Spectroscopic Studies of Intramolecular Hydrogen Bonding in Gramicidin S

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Abstract: Dispersion in amide proton exchange rates provided a means of synthesizing variably N-deuterated gramidicin S analogues, the isotopic composition of which were determined quantitatively by NMR spectroscopy. Difference IR spectroscopy of Me_2SO solutions then afforded isolation of residue-specific peptide group vibrations. There are two pairs of equivalent intramolecular hydrogen bonds (H-bonds) with donors (i) Leu NH ($\bar{\nu}_A$ = 3281 cm^{-1}; $\Delta\bar{\nu}_{1/2}$ = 56 cm^{-1}) and (ii) Val NH ($\bar{\nu}_A$ = 3334 cm^{-1}; $\Delta\bar{\nu}_{1/2}$ = 75 cm^{-1}). The IR difference spectra and hydrogen exchange kinetics are consistent with estimates of r_{NO} = 2.85 ± 0.05 Å, $-\Delta H°$ = 3.5 ± 0.5 kcal/mol, and $-\Delta G° \simeq$ 2.0 kcal/mol for H-bond pair (i) and r_{NO} = 2.95 ± 0.05 Å, $-\Delta H°$ = 3.7 ± 0.5 kcal/mol, and $-\Delta G° \geq$ 2 kcal/mol for H-bond pair (ii). H-bonding accounts entirely for the apparent kinetic barrier to proton exchange for Leu NH, while for Val NH ≤30% is of steric origin. Fermi resonance was observed in the amide A′ band, and of the internal amides ν_{ND}(Val) exhibits the stronger coupling to the deformation modes. These results represent the first selective characterization of intramolecular H-bonds in a heteropolypeptide in solution.

The identification of intramolecular hydrogen bonds (H-bonds) in solution has been a major aim of conformational studies of peptides. A variety of experimental methods have been applied to the task,[1] including high-resolution NMR spectroscopy, IR spectrophotometry, and hydrogen exchange (HX) kinetics. Both NMR spectroscopy and HX can be highly selective, but these methods do not differentiate between intramolecular H-bonding and solvation differences in a particular solvent.[2a,b] While IR spectroscopy is exquisitely sensitive to the effects of H-bonding on vibrations of both the donor and the acceptor moieties,[3] there is generally insufficient dispersion of frequencies for a given vibrational mode to render the method useful in any but the simplest oligo- and homooligopeptides,[4,5] and even in these compounds band assignment is not straightforward.[2a] We report here a novel combination of all these three methods which affords the necessary sensitivity and selectivity to provide definitive assignment of vibrational bands and a direct view of residue-specific intramolecular H-bonding in the cyclosymmetric decapeptide antibiotic, gramicidin S (GrS; Figure 1).

Experimental Section

Gramicidin S dihydrochloride (Sigma) was dissolved in H_2O/dioxane (5:2, v/v), filtered, and lyophilized prior to use. It yielded a single spot on TLC in *n*-BuOH/AcOH/H_2O, 4:1:5 (upper phase), and $CHCl_3$/MeOH/AcOH/H_2O, 25:15:4:2. NMR spectra were identical with those published previously.[6] It was therefore judged >99% pure.

Reagent grade Me_2SO (Baker) was distilled under reduced pressure over CaH_2. *p*-Dioxane (Eastman) was distilled over Na metal in the presence of benzophenone. *p*-Dioxane-d_8 99.5% g·atom ^2H (Merck) was used without further purification.

Exchange of ^2H for ^1H was accomplished by the addition of D_2O/dioxane (5:2, v/v), adjusted to the desired glass electrode reading with DCl and NaOD, to a sample of the lyophilized peptide. The final peptide concentration was 0.016 M. The solvent mixture was selected because of the availability of HX rate data for GrS[7] and the high solubility of the peptide. Exchange was terminated after 30 min by freezing in a dry ice–EtOH bath. The frozen samples, representing a distribution of pH's, were placed in a single vacuum flask and lyophilized. The flask was removed from the lyophilizer without venting and transferred to a glove box under He (Vacuum Atmospheres Co.). Preparation of all samples for spectroscopic measurements were performed in this environment.

For exchange of ^1H for ^2H, the peptide was initially per-N-deuterated in D_2O dioxane and lyophilized. Aliquots were divided out under He, sealed to exclude moisture, and removed from the glove box. The procedure was thereafter identical with that outlined above, save that protonated solvents were used.

^1H–^2H exchange rates of poly(D,L-alanine), type I, (Sigma) in D_2O/dioxane-d_8 (5:2, v/v) were obtained by monitoring the integrated intensity of the amide proton NMR resonance as a function of time at 24 °C. The peptide concentration was 2 mg/mL. The kinetic analysis was carried out as previously described.[8]

^1H NMR spectroscopy was performed on a Bruker WM-500 spectrometer operating in the Fourier transform mode. The lock signal was provided by the solvent. The peptide concentration was typically 8 mM GrS in Me_2SO-d_6 or methanol-d_4. The chemical shifts of the NH protons, in ppm relative to internal Me_4Si, were as follows: In Me_2SO-d_6, Val, 7.22; Leu, 8.34; Orn N′H, 8.68; Orn N^{δ}H, 8.07; D-Phe, 9.11; in methanol-d_4, Val, 7.73; Leu, 8.80. The determination of isotope composition was based on the integrated intensities of the proton resonances.

IR spectra were obtained on a Beckman 4240 grating spectrophotometer calibrated against polystyrene. Resolution was better than 1.4 cm^{-1} at 3000 cm^{-1}. Adjustable pathlength CaF_2 cells (Perkin-Elmer) were employed for the measurement of transmittance between 4000 and 1200 cm^{-1}. Absorbances were obtained by logarithmic scaling. In a typical difference spectrum, the peptide concentration was 0.037 M in Me_2SO and the path length was 0.085 cm.

For the determination of integrated IR band intensities, a Perkin-Elmer 180 grating spectrophotometer was employed and the path length reduced to 0.045 cm to ensure linearity. The intensities were measured by cutting and weighing the peaks obtained by subtraction of the digitized spectra. Interference from solvent absorbances in spectral regions of interest was negligible, and there was no measurable absorbance change indicative of rehydration during IR spectral acquisition.

Results

The dispersion in base-catalyzed amide proton exchange rates[7] provided a means of synthesizing variably N-deuterated GrS analogues, the isotopic composition of which were determined quantitatively by NMR spectroscopy. Difference IR spectroscopy of Me_2SO solutions of these analogues then afforded isolation of residue-specific amide group vibrations. Me_2SO was selected because (i) it is aprotic, (ii) it has been employed freuently in studies of the solution structure of GrS,[2,9-15] and (iii) non-H-

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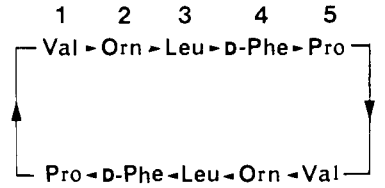

Figure 1. Primary structure of gramicidin S.

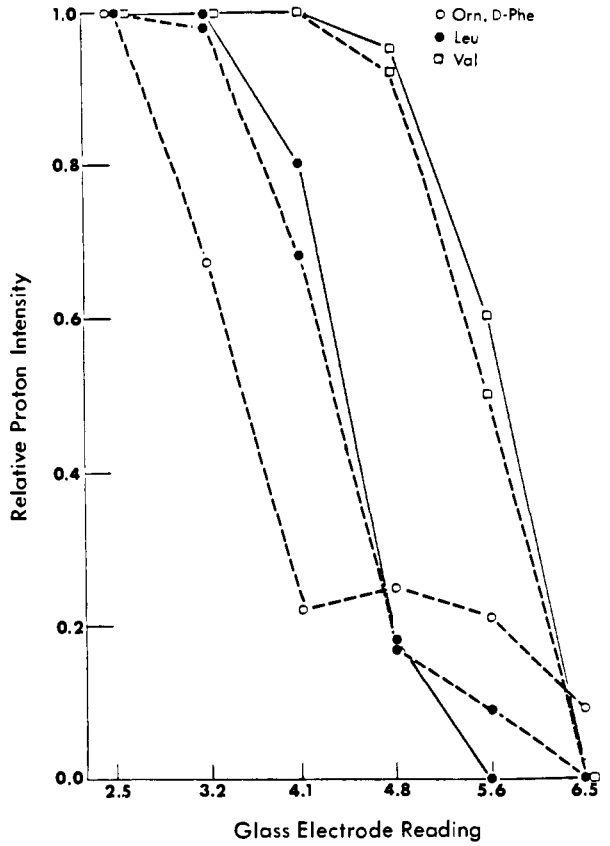

Figure 2. Isotope dispersion in a single run. Plot of integrated NMR peak intensities for the indicated NH protons in methanol-d_4 (solid line) or Me_2SO-d_6 (broken line) vs. pH of the exchange medium. A relative intensity of 1.0 corresponds to 2.0 equiv mol^{-1} for each of Leu and Val NH and 2.2 equiv mol^{-1} for D-Phe NH, Orn NH, and Orn $N^{\delta}H_3^+$ collectively.

bonded, intramolecularly H-bonded, and solvent H-bonded peptide NH groups are readily differentiated by their amide A[16] frequencies.[17,18]

HX conditions were adjusted so that for a given pair of isotopic analogues as large a difference in degree of N-deuteration as possible be achieved for a specific residue, with minimal differences incurred in the remainder. Rate constants for base-catalyzed HX are known to increase in the order k_{Val}, k_{Leu}, k_{Orn}, k_{Phe}[7] because of the combined influences of conformation and the inductive effects of side chain substituents. The rate law above pH 3 (uncorrected glass electrode reading) is $-d[NH]/dt = k[NH]/[H]$. If f_V is defined as the fraction of Val NH not deuterated in the synthesis of an analogue in which all NH groups are deuterated save that of Val, then at a time t after initiation of HX, $f_V = \exp(-k_{Val}t/[H])$. Similarly, for Leu, $f_L = \exp(-k_{Leu}t/[H]) = \exp[(k_{Leu} \ln (f_V/k_{Val})]$. Thus if $k_{Leu}/k_{Val} \equiv \rho$, $f_L = f_V^{\rho}$, and it is necessary just to maximize $f_V - f_L$ with respect to f_V to determine the optimal exchange conditions, assuming that deuteration of all residues exchanging more rapidly than Leu will be essentially complete. This analysis yields $pH_{optimal} = \log (\ln \rho/[(\rho - 1)k_{Val}t)]$; with the available data for k_{Val} and k_{Leu}, allowing HX to proceed 30 min at 25 °C requires that the pH be 5.4 for a product 93% deuterated at Leu and 21% deuterated at Val NH. The actual isotope dispersion in a typical run in displayed in Figure 2.

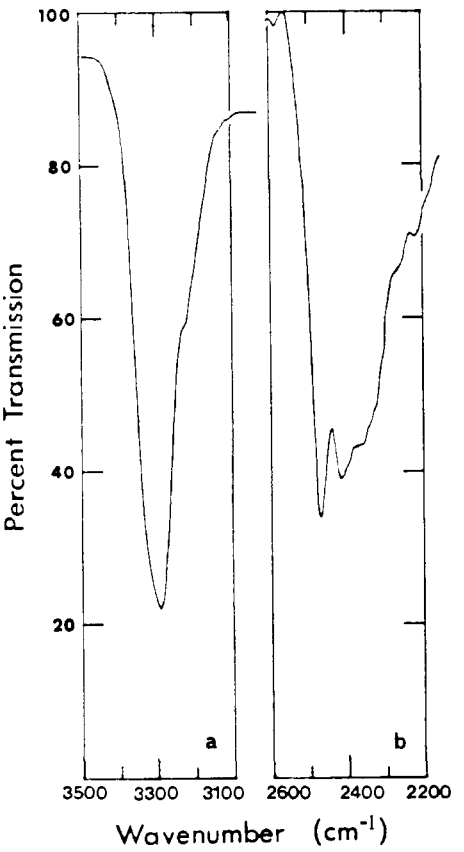

Figure 3. IR difference spectra between the spectra of GrS (i) and per-N-deuterated GrS (ii). (a) i - ii, displaying the complete amide A band; (b) ii - i, displaying the complete amide A′ band.

Figure 3a shows the difference spectrum between GrS and per-N-deuterated GrS across the entire amide A band. It reveals a featureless resonance centered at 3300 cm^{-1} with a bandwidth $\Delta\nu_{1/2} = 120$ cm^{-1}. This is similar to the spectra previously recorded for the bis(*N*-acetylornithyl)[19] and bis(*N*-benzyloxycarbonylornithyl)[20] derivatives in $CHCl_3$. The amide A′ band at 2300–2500 cm^{-1} obtained by subtracting the spectrum of GrS from the spectrum of per-N-deuterated GrS (Figure 3b) is broader and appears more complex.

Because of intense absorption by methyl CH near 3000 cm^{-1}, the amide B band was not observed; it was assigned to the shoulder

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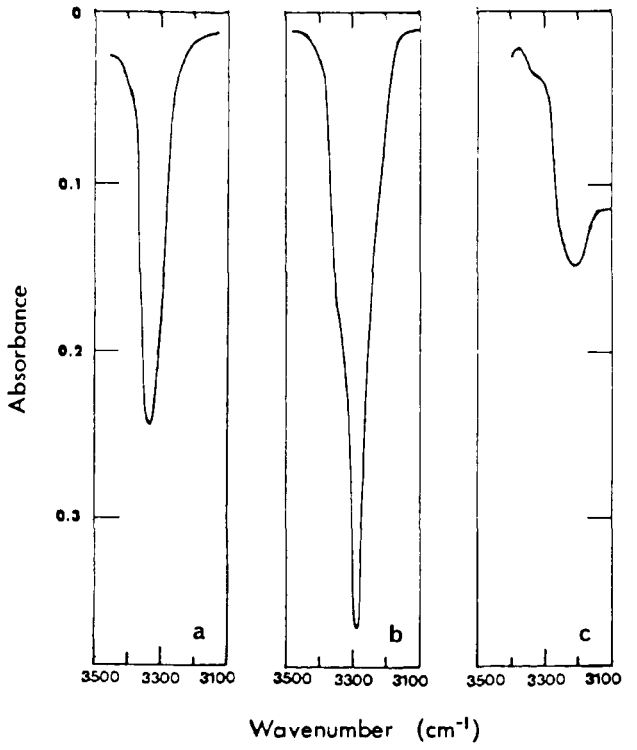

Figure 4. IR difference spectra between the spectra of partially deuterated GrS analogues prepared at pH 6.5 (i), 5.5 (ii), 4.8 (iii), 4.0 (iv), 3.2 (v), and 2.5 (vi). See text for details. (a) ii – i, amide A (Val); (b) iv – iii, amide A (Leu); (c) vi – v, Amide A (D-Phe, Orn).

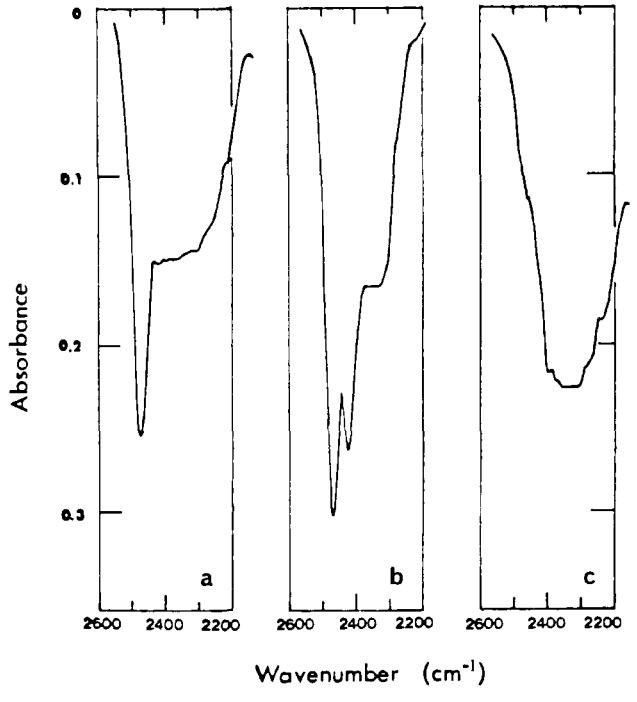

Figure 5. IR difference spectra; labeling as in Figure 4. (a) i – ii, amide A′ (Val); (b) iii – iv, amide A′ (Leu); (c) v – vi, amide A′ (D-Phe, Orn).

appearing at 3050 cm^{-1} in the simple spectrum. The sharp ($\Delta\bar{\nu}_{1/2}$ = 25 cm^{-1}) amide I band shifts from 1655 to 1650 cm^{-1} on complete deuteration.

Amide vibrations for specific residues were obtained from the IR difference spectra of partially deuterated analogues (Figures 4 and 5). The spectrum displayed in Figure 4a was obtained by subtraction of the spectrum of an analogue prepared at pH 6.5, which was totally N-deuterated, from the spectrum of an analogue prepared at pH 5.5, which was N-deuterated at Leu, Orn, and D-Phe and partially N-deuterated at Val. The two analogues

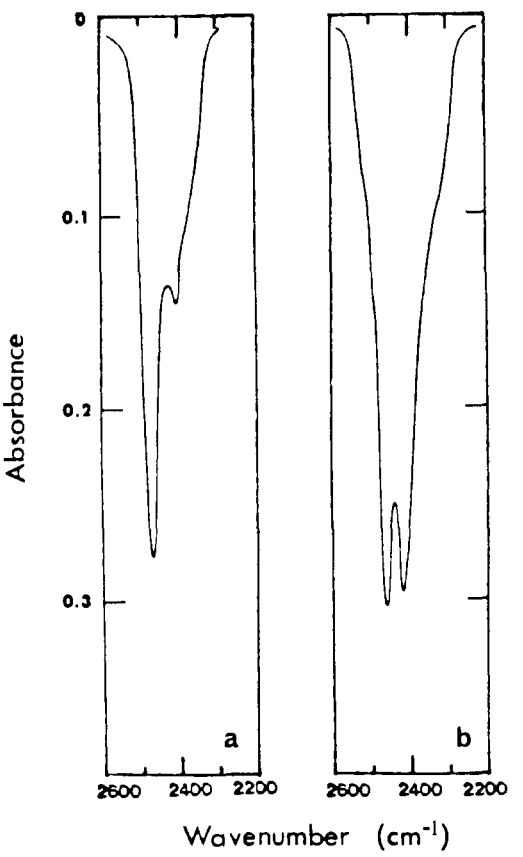

Figure 6. IR difference spectra between the spectra of partially deuterated GrS analogues prepared by exchange of ^1H following complete N-deuteration, to facilitate observation of Fermi splitting, at pH 6.5 (i), 5.6 (ii), 4.7 (iii), 4.0 (iv). (a) i – ii, amide A′ (Val); (b) iii – iv, amide A′ (Leu).

differed only in the degree of deuteration at Val NH, and the amide A band calculated in the difference spectrum was unambiguously assigned to this residue. This band is Gaussian, with $\bar{\nu}_A$(Val) = 3334 cm^{-1} and $\Delta\bar{\nu}_{1/2}$(Val) = 75 cm^{-1}. To calculate the spectrum shown in Figure 4b, the spectrum of an analogue synthesized at pH 4.8 was subtracted from the spectrum of an analogue synthesized at pH 4.0. These analogues differed in degree of N-deuteration at Leu (90%) and Val (10%). It is clear that, relative to Val, the valence band assigned to Leu (Figure 4b) is substantially narrower and more red shifted: $\bar{\nu}_A$(Leu) = 3281 cm^{-1} and $\Delta\bar{\nu}_{1/2}$(Leu) = 56 cm^{-1}. The amide A bands of D-Phe and Orn, isolated together (Figure 4c) in the difference spectrum of analogues synthesized at pH 3.2 and 2.5, lie at lower frequency than those of Val or Leu. Accurate recording or the amide A band shape for D-Phe and Orn was hindered by the strong methyl CH absorbance.

Isolation of the amide A′ bands (Figure 5) was accomplished in similar fashion, except that the spectrum of the analogue prepared at the lower pH was subtracted from that of the analogue prepared at the higher pH. The broad absorbance centered at 2350 cm^{-1} appearing in difference spectra in which D-Phe and Orn were N-deuterated (Figure 5a,b) was attributed to error in the computation of transmission differences when sample and reference absorbances were high and was eliminated by initially per-N-deuterating the peptide and thereafter exchanging in ^1H (Figure 6). In this manner, it was possible to delineate the band structure to the low frequency side of the Val (Figure 6a) and Leu (Figure 6b) amide A′ vibrations.

The complete set of IR band assignments is summarized in Table I. D-Phe and Orn are clearly differentiated from Leu and Val on the basis of the valence vibration frequencies $\bar{\nu}_A$ and $\bar{\nu}_{A'}$. For the former, $\bar{\nu}_A$ is close to that reported for secondary amides in Me_2SO,[18] and it is concluded that these NH groups are completely H-bonded to solvent. The amide A frequencies of Leu

Table I. Specific Peptide Group Vibrations in Gramicidin S in Me_2SO Solution[a]

vibrational mode	Leu			Val			D-Phe, Orn	
	$\bar{\nu}$	$\Delta\bar{\nu}_{1/2}$	A	$\bar{\nu}$	$\Delta\bar{\nu}_{1/2}$	A	$\bar{\nu}$	$\Delta\bar{\nu}_{1/2}$
A	3281	56	2.2	3334	75	2.4	~3250	
A′	2423, 2465	39		2410, 2477	61		~2350	>200
II	1535	62		1521	32			
II′				1460	38			

[a] Observed positions $\bar{\nu}$ and bandwidths $\Delta\bar{\nu}_{1/2}$ are in cm^{-1}; intensities A are in 10^7 mol^{-1} cm.

and Val are characteristic of NH—O=C H-bonds.[17] The latter set of bonds must be intramolecular, since ^{13}C NMR relaxation times measured[15] at higher peptide concentrations than employed here demonstrate a lack of intermolecular association in this solvent. In no instance was a band assignable to a non-H-bonded NH group observed.

Discussion

The novel combination of proton exchange, NMR, and IR spectroscopy employed here has afforded the unambiguous demonstration of two pairs of equivalent intramolecular H-bonds in GrS, with donor groups provided by Leu and Val NH, distinguishable on the basis of characteristic vibrational frequencies when isolated isotopically in solution. The selective delineation of the vibrational bands in this decapeptide not only serves to identify these intramolecular H-bonds but also permits us to characterize them in terms of their geometry and thermodynamics.

H-bond Lengths. It is possible to estimate the NO distance, r_{NO}, from $\bar{\nu}_{NH}$ through application of an empirical relationship proposed for NH–O H-bonds[21] once the observed amide A frequency is corrected for the effects of Fermi resonance, which influences the ν_{NH} fundamental through interaction with the first overtone of the amide II vibration (δ_{02}).[16] Assignment of the amide B band by difference IR was not possible, and the unperturbed δ_{02} frequency is unknown. An approximate correction can be made if δ_{02} is taken as twice the observed amide II fundamental[22] (Table I) and $\bar{\nu}_B$ = 3050 cm^{-1} (see Results), in which case the unperturbed stretch frequency $\bar{\nu}_{NH}$(Leu) is ~20 cm^{-1} lower than $\bar{\nu}_A$, while the Fermi resonance effect on $\bar{\nu}_{NH}$(Val) is negligible. Invoking the relationship proposed by Pimentel and Sederholm[21] and using $\bar{\nu}_{NH}$(free) = 3457 cm^{-1} for EtNHCOEt,[23] we obtained r_{NO} = 2.85 ± 0.06 Å for Leu and 2.98 ± 0.06 Å for Val. These estimates fall near the mode of the distribution function of collected values of r_{NO} in crystalline peptides.[24]

Previous NMR studies of GrS in solution[6,9-15,25,26] have generally supported a cyclosymmetric conformation of the antiparallel β-pleated type containing extended Val–Orn–Leu sequences connected to D-Phe-Pro fragments forming β-II′ turns.[27] (Studies of the crystal structure[28] confirm the symmetry property but have not provided estimates of interatomic distances.[29]) It is thus useful to consider the H-bond parameters in light of a model structure of the β sheet type[13,28] containing a maximum of four transannular H-bonds: one pair with donor Leu NH and acceptor Val C=O (Leu NH—O=Val) and a second pair, closing the β turns, with donor Val NH and acceptor Leu C=O (Val NH—O=C Leu).[30]

Several examples of geometrically similar H-bonds which have been well elucidated structurally and spectroscopically are to be found in the literature. The NH stretch frequency of Leu observed here is typical of polypeptides in the β conformation.[17] Crystallographically determined NO distances are 2.83, 2.76, and 2.88 Å in (L-Ala)$_n$ in the β form,[23] silk fibroin,[31] and dimeric *t*-BuCO-Gly-NH-*i*-Pr,[32] respectively; in solid MeCO-Gly-NHEt, $\bar{\nu}_A$ = 3276 cm^{-1}.[33] A survey of antiparallel β sheet structures in oligopeptide crystals[34] yielded an average r_{NO} of 2.88 ± 0.09 Å (IR data not available). These H-bonds tend to be linear, with the NHO angle, θ, greater than 170°.[30,32,35] Overall, good agreement is found with the projected r_{NO}, warranting an estimate of 2.80–2.90 Å for the Leu NH—O=C Val H-bond.

For Val NH, the angular dependence of the H-bonding interaction must be taken into account. Nonlinearity is characteristic of intramolecular H-bonds closing β turns. For the 10 lowest energy minima found[36] for closed (r_{NO} < 3.2 Å) β-II turns in a blocked LD-type dipeptide, $\langle r_{NO}\rangle$ = 2.96 ± 0.06 Å and $\langle\theta\rangle$ = 156 ± 10° (calculated for r_{NH} = 1.00 Å). If crystalline peptides (excluding depsipeptides) containing closed β-II or β-II′ turns for which published values of θ[35,37] or the dihedral angles φ and ψ within the turn[38,39] necessary for the calculation[40] of θ are available are considered, $\langle\theta\rangle$ = 149 ± 5°. This degree of distortion should be accompanied by a 10–20-cm^{-1} increase in $\bar{\nu}_{NH}$ above the value expected for a linear H-bond with r_{NO} on the usual range.[41a,b] Unfortunately, accompanying IR measurements are seldom available, primarily because of the difficulty of making specific band assignments in the amide A region. An additional complication is that C_2 symmetry is sometimes lost in crystalline cyclosymmetric peptides.[35,42]

The most useful comparisons were noted for the following compounds. *cyclo*-(Gly$_6$) retains C_2 symmetry in the solid state[38] and contains transannular 4→1 H-bonds (r_{NO} = 2.96 Å; θ_{calcd} = 155°), which most likely correspond to the strong 3333-cm^{-1} band observed in the IR spectrum of the Nujol mull.[43] Dihydrochlamydocin[44] has two 3→1 H-bonds with r_{NO} = 2.95 and 2.84

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Å and θ = 145 and 138°, respectively. [Ala⁴]-desdimethylchlamydocin has similar secondary structure[45] and demonstrates a bulk $\bar{\nu}_A$ in $CHCl_3$ of 3335 cm^{-1}. NH stretch frequencies of hydrated tropocollagens and isomorphic synthetic polytripeptides[46,47] range from 3320 to 3345 cm^{-1}, attributed in part to deviation from linearity[47] since r_{NO} = 2.90 Å for the collagen model[48] and 2.96 Å for $(Gly\text{-}Pro\text{-}Pro)_n$.[49] For these peptides, r_{NO} is consistently shorter than predicted by the Pimental–Sederholm relationship. If θ assumes a value typical of 4→1 intramolecular H-bonds, r_{NO} for the Val NH—O=C Leu bonds closing the β turns in GrS should lie between 2.90 and 3.00 Å.

Enthalpies. While H-bond enthalpies have been determined spectroscopically for complexes of simple amides,[3] difficulties arise when attempting to measure $\Delta H°$ from the temperature dependence of the association constant in polypeptides containing intramolecular H-bonds. GrS demonstrates no free ν_{NH} resonance for Leu or Val at 25 °C. Peptide unfolding is a cooperative process, and the temperature dependences should be complex. GrS is soluble only in polar media, this introducing the additional complications of specific interactions with the solvent[50] and hydrophobic effects.[51]

Spectroscopic–thermodynamic correlations, such as the regression of $\Delta H°$ on the bandshift of $\Delta\bar{\nu}_{XH} \equiv \bar{\nu}_{XH}$(H-bonded) − $\bar{\nu}_{XH}$(free) for analogous complexes,[52] can provide estimates of $\Delta H°$ and circumvent the difficulties of direct measurement. However, the Badger–Bauer relationship is not established for trans secondary amides. Amide A bands of the intermolecular complexes of suitable model compounds are characteristically broad ($\Delta\bar{\nu}_{1/2}$ > 120 cm^{-1}) and non-Gaussian,[53] and it is difficult to assign $\bar{\nu}_{NH}$(H-bonded) precisely.[54,55] A survey of experimentally determined enthalpies for complexes of model amides[56-60] ranging from −2.1 to −4.7 kcal mol^{-1} revealed no systematic variation with $\Delta\bar{\nu}_{NH}$[53,54,58,59,61] of predictive value.

A second approach to the estimation of $\Delta H°$ from IR data for model compounds is based on the integrated band intensity, A. Iogansen[62] successfully applied the relation $-\Delta H° = C\Delta\Gamma^{1/2}$ to a series of phenol adducts, where $\Gamma \equiv (cl)^{-1}\int_\nu \ln(I_0/I)d(\ln\bar{\nu}) \simeq A/\bar{\nu}_{XH}$, $\Delta\Gamma^{1/2} = \Gamma^{1/2}$(H-bonded) − $\Gamma^{1/2}$(free), and C is an empirically derived constant. It was proposed that Γ is proportional to the square of the dipole moment of the XH valence fundamental, to which the H-bond makes an additive contribution.[62] This method is advantageous in that the entire envelope of the valence band, rather than the point of maximal absorptivity alone, is utilized. Previous intensity measurements in simple amides[53] show a direct variation with $-\Delta H°$.[57,58,60]

Table II. Hydrogen Exchange Kinetics of the Amide Protons of Gramicidin S in Aqueous Media[a]

	solvent					
	D_2O/dioxane-d_8 (5:2 (v/v), 25 °C; ϵ 52.6[b])			D_2O (20 °C; ϵ 79.8[b])		
residue	R'_{obsd}[c]	R'_{pred}[d]	$\Delta\Delta G^{\ddagger}$	R'_{obsd}[e]	R'_{pred}[f]	$\Delta\Delta G^{\ddagger}$
Val	0.0106	2.22	3.15	0.128	5.97	2.23
Orn	0.495	1.32	0.58	2.33	3.60	0.25
Leu	0.0256	1.98	2.57	0.307	5.32	1.65
D-Phe	1.77	1.57	0	4.49	4.24	0

[a] Definitions: $R'_{obsd} \equiv 2(k_D k_{OD} K_w)^{1/2}$, in $(10^2\ min)^{-1}$, is the minimum HX rate as a function of pD at a given temperature.[8] R'_{pred}, in $(10^2\ min)^{-1}$, is the projected minimum HX rate for an hypothetical random coil peptide of corresponding primary structure.[65] $\Delta\Delta G^{\ddagger} \equiv -RT\ln(k_{obsd}/k_{pred}) \simeq -RT\ln(R'_{obsd}/R'_{pred})$, in kcal mol^{-1}. [b] Reference 67. [c] Reference 7. [d] Based on poly(D,L-alanine) HX parameters measured in the present study: k_D = 16.0 $mol^{-1}\ min^{-1}$, $k_{OD}K_w = 6.37 \times 10^{-6}$ mol min^{-1} at 24 °C. [e] Reference 68. [f] The average of several earlier determinations of R' for poly(D,L-alanine) was used in the calculation.

In GrS, band intensities for Leu and Val are readily determined from the IR difference spectra (Table I) since the amide A bands are discrete and closely approximated by Gaussians. Using values of 3.9×10^6 mol^{-1} cm and 73 cal $mol^{-1/2}$ cm^{-1}, respectively, for A(free) and C suggested by the model compound spectra[53] and $\bar{\nu}_{NH}$(free) = 3457 cm^{-1}, we obtain estimates of $-\Delta H°$ = 3.5 (Leu NH—O=Val) and 3.7 kcal mol^{-1} (Val NH—O=C Leu) from the Iogansen equation. The error in the estimates is ≃0.5 kcal mol^{-1} and is attributable primarily to uncertainties in $\Delta H°$ for the model compounds. The intramolecular H-bonds in GrS are thus of moderate strength.[52]

Free Energies and the HX Barriers. Estimates of the free energies of formation of the transannular H-bonds in GrS, $\Delta G°_{hb}$, can be obtained by joint analysis of the HX kinetics of the peptide protons and the IR data. It is then possible to discuss quantitatively the contributions of intramolecular H-bonding and steric hindrance to the measured kinetic barriers to HX at Leu and Val NH.

For a two-state system in the high motility limit,[63,64] the observed HX rate constant, k_{obsd}, is given by[8] eq 1 where k_u and k_r

$$k_{obsd}/k_u = [(k_r/k_u)K + 1]/(K + 1) \quad (1)$$

are the pseudo-first-order rate constants for the unrestricted (U) and restricted (R) conformers and $K \equiv [R]/[U]$. Here, k_u is the random-coil HX rate constant, which is calculable from model compound data[65] and $k_r < k_u$. In the case of intramolecular H-bonding, $k_r \ll k_u$[66] and $k_{obsd}/k_u \simeq (1 + K)^{-1}$.[63] Defining $\Delta\Delta G^{\ddagger} = -RT\ln(k_{obsd}/k_u)$ as the apparent kinetic barrier to HX, we obtain, for an H-bonded system with large K

$$\Delta\Delta G^{\ddagger}_{hb} \simeq -\Delta G°_{hb} \quad (2)$$

If, on the other hand, HX is blocked in the R state by steric factors in the absence of intramolecular H-bonding, this approximation cannot generally be made, and eq 1 holds.[8]

It is very likely that both H-bonding and steric hindrance will contribute to reduce k_{obsd}. To treat this situation we shall consider a simple model in which four formally discrete conformational states are in rapid equilibrium, each with a characteristic rate constant: fully solvent exposed (U; k_u), H-bonded (R_1; k_1), solvent shielded (R_2; k_2), and both H-bonded and solvent shielded (R_{12}; k_{12}). Defining $K_1 = [R_1]/[U] \simeq [R_{12}]/[R_2]$ and $K_2 = [R_2]/[U] \simeq [R_{12}]/[R_1]$, and allowing that k_1/k_u, $k_{12}/k_u \ll 1$,

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$$\frac{k_{obsd}}{k_u} = \frac{(k_2/k_u)K_2 + 1}{K_1 + K_2 + K_1K_2 + 1} \quad (3)$$

Combining eq 2 and 3, we obtain

$$\Delta\Delta G^{\ddagger} \simeq RT \ln \left[\frac{K_2 + 1}{(k_2/k_u)K_2 + 1} \right] - \Delta G^{\circ}_{hb} \quad (4)$$

indicating that in the general case the experimentally determined $\Delta\Delta G^{\ddagger}$ is an upper limit to $-\Delta G^{\circ}_{hb}$.

It is shown previously[7] that the HX kinetics of GrS, summarized in Table II, are consistent with the high motility limit. It had not been possible to account for the substantially reduced HX rates at Leu and Val NH in terms of specific conformational interactions, however, since k_{obsd}/k_u must depend on k_2, K_1, and K_2, which were unknown. In this context, the following conclusions based on the IR data greatly facilitate the interpretation of the HX kinetics: (i) Leu and Val NH participate in intramolecular H-bonds. (ii) The absence of a non- or solvent-H-bonded vibrational band for these residues requires that $K_1 > 30$, given the quantitative precision of the absorbance measurements. Thus a *lower* limit of 2 kcal mol^{-1} to $-\Delta G^{\circ}_{hb}$ is imposed by the IR difference spectra. (iii) $-\Delta H^{\circ}_{hb}$(Leu) $\simeq$ 3.5 kcal mol^{-1} and $-\Delta H^{\circ}_{hb}$(Val) $\simeq$ 3.7 kcal mol^{-1}. These are significantly greater in magnitude than $\Delta\Delta G^{\ddagger}$ (Table II) for each residue. Since the activation energy difference $\Delta G^{\ddagger}_1 - \Delta G^{\ddagger}_u$ for HX between the R_1 and U states should vary directly with the strength of the internal H-bond,[66] $k_1K_1/k_u \ll 1$, and the use of eq 2–4 is justified for GrS.

For the Leu NH—O=Val bond, the upper (1.65–2.57 kcal mol^{-1}) and lower (2 kcal mol^{-1}) limits indicated by the HX and IR experiments, respectively, establish that ΔG°_{hb}(Leu) $\simeq$ 2.0 kcal mol^{-1}. H-bond cleavage therefore accounts entirely for the apparent kinetic barrier to HX at Leu NH.

ΔG°_{hb}(Val) is not as well specified, since $\Delta\Delta G^{\ddagger} > 2$ kcal mol^{-1}. To characterize better the components of the kinetic barrier, the effect of variation of the dielectric constant,[67] ϵ, on HX kinetics[68] was investigated (Table II). As expected,[51] $\Delta\Delta G^{\ddagger} \simeq \Delta G^{\circ}_{hb}$ varies as ϵ^{-1} in the case of Leu NH. The sensitivity of $\Delta\Delta G^{\ddagger}$(Val) to ϵ^{-1} is slightly less but sufficient to show that H-bond cleavage contributes the major portion of the kinetic barrier at Val NH. Modeling of the β turn region of GrS shows the Val NH proton to be shielded from solvent by the peptide group linking D-Phe and Pro and the side chains of Leu and Val. A small steric term accounting for $\leq$1 kcal mol^{-1}, or $\sim$30%, of $\Delta\Delta G^{\ddagger}$(Val) is consistent with the IR data and HX kinetics.

Fermi Resonance. The pair of peaks assigned to each of Leu and Val in the amide A′ region (Figure 6) could represent separate conformers or Fermi interaction of ν_{ND} with other amide vibrations. The first possibility seems unlikely, since the NH stretch fundamentals are not doubled. Alternatively, Fermi resonance of ν_{ND} with the combination mode amine II′ + III′ has been demonstrated in N-deuterated amides and *trans*-polyamides.[69,70] The occurrence of $\bar{\delta}_{ND}$(Val) at 1460 cm^{-1} suggests that the amide II′ frequencies do not differ appreciably from the model compounds.[16] When Fermi splitting is present the frequencies of the unperturbed vibrations are calculable from the intensity ratio, B, of the unperturbed peaks[69] as long as the intensity of the unperturbed combination band is much less than that of the unperturbed fundamental.[71] If $\bar{\nu}_1$ and $\bar{\nu}_2$ are the perturbed (measured) frequencies for a given residue, $\bar{\nu}_1 > \bar{\nu}_2$, and $\bar{\nu}_f$ and $\bar{\nu}_c$ are the unperturbed fundamental and combination mode frequencies, then[63] $B = (\bar{\nu}_1 - \bar{\nu}_f)/(\bar{\nu}_f - \bar{\nu}_2)$ and $\bar{\nu}_1 - \bar{\nu}_f = \bar{\nu}_c - \bar{\nu}_2$. This gives for Leu ($B \simeq 1$) $\bar{\nu}_f \equiv \bar{\nu}_{ND} = 2444$ cm^{-1}, $\bar{\nu}_c \equiv \bar{\delta}_{ND} + \bar{\gamma}_{ND} = 2444$ cm^{-1}, and for Val ($B \simeq 0.4$) $\bar{\nu}_{ND} = 2458$ cm^{-1}, $\bar{\delta}_{ND} + \bar{\gamma}_{ND} = 2429$ cm^{-1}, $\bar{\gamma}_{ND}$(calcd) = 969 cm^{-1}. The isotope ratio $\bar{\nu}_{NH}/\bar{\nu}_{ND}$ calculated for the corrected valence vibration frequencies is 1.33 for Leu and 1.35 for Val (free NH oscillator, 1.37). These suggest that the vibrational potential in which the H atom resides is of the single minimum type in both complexes.[72,73] Study of the overtone region is, however, essential to elucidating the shape of the potential.[72]

Fermi resonance arises when stationary states of the unperturbed Hamiltonian are connected by an anharmonic perturbing potential, which here is proportional to the normal coordinates of the coupled vibrations.[74] If h' is the off-diagonal matrix element of the perturbation connecting the ND valence vibration with the amide II′ + III′ combination mode, it can be shown from first-order perturbation theory that $h' = (\bar{\nu}_1 - \bar{\nu}_2)B^{1/2}/(B + 1) = 21$ cm^{-1} for Leu and 30 cm^{-1} for Val. These are comparable to values of h' calculated for N-deuterated amides for which $\bar{\delta}_{ND}$ and $\bar{\gamma}_{ND}$ are known.[69]

Bandwidths. Comparison of the bandwidths reveals that $\Delta\bar{\nu}_{1/2}$(Val) > $\Delta\bar{\nu}_{1/2}$(Leu) for amide A and A′. Larger bandwidths in H-bonded complexes of weak to moderate strength have been attributed[75] to greater degrees of mechanical anharmonicity in the valence vibration. However, an increase in anharmonicity is generally associated with a larger fractional bandshift;[76] in GrS, $\Delta\bar{\nu}_{NH}$(Leu) > $\Delta\bar{\nu}_{NH}$(Val). It is possible to rationalize this apparently paradoxical result in terms of stochastic processes modulating the spectral linewidths in solution[77] and conformational constraints. The NH stretch vibration is strongly coupled to the σ_{NH-O} mode of the complex in the far-IR region. This, in turn, is perturbed by random intermediate potentials as well as, in GrS, slow conformational librations. The correlation times involved are long on the IR timescale[77] so that what is observed is a statistical ensemble of wavenumber shifts produced by the couplings, approximately normally distributed about the most probable value.[76] One source of the dispersion is induced fluctuation in θ. In intermolecular complexes, θ can assume any value with $\langle\theta\rangle$ = 180° (for acyclic amides), and $\Delta\bar{\nu}_{1/2}$ is dominated by the anharmonicity. In GrS, θ is constrained to possess different equilibrium values for the two pairs of H-bonds, and $|d\bar{\nu}/d\theta|$, which increases rapidly with decreasing θ[41a] determines the relative linewidths. The observed trend in $\Delta\bar{\nu}_{1/2}$ may thus be consistent with nonlinearity of the Val NH—O=Leu bonds. The $\mu^{-1/2}$ dependence of $\Delta\bar{\nu}_{1/2}$ (μ = reduced mass) predicted by the strong coupling theories[78] is verified by comparison of the amide A and A′ bandwidths.

The amide II frequencies confirm the $\bar{\nu}_{NH}$ bandshift difference between the H-bonds.[76] The composite nature of the in-plane deformation mode[16] precludes for the present a straightforward interpretation of the observed disparity in the amide II bandwidths.

Conclusion

Our approach to the identification of intramolecular H-bonds in peptides should, at the outset, resolve once and for all a persistent uncertainty associated with the solution conformation of GrS. ^{1}H nuclear Overhauser enhancements (NOE's)[9,10,14,25] had abundantly corroborated the β sheet structure for very short interproton

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vectors, but the r^{-6} dependence of the NOE the problem of spin diffusion[10] precluded quantitation of the distance between the Val-Orn-Leu strands. HX rates,[7,13,68] NMR chemical shift temperature coefficients,[2,68] and the solvent variation of NMR chemical shifts[2,12] were not definitive, since all were equally consistent with transannular H-bonding or steric shielding of Val and Leu NH from bulk solvent. Consequently, the important question of whether the conformation was constrained by one or two pairs of intramolecular H-bonds[79] remained, until the present study, unsettled.

In peptides less thoroughly investigated than GrS, the identification of specific intramolecular H-bonds by vibrational spectroscopy should clearly provide a powerful constraint in conformational analysis. We have shown that the IR spectrum of a peptide bond thus isolated readily furnishes information concerning the structure and energetics of the H-bond not obtainable by methods conventionally employed with polypeptides. Thus, the experimental protocol outlined here should form a basis for the systematic study of the role of intramolecular H-bonding in the stabilization of polypeptide conformation. GrS, which possesses two types of H-bond known to be ubiquitous in peptides and proteins,[80] one typical of β sheets and the other of 4→1 reverse turns, may prove a very useful prototype in this endeavor.

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Multiphoton Ionization Time-of-Flight Mass Spectrometry of Transition-Metal Complexes: $Mn_2(CO)_{10}$ and $Re_2(CO)_{10}$

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Abstract: Pulsed laser irradiation of gas-phase $Mn_2(CO)_{10}$ at selected wavelengths (e.g., 511 and 483 nm) induces extensive photofragmentation, followed by multiphoton ionization of the Mn, Mn_2, and MnCO photoproducts. Subsequent ion–molecule reactions of MPI-generated Mn^+ with $Mn_2(CO)_{10}$ ($\sim 10^{-5}$ torr) and $Re_2(CO)_{10}$ ($\sim 10^{-6}$ torr) yield adduct ions $Mn_3(CO)_{10}^+$ and $MnRe_2(CO)_{10}^+$ and their daughter ions.

The challenge of understanding a new class of molecules, i.e., complexes containing transition-metal cores, with potential practical applications has prompted a grat deal of activity by organometallic chemists in the areas of synthesis and characterization.[1] The rich photochemistry of these compounds[2] presents interesting problems concerning their photofragmentation and the spectroscopy of the resulting fragments. The lack of detailed gas-phase experimental data has hindered the development of a theoretical picture of bonding in these compounds.[3]

Recently, methods for the study of photofragmentation and photoionization of isolated molecules have become available. For example, resonance enhanced multiphoton ionization (REMPI) studies[4,5] have been successfully applied to a variety of polyatomic organic molecules. The process observed consists of concerted absorption of several photons to a resonant intermediate electronic state of the molecule followed by absorption of additional photons to produce ionization and then fragmentation. However, recent gas-phase multiphoton ionization (MPI) experiments[6,7] on transition-metal compounds indicate that excitation of these molecules results in efficient photofragmentation followed by ionization of the neutral photoproducts.

The present paper reports MPI-mass spectrometry of transition-metal complexes aimed at elucidating their photodissociation, photoionization, and the gas-phase chemistry of resulting photofragments. The model system $M_2(CO)_{10}$ (M = Mn and Re) chosen exemplifies transition-metal complexes containing metal–metal bonds.[2] Most of the experimental information to date on the photochemistry of these molecules comes from condensed-phase experiments. Kinetic studies in solution[7] identify M–M homolytic cleavage as the first step in the light-initiated solution chemistry of $M_2(CO)_{10}$. In the vapor phase, luminescence after UV laser photodissociation of $Mn_2(CO)_{10}$ has been observed.[8]

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