Ala-OH, with manganese and several other mono- and divalent metals have been used in our laboratory in this connection. They are readily prepared by mixing the N-protected amino acid and the metal bicarbonate (or carbonate for divalent metals) in water, allowing CO_2 evolution to cease, and evaporating solvent. (C. M. Deber, unpublished results.)
(26) V. Madison, unpublished results.

A Proton Magnetic Resonance Investigation of the Preferred Conformation and the Barrier to Internal Rotation of Phenylcyclopropane

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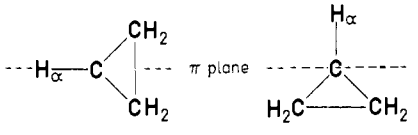
Abstract: Analysis of the proton magnetic resonance spectrum of phenylcyclopropane in CS_2 solution yields -0.23 ± 0.02 Hz for the spin–spin coupling constant over six bonds between the para proton in the phenyl group and the α proton in the cyclopropyl moiety. The assumption that this coupling is dominated by a σ–π electron mechanism, combined with a hindered rotor treatment, indicates a barrier to internal rotation about the C–C bond of 2.0 ± 0.3 kcal/mol. This value assumes a predominantly twofold barrier and implies that the low-energy conformation is that in which the C–H_α bond prefers the plane of the phenyl group. The result is compared with theoretical and other experimental data. The phenyl proton chemical shifts are consistent with electron donation from cyclopropyl to phenyl.

The Walsh model[2] of the orbitals in cyclopropane suggests that maximum conjugative interaction between pπ orbitals of the substituent and the pseudo-π orbitals of the cyclopropyl group in cyclopropane derivatives occurs for a conformation, **1**, in which the C–H_α bond of the cyclopropyl group lies in the benzene plane. If the conformation of the cyclopropyl derivative is determined by this conjugation, then **1** will be of lower energy than **2** and the barrier to internal rotation may well be predominantly twofold in nature.



(1)

(2)

The model is consistent with experiment[3–5] in that the π planes of the NO_2, CHO, COF, and COCl groups in the corresponding derivatives of cyclopropane do prefer an orientation corresponding to **1.** Furthermore, the twofold component in the barrier to internal rotation is 3.3 ± 1.5 kcal/mol for the nitro compound[3] and is 4.4 ± 0.4 kcal/mol in the aldehyde.[4]

Measurements[6] of the temperature dependence of the chemical shift between the ortho and meta protons in *p*-deuteriophenylcyclopropane suggest that **1** is 1.4 kcal/mol lower in energy than **2,** although no error limits were assigned. The torsion frequencies[7] indicate a barrier of 5.8 kcal/mol but the same method yielded rather large internal barriers in molecules like biphenyl and stilbene.

Classical calculations[8] of the conformational energies of **1** and **2** gave ambiguous results. The electron diffraction pattern[8] was consistent with a preferred conformation **1.**

On the other hand, dipole moment[9] and infrared intensity data[10] are interpreted to mean that the cyclopropyl group behaves like an alkyl group in donating electrons to the aromatic ring, its Hammet σ constant being near -0.1.

In this paper an analysis of the proton magnetic resonance spectrum of phenylcyclopropane in CS_2 solution yields long-range coupling constants between the ring protons and α proton on the cyclopropyl group. The assumption of a predominantly twofold barrier to internal rotation allows the deduction of its magnitude from the long-range coupling. Semiempirical and ab initio molecular orbital calculations are presented for **1** and **2.** The ring proton chemical shifts are compared with those expected in toluene.

Experimental Section

A 10 mol % solution of phenylcyclopropane (Aldrich, 97%) in CS_2, containing a little tetramethylsilane, was degassed by the freeze–pump–thaw technique. The proton magnetic resonance spectrum was calibrated at 305K in the frequency sweep mode on an HA100 spectrometer.

INDO MO FPT calculations[11] were performed for conformations **1** and **2** using the geometry based on the electron diffraction data. Ab initio minimal basis set molecular orbital calculations at the STO-3G level[12] were also performed on an IBM 370/158 system.

Results and Discussion

Spectral Analysis. The spectrum corresponds to an ABB′-CC′RXX′YY′ spin system (see **3**) and, as such,[13] cannot be