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Conformational Analysis of Linear Peptides. 3. Temperature Dependence of NH Chemical Shifts in Chloroform

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Abstract: The following conclusions concerning NH chemical shifts for peptides in chloroform are based on numerous observations on small model peptides reported here and also on data in the literature. (1) The temperature dependence of chemical shift for an amide NH proton exposed to solvent in chloroform solutions is 0.0024 ± 0.0005 ppm K^{-1}. (2) Small temperature dependencies can also be observed if the NH group is shielded from solvent and remains shielded over the temperature range of the ^1H NMR measurements. (3) Larger temperature dependencies are observed if the NH group is shielded from solvent initially but becomes exposed with increasing temperature, a situation which holds when intermolecular self-association is significant or when intramolecularly hydrogen-bonded conformations unfold as the temperature is increased. Previously proposed conformations based on the presumption that NH chemical shifts display in chloroform solution the same behavior as in strongly polar solvents are herein reexamined.

In addition to the very large number of studies of peptide conformation in strongly polar solvents, including water, there is a small but growing literature dealing with peptides in less polar solvents. Our own interests are in linear peptides in nonaqueous environments, as in the lipid regions of membranes. Previous work from our laboratories includes a ^1H NMR[3] study of Nva oligopeptides in chloroform[4] and a study of self-association in chloroform applying vapor-pressure osmometry.[5]

In our application of ^1H NMR to linear peptides in chloroform we wished to use, to the extent possible, that method of interpreting the temperature dependence of NH chemical shift which had proved so useful to the study of peptides in water. That method is based on the empirical observation that in aqueous solutions, the chemical shift of an NH exposed to the solvent displays a generally higher temperature dependence (ca. 0.005 ppm K^{-1} or more) than does the chemical shift of an NH shielded from the solvent. We were concerned, however, that modifications of interpretation might be necessary in chloroform. In previous ^1H NMR studies of linear peptides in chloroform[6-13] or carbon tetrachloride,[14] the presumption was sometimes, but not always, made that the temperature dependence of NH chemical shift varies in chloroform the same as it does in aqueous solution. In the present work we describe our work on model compounds that shows there are significant differences, and we outline a means of extracting conformational information applicable to chloroform solutions.

Table I. Temperature Dependence of Chemical Shift of NH Resonances in $CDCl_3$ (in ppm K^{-1})

compd	concn, M	N(1)H	N(2)H
Ac-Gly-OMe	0.007	0.0026	
	0.018	0.0022	
t-Boc-Gly-OMe	0.005	0.0028	
	0.020	0.0027	
Ac-L-Ala-OMe	0.011	0.0031	
	0.044	0.0031	
t-Boc-L-Ala-Ome	0.009	0.0031	
Ac-L-Nva-OMe	0.030	0.0020	
t-Boc-L-Nva-OMe	0.030	0.0020	
	0.300	0.0020	
Ac-L-Val-OMe	0.004	0.0025	
	0.006	0.0021	
t-Boc-L-Val-OMe	0.003	0.0020	
	0.012	0.0024	
t-Boc-L-Ala-L-Ala-OMe	0.003	0.0024	0.0024
	0.012	0.0027	0.0026
	0.025	0.0028	0.0025
	0.080	0.0025	0.0028
t-Boc-L-Val-L-Val-OMe	0.004	0.0025	0.0014
	0.016	0.0034	0.0024
t-Boc-L-Met-L-Met-OMe	0.027	0.0020	0.0019
t-Boc-L-Nva-L-Nva-OMe	0.009	0.0024	0.0020
t-Boc-D-Chg-D-Chg-OMe	0.003	0.0028	0.0015
	0.013	0.0030	0.0013

Experimental Section

Ac-Gly-OMe.[15a] This compound was prepared from acetyl chloride and HCl·H-Gly-OMe in anhydrous chloroform by using *N*-methylmorpholine to deprotonate the ammonium group: yield 55%; oil (from ethyl ether–petroleum ether). Anal. Calcd for $C_5H_9NO_3$: C, 45.8; H, 6.9; N, 10.7. Found: C, 45.3; H, 6.9; N, 10.6.

***t*-Boc-Gly-OMe.**[15b] This compound was prepared by *t*-Boc-N_3 and HCl·H-Gly-OMe in anhydrous ethyl acetate by using *N*-methylmorpholine to deprotonate the ammonium group: yield 57%; oil (from ethyl acetate–petroleum ether). Anal. Calcd for $C_8H_{15}NO_4$: C, 50.8; H, 8.0; N, 7.4. Found: C, 50.2; H, 7.9; N, 7.3.

Ac-L-Ala-OMe.[15c,d] This compound was prepared as described above for Ac-Gly-OMe, using HCl·H-L-Ala-OMe as the amino component: yield 60%; oil (from ethyl ether–petroleum ether); $[\alpha]^{20}_D = -62.2°$ (*c* =

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(3) The abbreviations used are as follows: ^1H NMR, proton magnetic resonance; ^{13}C NMR, ^{13}C magnetic resonance; IR, infrared; Ac, acetyl; *t*-Boc, *tert*-butyloxycarbonyl; OMe, methoxy; Gly, glycine; Ala, alanine; Val, valine; Nva, norvaline; Met, methionine; Chg, α-cyclohexyl glycine; Pro, proline.

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Table II. Temperature Dependence of Chemical Shift of NH Resonances in $CDCl_3$ (in ppm K^{-1})

compd	concn, M	N(1)H	N(2)H
Ac-L-Nva-OMe	0.030	0.0020	
	0.300	0.0050	
t-Boc-L-Nva-L-Nva-OMe	0.009	0.0024	0.0020
	0.480	0.0030	0.0059
Ac-L-Val-Gly-OMe	0.003	0.0033	0.0033
	0.006	0.0034	0.0042
	0.009	0.0052	0.0057
	0.017	0.0065	0.0079

Table III. Temperature Dependence of Chemical Shift of NH Resonances in $CDCl_3$ (in ppm K^{-1})

compd	concn, M	N(1)H	N(2)H	N(3)H
t-Boc-Gly-L-Val-Gly-OMe	0.002	0.0024	0.0021	0.0041
	0.004	0.0034	0.0027	0.0061
	0.006	0.0037	0.0039	0.0077
	0.018	0.0038	0.0038	0.0073
	0.020	0.0043	0.0041	0.0081
	0.023	0.0040	0.0049	0.0082

1.3; methanol); $[\alpha]^{20}_D = -78.4°$ (c = 1.6; water). Anal. Calcd for $C_6H_{11}NO_3$: C, 49.6; H, 7.6; N, 9.6. Found: C, 49.2; H, 7.5; N, 9.5.

The synthesis and physical properties of *t*-Boc-L-Ala-OMe,[15e] *t*-Boc-L-Ala-L-Ala-OMe,[16a] Ac-L-Nva-OMe,[4] *t*-Boc-L-Nva-OMe,[4] *t*-Boc-L-Nva-L-Nva-OMe,[16b] Ac-L-Val-OMe,[5] *t*-Boc-L-Val-OMe,[5] *t*-Boc-L-Val-L-Val-OMe,[17] *t*-Boc-L-Met-L-Met-OMe,[18a] *t*-Boc-D-Chg-D-Chg-OMe,[18b] Ac-L-Val-Gly-OMe,[5] and *t*-Boc-Gly-L-Val-Gly-OMe[5] have been described by us previously.

Weighed amounts of peptide were placed directly into NMR sample tubes, and a known volume of solvent was added. $CDCl_3$ (99.96%) from Wilmad Co., Buena, NJ was used. Spectra were obtained at the Southern New England High Field NMR Facility, New Haven, CT on a Bruker 270 HX spectrometer. Up to 2000 scans were accumulated requiring 53 min. Me_4Si was the internal standard.

All variations of chemical shift with temperature change observed in this study were linear. In dipeptides and in the tripeptide we numbered the amino acid residues from the N end of the peptide chain, so that the proton attached to the nitrogen of the N-terminal residue is labeled N(1)H.

We assigned the high-field NH resonance to the N-terminal amino acid residue, N(1)H, of urethane N-protected dipeptides.[4] Assignments of NH resonances in *t*-Boc-Gly-L-Val-Gly-OMe have previously been reported.[8,10]

Infrared absorption spectra were recorded with a Perkin-Elmer Model 580 spectrophotometer.

Results

Table I shows the temperature dependence of NH chemical shifts we measured at low concentrations. We estimate the accuracy of each individual temperature dependence to be ±0.0005 ppm K^{-1}. The average of the 34 values in Table I is 0.0024 ppm K^{-1} and the standard deviation 0.0005 ppm K^{-1}. Thus, to within the precision of the measurements, the temperature dependencies in Table I are equal.

Table II shows results for cases in which we observed increased temperature dependencies as the concentration was increased and, at the lowest concentration, a temperature dependence approaching the average of the measurements in Table I, e.g., 0.0024 ± 0.0005 ppm K^{-1}.

Table III shows results for *t*-Boc-Gly-L-Val-Gly-OMe in which we observed a concentration dependence, but the lower limiting concentrations gave temperature dependencies that did not approach the value of 0.0024 ppm K^{-1} for all NH resonances.

Discussion

On the basis of our vapor-pressure osmometry study of self-association in chloroform,[5] we can conclude that for *t*-Boc-L-Nva-OMe and *t*-Boc-L-Val-OMe the osmotic nonideality of chloroform solutions is too small to attribute to intermolecular associations. In the cases of Ac-L-Nva-OMe and Ac-L-Val-OMe, molal dimerization constants at 36 °C were measured to be 1.18 and 1.33 m^{-1}, respectively,[5] so that at concentrations of 0.004 and 0.006 M, intermolecular association in Ac-L-Val-OMe is insignificant and at 0.030 M concentration, over 90% of the Ac-L-Nva-OMe is unassociated. On the basis of osmotic pressure measurements the association constants of the dipeptides in Table I are expected to be less than that for *t*-Boc-Gly-L-Val-Gly-OMe. Therefore, in assessing the accessibility of the NH groups to solvent, we can exclude intermolecular hydrogen bonds from consideration for the compounds in Table I.

In the case of the blocked monomers in Table I, a most straightforward argument can be made that the NH group is exposed to solvent. The only interaction of the NH group that competes with solvent interactions is that with the CO group attached to the C^α atom of the same molecule, as in an extended C_5 structure.[18c,d] Even in that structure the NH group remains largely accessible to bulk solvent.

We have obtained IR absorption data on all blocked monomers in Table I at 0.030–0.050 M concentration in deuteriochloroform.[5,19a] Bands are observed in the 3455–3435-cm^{-1} region,[15d] indicative of exposed NH groups. No bands are visible in the 3425–3400-cm^{-1} region, suggesting the absence of the C_5 structure. Only in the case of the N-acetyl derivatives are extremely small bands seen in the 3370–3310-cm^{-1} region, indicating that any association that occurs is at the limits of detection by IR absorption at that concentration. The mode of self-association of Ac-L-Ala-OMe has been examined by ^1H NMR and ^{13}C NMR spectroscopies at very high concentrations in $CDCl_3$ solution.[19b]

Below 5×10^{-3} M concentration in deuteriochloroform *t*-Boc-L-Ala-L-Ala-OMe,[19a] *t*-Boc-L-Val-L-Val-OMe,[19a] and *t*-Boc-D-Chg-D-Chg-OMe[18b] have their IR maximum near 3435 cm^{-1}, indicating largely prevailing conformations in which the NH group is unperturbed and/or conformations in which the NH group is spatially close to the β-carbon of the side chain.[20] Hydrogen-bonded species represent less than 10% in the conformational equilibrium mixture. The case of *t*-Boc-L-Nva-L-Nva-OMe has been described by us earlier.[4] *t*-Boc-L-Met-L-Met-OMe has been studied by Ribeiro et al. with IR and ^1H NMR spectroscopy.[13] IR spectra indicated only a small amount (12%) of hydrogen-bonded NH groups and small temperature coefficients for the NH chemical shifts were observed (0.0030 and 0.0024 ppm K^{-1} for N(1)H and N(2)H, respectively, at 0.005 M).

Our conclusion that an NH group exposed to $CDCl_3$ displays a low-temperature coefficient of chemical shift is not in conflict with most ^1H NMR studies of peptides in chloroform. Specifically, it is not in conflict with the observation of others that an NH group intramolecularly hydrogen bonded in a small cyclic peptide also displays a small temperature coefficient of chemical shift. For example, Pease and Watson,[21] in their study of *cyclo*-(Gly-L-Pro-Gly-D-Ala-L-Pro) in $CDCl_3$, concluded that Gly(1) NH and D-Ala NH were intramolecularly hydrogen bonded and displayed small temperature coefficients of 0.0016 and 0.0032 ppm K^{-1}, respectively. Their structure is strongly supported by consistent data from solvent titrations, ^1H NMR temperature coefficients, ^{13}C NMR, and X-ray data. Presumably, the small coefficients in such a case are related to the conformational stability imposed by the cyclic constraints. The detailed origin of the small coefficient for NH groups exposed to chloroform is not known, but more likely it is better explained in terms of a near balance of

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opposing influences on chemical shift, from different mechanisms, rather than the simpler (and probably less correct) explanation that the various types of interaction of the NH group with chloroform are separately small.

Pease and Watson[21] also observed a larger temperature coefficient of 0.0139 ppm K^{-1} for the Gly(2) NH which is directed away from the cyclic structure and attributed that large value to intermolecular hydrogen bonding, since the concentration was 0.07 M. In Table II we report concentration dependence studies, indicating that, indeed, increased temperature dependencies of chemical shift can result from intermolecular associations. Moreover, the two N-acetyl compounds described in Table II were studied previously by us in vapor-pressure osmometry determinations of association constants.[5] Those measurements allow independent and quantitative statements to be made about the extent of intermolecular association at the concentrations of the ^1H NMR measurements. For example, from the dimerization constant for Ac-L-Nva-OMe (K_2 = 1.18 m^{-1}), we can determine that approximately 7% of the molecules are present in dimeric form at 0.03 m concentration, whereas 32% are in dimers at 0.30 m. Table II indicates the larger temperature dependence of NH chemical shift under conditions of greater intermolecular association. The much smaller tendency of *t*-Boc-L-Nva-OMe to associate, as measured by vapor-pressure osmometry,[5] also explains the absence of a concentration dependence in that compound (see Table I).

On the other hand, the large tendency of Ac-L-Val-Gly-OMe to aggregate[5] accounts for the substantial concentration dependence in temperature variation of both NH groups in that peptide. Renugopalakrishnan et al.[10] reported the 220-MHz ^1H NMR spectrum of the same compound measured in $CDCl_3$ at 0.03 M. Temperature coefficients of 0.0056 and 0.0104 ppm K^{-1} were reported for N(1)H and N(2)H, respectively, and the lower value of N(1)H chemical shift, relative to N(2)H, was interpreted as indicating greater *shielding* of that proton, leading those workers to conclude the existence of the unusual C_8 structure with concomitant nonplanarity and cis configuration of the peptide moiety.[18c,d] A D_2O-exchange experiment was cited[10] as additional evidence for the existence of the C_8 structure by virtue of a slower exchange rate being observed for the Val N(1)H proton.

However, Ac-L-Val-Gly-OMe is highly aggregated in chloroform at 0.03 M (see ref 5 and Table II). The temperature dependencies measured at that concentration reflect the changing degree of aggregation rather than a preferred structure for the individual molecules. (The D_2O experiment[10] also reflects the breakup of intermolecular aggregates rather than unfolding of individual molecules.) Our data at 0.003 M (Table II) show temperature dependencies for the two NH resonances to be small and equal. We conclude that Ac-L-Val-Gly-OMe does not adopt a preferred structure in chloroform; rather, it is flexible with the two NH groups approximately equally solvated.

Our data in Table III for *t*-Boc-Gly-L-Val-Gly-OMe show that an observable concentration dependence persists in that peptide to concentrations below 0.01 M. The dimerization constant for that compound in chloroform,[5] 6.59 m^{-1}, indicates that approximately 20% of the molecules are in intermolecularly hydrogen-bonded dimers at 0.02 M. The temperature coefficients previously reported for 0.02 M solutions[8] therefore reflect changes in states of aggregation. Our measurements (Table III) extend to 0.002 M where less than 2% of the molecules are in associated species.[5] These temperature dependencies reflect behavior of the NH resonances of the unassociated molecules. Two of the values are insignificantly different from that of an exposed NH group. For N(3)H, however, that of the C-terminal Gly residue, the temperature dependence (0.0041 ppm K^{-1}) is different by more than three standard deviations from that of an exposed NH group (0.0024 ppm K^{-1}). Since a large temperature dependence is known to occur when an NH group, initially shielded, is transferred into a solvated environment with temperature increase, as demonstrated for dissociating aggregates in Table II, we take the high value for N(3)H also to reflect a transfer of that NH group from an initially shielded environment to a solvated one.

t-Boc-Gly-L-Val-Gly-OMe has two possible structures involving the N(3)H group. One is the standard β turn in which the hydrogen bond is with the carbonyl group of the *t*-Boc moiety, forming a C_{10} ring.[18c,d] The second is a C_7 structure formed with the carbonyl of the Gly(1) residue, but since no C_7 structure was found with Ac-L-Val-Gly-OMe, a similar sequence (see above), we conclude that in chloroform *t*-Boc-Gly-L-Val-Gly-OMe adopts in part the usual C_{10} β turn as the only folded form. Our IR data in $CDCl_3$, previously reported,[5] are in accord with the above conclusions.

Our ^1H NMR data for this tripeptide are essentially analogous to those reported earlier at 0.020 M.[8] In the earlier work, however, the temperature dependence of 0.0043 ppm K^{-1} for N(1)H, being the lowest of the three measured, was taken to mean that the N(1)H group was shielded, following the behavior of NH resonances in aqueous solutions. On that basis the data were taken as evidence of the existence of the rather unusual C_{11} structure. We do not believe the C_{11} structure exists to any significant extent in chloroform solutions of this peptide.

Our ^1H NMR data at 0.002 M concentration indicate nonequivalent methylene protons in Gly(1), but there is no evidence of nonequivalence in Gly(3) at that concentration. The nonequivalence observed in Gly(3) at higher concentration[8] may have arisen from aggregates. Furthermore, Gly(1) is *inside* the proposed C_{10} structure, and the nonequivalence is expected. In a C_7 conformation involving the central residue of the tripeptide, both Gly residues are outside the ring structure and neither pair of methylene protons would be expected to show nonequivalence.

On the basis of the results of the present work and data in the literature, we can draw the following conclusions which offer a means of extracting conformational information from ^1H NMR measurements of peptides in chloroform:

(1) The temperature dependence of NH chemical shifts for NH protons exposed to chloroform is small (0.0024 ± 0.0005 ppm K^{-1}).

(2) The temperature dependence of NH chemical shifts for NH protons which are shielded (hydrogen bonded or buried) and remain shielded over the course of temperature variation is also small. Most examples of this case have been and will be found in cyclic peptides.

(3) The temperature dependence of NH chemical shifts for NH protons initially shielded but transferred to an exposed (solvated) environment in the course of temperature variation is significantly larger than 0.0024 ± 0.0005 ppm K^{-1}.

Thus, contrary to the case of aqueous solutions, a measured small temperature dependence does not mean the NH group is shielded nor does a large temperature dependence mean the NH group is exposed; i.e., there is no simple one-to-one correspondence between the magnitude of temperature dependence and the degree of exposure to solvent over the temperature range of the ^1H NMR measurements. A small temperature dependence can reflect either an NH group exposed to solvent or an initially and permanently shielded NH group. A large temperature dependence, although always reflecting an initially shielded NH group which is transferred to an unshielded environment, can be related either to dissociating aggregates or to unfolding species.

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