Proton Magnetic Resonance Study of Conformational Dynamics, Coordinated Internal Motions, and Chemical Shifts of Tocinamide†

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ABSTRACT: All proton magnetic resonance lines of the $NH{\cdot}CH^{\alpha}{\cdot}CH_2^{\beta}$ fragments of five amino acid residues of the heterodetic peptide, tocinamide, have been analyzed, including reassignment of certain NH, CH^{α}, and CH_2^{β} resonances. The ^1H NMR spectral parameters evaluated from this analysis include (a) all chemical shifts, (b) $\langle {}^3J_{NHCH} \rangle$, $\langle {}^3J_{\alpha\beta} \rangle$, and $\langle {}^2J_{\beta\beta} \rangle$ values, and (c) temperature dependencies of these coupling constants and chemical shifts—the latter includes aliphatic and aromatic CH protons. The $\langle {}^3J_{\alpha\beta} \rangle$ coupling constants yielded C_{α}-C_{β} rotamer populations, p_i, for five residues and these were used to explore conformational dynamics and coordinated internal motions of tocinamide. The rotamer populations and their temperature dependencies established, (1) that all C_{α}-C_{β} bonds exhibit extensive internal rotation; (2) that rotamer preferences exist for every residue; (3) that the 162 conformations possible for completely free rotation around every bond of the $C_{\alpha}{}^1$-$C_{\beta}{}^1$-S^1-S^6-$C_{\beta}{}^6$-$C_{\alpha}{}^6$ disulfide fragment can be reduced to two or three if the concept of coordinated internal motion is introduced. We reject the possibility of a frozen conformation for the Cys^1-Cys^6 fragment in the eclipsed, staggered, or nonclassical rotamer states and propose the following possibilities: (a) three classical rotamers populated in the ratio 60:25:15, (b) three nonclassical rotamers not differing by ±15° from the classical values of 180°, +60°, and −60°, or (c) two nonclassical but noneclipsed rotamers with a third rotamer essentially zero, and (4) that the C_{α}-C_{β} rotamer populations of the Tyr^2-Cys^1-S-S-Cys^6 fragment are consistent with coordinated internal motions of the C_{α}-C_{β} bonds of all three residues. The existence of temperature coefficients, $\Delta\delta/\Delta T$, of ±2 ppb/°C for aliphatic protons can be accounted for, in part, by postulating intrinsic rotamer chemical shifts which are temperature independent. $\Delta\delta/\Delta T$ values should therefore be useful for investigating conformational dynamics. A further consequence of this interpretation is that greater care should be exercised in using $\Delta\delta/\Delta T$ values to assign hydrogen-bonded amide protons, especially when $\Delta\delta/\Delta T \approx -2$ to -4 ppb/°C.

With few exceptions, proton magnetic resonance studies of polypeptide hormones have been restricted to evaluation of backbone conformations, notably by the development of criteria for hydrogen bonding (Urry et al., 1975, and references cited therein), by the investigation of methods of simplifying spectra (Gibbons et al., 1975), and by the interpretation of $\langle {}^3J_{NHCH} \rangle$ coupling constant values in terms of backbone angles (Kopple et al., 1973). Recently, it became obvious that many peptides do not have unique conformations (Stanley et al., 1975; Ivanov et al., 1975) but exhibit conformational averaging (Walter et al., 1974; Jones et al., 1976), the extent of which depends on the constraints placed on the molecule by both covalent and nonbonded interactions.

That internal rotation about C_{α}-C_{β} bonds occurs extensively in polypeptides of molecular weight 800–2000 has been clearly shown by ^{13}C relaxation studies (Deslauriers and Somerjai, 1976; Allerhand and Komoroski, 1973). Since the time scale for such rotations is of the order of 10^{-10}–10^{-11} s (Allerhand and Komoroski, 1973), high resolution magnetic resonance spectra of these systems are in the fast exchange limit; consequently, the spectra of the individual C_{α}-C_{β} rotamers are not seen.

A preliminary ^1H NMR[1] study of side-chain rotations for gramidicin S has appeared (Wyssbrod and Gibbons, 1973) and detailed studies of pentagastrin have been reported (Feeney et al., 1972). A preliminary side-chain rotamer analysis of several residues of oxytocin and penicillamine oxytocin has also been published (Meraldi et al., 1975).

Here we report a full ^1H NMR spectral analysis and temperature study of five of the six residues of tocinamide (H-Cys-Tyr-Ile-Gln-Asn-Cys-NH_2). This analysis leads to the following conclusions: (1) all three classical rotamers (Figure 1) of each residue are significantly populated over the temperature range studied, (2) in all cases preference is shown for the population of one rotamer at the expense of the others. Coordinated internal motion in the region around the disulfide bridge is postulated.

Detailed study of the ^1H NMR spectrum of tocinamide has allowed us to suggest corrections to certain of the previous assignments (Brewster et al., 1972) and in addition to determine accurate values of the $\langle {}^3J_{NHCH} \rangle$ coupling constants (Table I).

Investigation of the dependence on temperature of each of the resonances was carried out, leading, in turn, to the following conclusions: (1) most of the amide protons show a very high-temperature dependence, (2) two amide proton chemical shifts are, as previously reported (Brewster et al., 1972), much less dependent on temperature and may therefore be involved in some form of solvent-shielding interaction, (3) the CH proton chemical shifts do vary with temperature, (4) it should be possible to relate internal motions and chemical shifts.

We also report the solid-phase synthesis of tocinamide, including the use of *N*-hydroxybenzotriazole to obviate problems associated with the incorporation of the asparagine residue.

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; DMF, dimethylformamide.

TABLE I: Chemical Shifts (in ppm) of the Tocinamide Protons, Measured at 40 °C from an Internal Standard of Me_4Si.

Residue	δNH	δα	δβ1	δβ2	δγ1	δγ2	δγ3	δδ	$^3J_{NHCH}$[a] (Hz)
Cys[1] [a]		3.38	3.25	2.63					
Tyr[2]	8.24	4.34	2.94	2.73					
Ile[3]	7.48	4.12	1.70		1.34	1.07	0.87	0.79	8.00
Gln[4]	8.37	3.86	1.81	1.81	2.11	2.11			3.56
Asn[5]	8.56	4.18	2.69	2.54					6.70
Cys[6]	8.31	3.98	3.27	3.18					7.32

[a] Values at 70 °C.

Experimental Section

Synthesis. Tocinamide was synthesized using the Merrifield solid-phase procedure (Merrifield, 1963, 1964). *N-tert*-Butoxycarbonylcysteine[2] (suitably protected amino acid derivatives were purchased from Bachem Inc.) was first attached to a chloromethylated copolymer of styrene and 1% divinylbenzene (as supplied by Bio-Rad Laboratories) using the tetramethylammonium salt method (Yamashiro and Li, 1973). After subjecting the product to one complete cycle of the solid-phase synthesis procedure, using *N-tert*-butoxycarbonylasparagine nitrophenyl ester for the coupling and recoupling stages of the cycle, a routine ninhydrin test (Kaiser et al., 1970) gave a strong positive result, indicating the presence of uncoupled amino groups. The recoupling stage was repeated a total of five times and a positive ninhydrin test was obtained after each repetition. An amino acid analysis of the resin-bound peptide (Westall et al., 1972) showed that even after five recoupling steps less than 50% incorporation of the asparagine had been achieved.

This problem does not arise in the corresponding step in the solid-phase synthesis of oxytocin (Manning, 1968). It therefore seems likely that the active ester coupling is sterically inhibited by the proximity of the polymer support in the case of tocinamide. *N*-Hydroxybenzotriazole has been used to enhance the rate of sterically hindered active ester coupling reactions in the solution phase synthesis of peptides (König and Geiger, 1973). Consequently, the synthesis of the resin-bound dipeptide was repeated using 1 equiv of *N*-hydroxybenzotriazole with DMF as solvent, in both the coupling and recoupling stages of the cycle. In this case, a negative-ninhydrin test was recorded and the synthesis of tocinamide was completed using the well-established procedures for the synthesis and purification of oxytocin-like compounds (Manning et al., 1973, and references cited therein). The product was obtained in an overall yield of 12.8%, based on the initial cysteine incorporation on the resin. The product gave a single spot when subjected to thin-layer chromatography with the upper phase of the solvent system butanol–acetic acid–water (4:1:5, v/v). Amino acid analysis gave: Asp, 1.00; Glu, 1.01; Cys, 1.90; Ile, 1.07; Tyr, 0.95.

Proton Magnetic Resonance. Proton magnetic resonance spectra were measured using a Bruker WH-270 spectrometer equipped with a Nicolet 1180 computer. The temperature of the probe was controlled to within ±2 °C. Tocinamide samples (1–5 mg) prepared in the protonated form were thoroughly dried before dissolving in dimethyl sulfoxide (99.9% d) (0.5 cm^3). Deuterium exchange was carried out by dissolving the sample in D_2O, lyophilizing, and drying before redissolving in dimethyl sulfoxide. Spectral simulations, to extract accurate coupling constant and chemical-shift data, were carried out using Nicolet 1080 and the Univac 1108 computers.

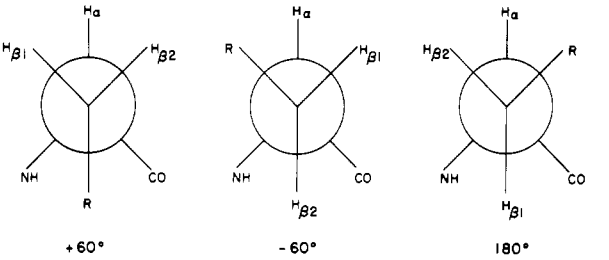

FIGURE 1: The three classical rotamer states for the C_α–C_β bond of a general amino acid.

Results and Discussion

Assignments. The spectrum of tocinamide in $[^2H_6]Me_2SO$ is shown in Figure 2. Although this spectrum is essentially identical to that published by Brewster and co-workers (Brewster et al., 1972), double-resonance experiments clearly show that their assignments of the Asn[5] and Cys[6] amide protons must be reversed. These corrected assignments are consistent with those published for the closely related deaminotocinamide molecule (Brewster et al., 1972).

Although previous work does not positively assign the protons for the Tyr[2] and Cys[6] residues, assignments were suggested for these two residues. We feel that the evidence available is more consistent with assignments shown in Figure 2, the reverse of those previously suggested.

In the absence of data from the synthesis of partially deuterated tocinamide, the assignments for the Tyr[2] and Cys[6] residues must be made on the basis of the observed positions and properties of the resonances. In oxytocin and its analogues, the tyrosine β protons are consistently assigned at higher field than those of Cys[6] (Brewster et al., 1973); this would be in accord with our assignments. The assignments for the C_α protons of Cys[6], Tyr[2], and Asn[5] in the oxytocin molecules are always made consecutively in the order shown between 4.3 and 4.9 ppm. Our assignments would preserve this pattern for Tyr[2] and Asn[5] but would assign the Cys[6] resonance at approximately 4.0 ppm; the previous assignments preserve the Cys[6] and Asn[5] positions assigning Tyr[2] at 4.0 ppm. It seems reasonable that the Cys^6C_α proton would be exceptional in tocins relative to oxytocins as in the former Cys[6] is the terminal residue adjacent to a primary amide rather than the three residue peptide tail found in the latter.

The ^1H NMR spectrum of tocinamide (Figure 2) shows one amide resonance which is considerably broadened in relation to the others. Amide resonances for 2-position amino acid residues in oxytocins have previously been observed to be broadened (Kopple et al., 1976, and references cited therein)

[2] All amino acids used were of the L configuration.

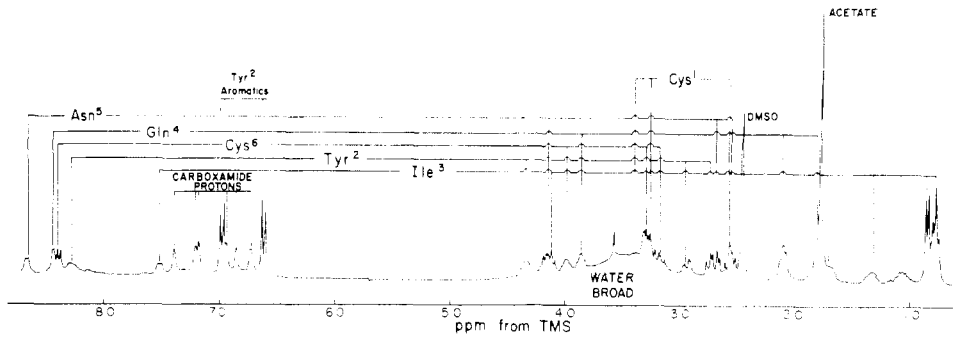

FIGURE 2: The ^{1}H NMR spectrum of tocinamide dissolved in [$^2H_6$]Me_2SO, recorded at 40 °C. Abbreviation used: DMSO, dimethyl sulfoxide.

TABLE II: The $\alpha\beta$ Coupling Constants for Tocinamide Together with the Rotamer Populations and Energy Differences Calculated from them.

Residue	Temp (°C)	$\langle^3J_{\alpha\beta}\rangle$ (Hz)	Rotamer Populations −60° or 180° (p_1)	180° or 60° (p_2)	+60° (p_3)	Boltzman Energy Differences (cal) p_1/p_2	p_1/p_3	p_3/p_2
Cys[1]	70	3.92 9.64	0.12	0.64	0.24	1140	470	670
	80	4.45 9.55	0.17	0.63	0.20	920	110	800
Tyr[2]	20	3.77 8.93	0.11	0.58	0.31	970	600	360
	30	4.26 9.60	0.15	0.66	0.19	890	140	750
	40	4.52 9.30	0.17	0.61	0.22	760	100	660
	50	4.51 9.60	0.17	0.64	0.19	850	70	780
	60	4.56 9.73	0.18	0.65	0.17	850	−40	880
	70	4.54 9.72	0.18	0.65	0.17	870	−40	910
	80	4.65 9.75	0.19	0.65	0.16	860	−120	980
Ile[3]	20	8.18	0.51[a]	0.49[b]				
	30	7.64	0.46	0.54				
	40	7.38	0.44	0.56				
	50	7.18	0.42	0.58				
	60	6.98	0.40	0.60				
	70	6.88	0.39	0.61				
	80	6.74	0.38	0.62				
Asn[5]	20	5.36 7.75	0.25	0.47	0.28	360	70	300
	30	5.56 7.86	0.27	0.48	0.25	350	−50	390
	40	5.63 7.70	0.28	0.47	0.25	310	−50	350
	50	5.81 7.90	0.29	0.48	0.23	320	−150	470
	60	5.77 7.74	0.29	0.47	0.24	320	−130	440
	70	5.86 7.72	0.30	0.47	0.23	310	−180	490
	80	5.92 7.58	0.30	0.45	0.25	280	−160	440
Cys[6]	40	4.93 9.22	0.16	0.60	0.24	920	250	560
	50	4.32 9.01	0.16	0.58	0.26	870	310	510
	60	4.41 9.20	0.17	0.60	0.23	830	200	630
	70	4.43 8.80	0.17	0.57	0.26	820	290	530
	80	4.53 8.74	0.18	0.56	0.26	800	260	540

[a] For trans isomer. [b] For sum of two gauche isomers.

and this would consequently support our assignments. This evidence could not be considered previously as this resonance had been erroneously assigned to Asn[5]. Further support for making this amide assignment comes from the fact that when performing difference double-resonance experiments, irradiating peaks overlaying the broad water band, saturation transfer from the water to this and only this made amide resonance was repeatedly observed. It can be shown (Jones and Gibbons, unpublished results) that saturation transfer from water occurs much faster to the amide proton of the second residue than to any of the other observed amide resonances, thus supporting our Tyr[2] amide assignment.

Double resonance showed that the Cys[1] β-proton chemical shifts are separated by 0.62 ppm and do not both occur at 2.63 ppm as previously suggested (Brewster et al., 1972). This, together with all the tocinamide chemical shift data and the values for the $\langle^3J_{NHCH}\rangle$ coupling constants, is shown in Table I.

Rotamer Analysis. Four of the six tocinamide amino acid residues (Cys[1], Tyr[2], Asn[5], and Cys[6]) are simplified to ABX spin systems when the amide protons are exchanged for deuterium atoms, where A and B are the β protons and X is the α proton. The Gln[4] residue is slightly more complex, since it has an A_2PQX spin system, whereas the Ile[3] residue exhibits an

AB spin system. We were able to perform spin–spin analyses for the residues 1, 2, 3, 5, and 6, but because of degeneracy in the Gln^4 PQ multiplet the analysis of residue 4 is not reported here.

The $\langle {}^3J_{\alpha\beta} \rangle$ values are shown in Table II together with the rotamer populations (p_i) and energy differences ($\Delta G°$) calculated from them (Pachler, 1964).[3] The analysis of the Cys^1 resonances could only be performed at the two highest temperatures, since at temperatures below 70 °C the X quartet appears as a broad unresolved band; in addition, analysis of the Cys^6 spin system was restricted to the temperature range 40–80 °C because of interference by the water peak at lower temperatures.

The rotamer populations, together with the fact that only one spectrum is observed for each multiplet of each residue, indicate that all five analyzed residues of tocinamide exhibit internal motion about the C_α–C_β bond, verifying a similar conclusion obtained from ^{13}C (T_1) studies. The ^1H NMR data thus provides a valuable complement to the ^{13}C T_1 measurements, particularly because discrete values for the populations have not been extracted from T_1 results. Consequently, the observation that $p_1 \neq p_2 \neq p_3 \neq \frac{1}{3}$ for any of the five amino acid residues is of considerable interest, indicating preferred conformations exist.

It has been proposed (Brewster et al., 1972) that the carboxamide carbonyl group of the Asn^5 residue is hydrogen bonded to the temperature-insensitive carboxamide proton of the C terminus. Model building shows that this hydrogen bond can only be formed for one of the two trans–gauche rotamers; it cannot be formed for the gauche–gauche rotamer. The fact that all three classical rotamers for Asn^5 are populated (Table II) suggests that the proposed hydrogen bond does not make a significant contribution to the solution conformation of tocinamide in Me_2SO.

Since the tocinamide ring is closed with a disulfide bond, the possibility that all three rotamer states for both Cys^1 and Cys^6 are significantly populated would require a considerable amount of flexibility in this region of the molecule. Indeed, the whole of this unit of the molecule may be exhibiting some form of coordinated motion.

Coordinated Motion in Tocinamide. If the internal motions of tocinamide, both backbone and side chain, were uncorrelated, then an exceedingly large number of solution conformations would coexist. This number will be reduced by a variety of interactions which have been previously documented (Lowe, 1968); these include the cyclic nature of the molecule, hydrogen bonding, torsional potentials, coulombic interactions, and van der Waals forces. However, a more dramatic reduction in the number of coexisting solution conformations would be brought about if the internal motions were in some way coordinated. Such correlations could occur throughout the entire molecule or alternatively be restricted to smaller independent sections of tocinamide.

Coordinated internal motions can be classified as: (1) intraresidue motions, which would involve coordination of ϕ, ψ, or χ motions within the same amino acid residue, and (2) interresidue motions which would involve coordination of ϕ, ψ, or χ motions from different residues. The data described above indicates that the side chains of tocinamide are in motion. How, or even if, these motions are correlated is not yet possible to determine conclusively, although some insight can be obtained by considering the rotamer population values.

If the rotation of the side chains of two or more amino acid residues is correlated (or geared) then the population of time coincident rotamer states for each residue must be identical. That is, $p_i{}^A = p_1{}^B$, $p_j{}^A = p_m{}^B$, and $p_k{}^A = p_n{}^B$ where A and B are two residues exhibiting coordinated side-chain motion. The detection of interrelationships between side chain and backbone is inherently more difficult but would, however, be reflected in the range of (ϕ,ψ) values allowed between each residue. The time scale for these motions is not directly available from our current ^1H NMR analysis, but the fact that a single rotamer-averaged spectrum is observed, rather than the combination of three superimposed spectra, allows us to set an upper limit of 10^{-3} s for the lifetime of an individual rotational state; presumably, the rotational rate is closer to the order of 10^{-10}–10^{-11} s.

The data in Table II shows that, within our experimental limitations, the classically derived rotamer populations for Cys^1 and Cys^6 are consistent with the existence of coordinated motion between the side chains of these two residues. The fact that these residues are covalently linked lends credibility to this possibility. On the other hand, the very existence of the disulfide bridge has led to speculation concerning the interpretation of $\langle J_{C_\alpha\text{-}C_\beta} \rangle$ data for linked cysteine residues (Kopple et al., 1976).

The disulfide bridge forms an integral part of the cyclic backbone of tocinamide and, consequently, one might consider that the conformation of the cysteine side chains could be relatively rigid. It is possible to assume that this portion of the backbone is rigid and use the appropriate Karplus curves (Kopple et al., 1973) to interpret the observed coupling constants in terms of fixed χ_1 angles. This analysis is not unequivocal and can give up to four possible χ_1 values for each residue. In this case, the same two possible angles were determined for both cysteine residues, either 0° or 120°. This means that if the disulfide bridge of tocinamide were to be in a fixed conformation then this conformation must be a totally eclipsed form. Neither calculations (Kotelchuck et al., 1972) nor crystallographic measurements (Parthasarathy, private communication) have provided any evidence that eclipsed conformations exist in peptide disulfides. Our studies of the temperature dependencies of the coupling constants and chemicals shifts also indicate that this is unlikely. Furthermore, these χ_1 values (0° or 120°) are consistent with the number average χ_1 values (5° or 114°) obtained by assuming that each cysteine residue populates the three classical rotamer states in the ratio 60:25:15.

It has been suggested (Feeney, 1975) that the dependence of $\langle {}^3J_{C_\alpha\text{-}C_\beta} \rangle$ on temperature may be used as a criterion for side-chain mobility. Unfortunately, only one of the four cysteine $\Delta\langle {}^3J_{C_\alpha\text{-}C_\beta} \rangle/\Delta T$ values for tocinamide can be obtained with any degree of accuracy from our spectra; however, this value (-12×10^{-3} Hz/°C for Cys^6) is not consistent with a rigid C_α–C_β side-chain conformation. Further evidence against disulfide bridge rigidity in the oxytocins, in general, is obtained by examining the cysteine $\langle {}^3J_{C_\alpha\text{-}C_\beta} \rangle$ values for a series of such molecules. The values obtained for tocinamide in dimethyl sulfoxide, for oxytocin in dimethyl sulfoxide (Gibbons et al., unpublished results), for oxytocin in water (Meraldi et al., 1975), and for the retro-tocinamide analogue in dimethyl sulfoxide (Kopple et al., 1976) vary considerably. Consequently, no single fixed disulfide conformation can exist for the oxytocin-like molecules under all these temperature, solvent, and molecular variations.

[3] The calculation of p_i values by the Pachler method assumes that only the three classical rotamer states, shown in Figure 1, contribute to the C_α–C_β stereochemistry. See later in the text for the discussion of other possible analyses.

TABLE III: Rotamer Populations for the Three Residues of Tocinamide Which May Exhibit Coordinated Motion.

Temp (°C)	Residue	p_1	p_2	p_3
70	Cys^1	0.12	0.64	0.24
	Tyr^2	0.18	0.65	0.17
	Cys^6	0.17	0.57	0.26
80	Cys^1	0.17	0.63	0.20
	Tyr^2	0.19	0.64	0.16
	Cys^6	0.18	0.56	0.26

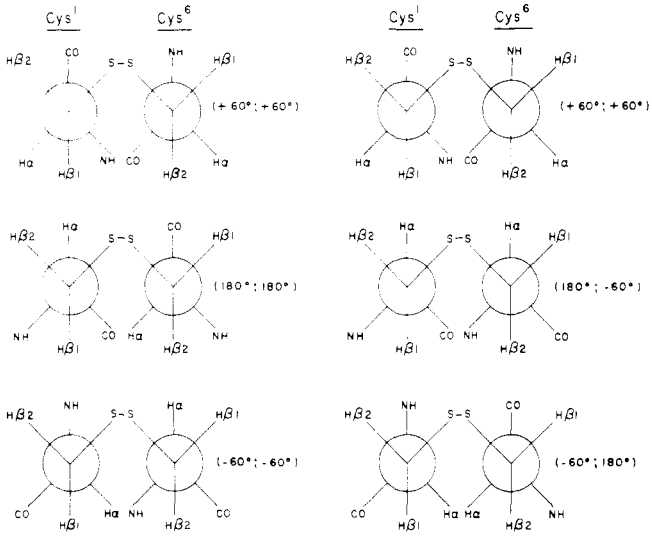

FIGURE 3: The two possible sets of rotamer states, one of which would exist if concerted motion were to occur about the tocinamide disulfide bridge, provided that the assumption of classical rotamers is valid.

Kopple and co-workers (Kopple et al., 1976), in their analysis of a retro-tocinamide analogue, consider an interpretation of their data whereby the cysteine side chain populates only two *classical* rotamers. We do not believe their observed coupling constants can be consistent with this conclusion. However, it is possible that rotational averaging can occur between nonclassical rotamer states; i.e., one could postulate three values of χ, as $(60 + x)°$, $(180 + y)°$, and $(-60 + z)°$, where x, y, and z may be positive or negative. By varying x, y, and z, together or independently one can obtain population figures for various coexisting nonclassical states; for each set of x, y, and z, two sets of solutions for p_1, p_2, and p_3 may be obtained.[4]

We have examined a number of nonclassical situations. The results show that under certain circumstances the population of one of the rotamer states may be close to zero. Consequently, it is possible that the observed coupling constants for tocinamide (or for that matter for any other disulphide bridge containing peptide) may be consistent with conformational averaging between three classical rotamer states or between two or three nonclassical rotamer states. In view of this, we feel that it is pertinent to further consider the possibility of the occurrence of coordinated motion for the classical case, bearing in mind that the principle of such motion could apply equally well to any nonclassical situation.

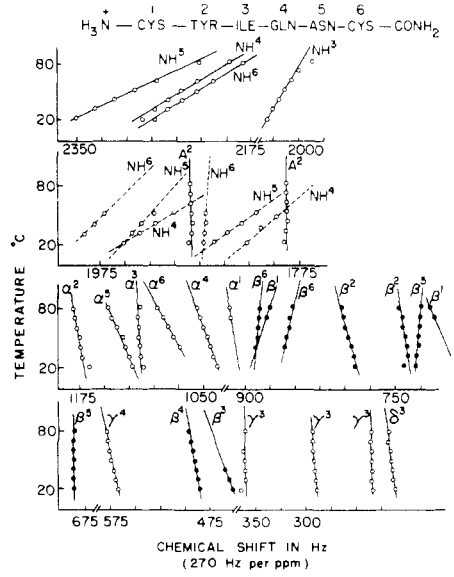

FIGURE 4: The variation with temperature of each of the resonances in the tocinamide spectrum. (- - -) The carboxamide protons. (A) An aromatic resonance.

There are 162 possible conformations for the disulfide bridge section of the ring if one includes rotations about the two C_β–S bonds, the S–S bond, and the two C_α–C_β bonds. This number could be reduced to three if the rotation about the two C_α–C_β bonds were to occur in a coordinated manner, causing the whole disulfide bridge to rotate in unison. For a classical analysis, all three rotamer states for each of the two cysteine residues are populated and the population distributions are, within the limit of our experimental determination (Table III), consistent with concerted motion. That is, the condition that $p_1^{Cys^1} = p_1^{Cys^6}$, $p_2^{Cys^1} = p_2^{Cys^6}$, and $p_3^{Cys^1} = p_3^{Cys^6}$ is fulfilled.[5] If there are only three solution conformations of the tocinamide disulfide bridge, we cannot, at present, unambiguously distinguish between the two possible sets which arise from our inability to absolutely assign the −60° and the 180° rotamer states. The two possible sets are shown in Figure 3.

Sugeta et al. (1972) found three conformer classes in disulfides attributable to C–S internal rotations. This indirectly supports our finding of three C_α–C_β rotamers and extensive motions of all the single bonds of the cystine bridge of tocinamide. Our contention of correlated motion and the classical vs. nonclassical nature of the three C_α–C_β and C–S rotamers remain to be tested.

Examination of molecular models shows that in order to allow geared rotation of the tocinamide disulfide bridge considerable flexibility is required in this region of the molecule. However, other parts of the molecule may be less flexible or, alternatively, part of a separate unit of coordinated motion. In fact, it is of particular interest to note that the Tyr^2 rotamer population values are also comparable to those for Cys^1 and Cys^6 (Table III); consequently, it seems reasonable that the Tyr^2 motion could be correlated with that of the disulfide bridge. If they exist, the correlated motions considered above would be classified according to the scheme proposed, as type (2).

[4] This is also true for the classical situation but in that case p_1 from one solution equals p_2 from the other and vice versa, leading to the fact that one cannot unambiguously distinguish the 180° and −60° rotamer population values.

[5] For this condition to be fulfilled, it is not required that $p_1^A = p_1^B$, $p_2^A = p_2^B$, and $p_3^A = p_3^B$, but merely that $p_i^A = p_1^B$, $p_j^A = p_m^B$, and $p_k^A = p_n^B$, for example, $P_1^A = p_2^B$, $p_2^A = p_3^B$, and $p_3^A = p_1^B$ would still be consistent with coordinated motion; however, in this case different rotamers would be moving in tandem.

TABLE IV: The Tocinamide Temperature Coefficients Expressed in ppb/°C.

Resonance	Temp Coeff.	Resonance	Temp Coeff.	Resonance	Temp Coeff.
$Cys^1\alpha$	+0.53	$Ile^3\gamma 3$	+0.10	$Cys^6\alpha$	+2.00
$Cys^1\beta 1$	−1.26	$Ile^3\delta$	+0.45	$Cys^6\beta 1$	−0.36
$Cys^1\beta 2$	+1.49	Gln^4NH	−5.67	$Cys^6\beta 2$	−0.81
$Tyr^2\alpha$	+0.64	$Gln^4\alpha$	+1.31	Aromatic	+0.04
$Tyr^2\beta 1$	+0.97	$Gln^4\beta$	+0.62	Aromatic	−0.05
$Tyr^2\beta 2$	+0.51	$Gln^4\gamma$	+0.77	Carboxamide	−3.85
Ile^3NH	−2.10	Asn^5NH	−7.35	Carboxamide	−3.39
$Ile^3\alpha$	+0.21	$Asn^5\alpha$	+1.52	Carboxamide	−6.32
$Ile^3\beta$	+1.27	$Asn^5\beta 1$	−0.32	Carboxamide	−0.37
$Ile^3\gamma 1$	+0.06	$Asn^5\beta 2$	−0.13	Carboxamide	−5.24
$Ile^3\gamma 2$	+0.28	Cys^6NH	−5.56	Carboxamide	−5.45

Chemical Shifts and Conformation. The problem inherent in the analytical approach to peptide conformation described above lies in the unavoidable inaccuracy associated with the $\langle {}^3J_{\alpha\beta}\rangle$ values and the p_i values calculated from them. To overcome these inaccuracies, the dependence on temperature of the chemical shifts of most of the resonances in the spectrum was investigated (Table IV and Figure 4). We are currently attempting a detailed correlation of this data with tocinamide conformations and internal motions. Here we point out certain general features not previously observed for peptides.

The chemical shifts of almost all the protons in tocinamide are dependent on temperature; the value of this temperature dependence ($\Delta\delta i/\Delta T$) varies considerably within the range ±7 ppb/°C. The number of different factors which determine the absolute value of the chemical shift of any particular proton can be large. However, under the conditions of these ^{1}H NMR experiments it seems reasonable to assume that the two major temperature-dependent influences on chemical shift will be solvent–solute interactions and intramolecular interactions related to magnetic anisotropy effects. Hence, it is instructive to attempt to explain the chemical-shift temperature dependencies in terms of these properties.

Toward this end, much work has been published relating to the temperature dependence of peptide amide protons (Urry et al., 1975). In such cases, the "high" (>−3 ppb/°C) temperature dependencies are explained in terms of solvent interaction, whereas the "low" (0 to −3 ppb/°C) values are believed to be due to intramolecular solvent shielding, e.g., the formation of hydrogen bonds.

The current data (Table IV and Figure 4) provide two interesting observations. First, the aromatic protons are observed to have zero temperature dependence, and, secondly, for this particular molecule the magnitude of temperature dependencies follows the general order $CH_\alpha > CH_\beta > CH_\gamma \sim CH_\delta$. These facts suggest that for protons attached to carbon atoms the solute–solvent interactions play virtually no role in determining $\Delta\delta/\Delta T$, whereas the magnetic anisotropies are of particular importance. Thus, the following picture may represent a simple but useful description of the dependence on temperature of peptide (or at least tocinamide) proton chemical shifts.

Exchangeable proton temperature dependencies, notably those of amides, are dominated by solvent–solute interactions. Removal or reduction of these interactions, e.g., in hydrogen bonding, leaves a chemical-shift temperature dependence of up to about |2| ppb/°C which is dependent on magnetic anisotropy effects. The temperature dependence of other, i.e., CH, protons is largely determined by intramolecular anisotropic effects which will vary according to the relative orientation of the protons within the molecule; i.e., temperature dependencies of CH protons, with values up to about |2| ppb/°C, are directly related to the internal motions and, hence, rotamer populations of the tocinamide molecule.

If the above interpretation is correct, even greater care must be exercised in assigning or not assigning hydrogen bonds, particularly as the sign of the magnetic anisotropy effects may be either positive or negative. The $\Delta\delta i/\Delta T$ values for amides are almost exclusively negative in sign; consequently, two amide protons having $\Delta\delta i/\Delta T$ equal to −4 and 0 ppb/°C, respectively, could have the same solvent-shielding characteristic of −2 ppb/°C combined with magnetic anisotropic effects of −2 and +2 ppb/°C, respectively. If the possibility of a magnetic anisotropic effect is ignored, the latter would be assigned as strongly hydrogen bonded and the former as non-hydrogen bonded. Obviously, this is an extreme case and many assumptions have been made; however, the need for care is obvious and, where possible, hydrogen bonding should be confirmed by at least two complementary techniques (Urry et al., 1975).

In contrast to the above difficulties, the detailed investigation of the $\Delta\delta i/\Delta T$ values for CH protons should lead to information about both conformation and internal motion. If, as is considered above, the magnetic anisotropies of neighboring groups make the major contribution to the CH chemical shift temperature dependencies, then it is not unreasonable to postulate that at a fixed temperature each rotamer state exhibits a fixed chemical shift for each proton position in the spin system of a given residue. The observed chemical shift for each proton in the system would then be determined by summing the population weighted contributions of these "intrinsic" chemical shifts, i.e., $\langle \delta^k \rangle_{obsd} = p_1\delta_1{}^k + p_2\delta_2{}^k + p_3\delta_3{}^k$, where $\delta_i{}^k$ are the intrinsic rotamer chemical shifts for each proton (k) in the three classical rotamers (1–3).

In a region of rigid backbone conformation, these intrinsic chemical shifts would be independent of temperature. Conversely, in a region of flexible backbone the intrinsic chemical shifts would be dependent on temperature *unless* rotation (χ motion) is coordinated with backbone (ϕ,ψ) motion, which it appears may well be the case, at least for certain residues in the tocinamide molecule. The investigation of these intrinsic chemical shifts forms a significant part of our current analysis.

Conclusion

We have completed a detailed ^{1}H NMR analysis of the population of the rotational states about the C_α–C_β bonds of five of the six amino acid residues of tocinamide. The results of this analysis may be summarized as follows:

(1) The use of difference double resonance has promoted the correction of some of the previous assignments.

(2) Computer simulation of five of the six tocinamide residues has allowed detailed analysis of their C_α–C_β spin systems. It is proposed that the p_i values so determined may be used for: (a) detection of internal motion in a manner simpler but complementary to ^{13}C relaxation methods, (b) detection of conformational preferences about the C_α–C_β bonds, (c) reduction of the number of possible coexisting solution conformations, (d) postulation of the existence of correlated internal motion in the region of the disulfide bridge and thus the possibility of the coexistence of three interconverting conformations in this region of the molecule of statistical weight about 60:25:15, (e) postulation of the existence of correlated internal motion of the contiguous Cys^1 and Tyr^2 residues.

(3) All tocinamide protons, except those of the tyrosine aromatic ring, exhibit temperature dependencies. The $C_\alpha H$ and $C_\beta H$ dependencies are probably dominated by rotamer population effects, but those of amide protons have additional, often dominating, contributions from solvent-solute interactions. Consideration of $\Delta\delta i/\Delta T$ effects stresses the need for exercising care when assigning amide protons to hydrogen bonds and leads to the concept of intrinsic rotamer chemical shifts for CH protons.

Finally, it becomes clear that each CH–CH_2 fragment in a peptide is characterized by the following parameters: (1) $\langle\delta i\rangle$, the observed α,β chemical shifts, (2) $\Delta\langle\delta i\rangle/\Delta T$, the variation in observed α and β chemical shifts with temperature, (3) $\Delta\beta\beta$, the difference in observed chemical shifts in the two β protons, and, possibly, (4) δi^j, the calculated intrinsic rotamer chemical shifts. The basic problem is to correlate each of these ^1H NMR parameters with the conformations and internal motions of the polypeptide. In the case of tocinamide, we are continuing in our attempts to do this.

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References

Allerhand, A., and Komoroski, R. (1973), *J. Am. Chem. Soc. 95,* 8228.

Brewster, A. I., Glasel, J.A., and Hruby, V. J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1470.

Brewster, A. I. R., Hruby, V. J., Glasel, J. A., and Tonelli, A. E. (1973), *Biochemistry 12,* 5294.

Deslauriers, R., and Somerjai, R. L. (1976), *J. Am. Chem. Soc. 98,* 1931.

Feeney, J. (1975), *Proc. R. Soc. London, Ser. A 345,* 61.

Feeney, J., Roberts, G. C. K., Brown, J. P., Burgen, A. S. V., and Gregory, H. (1972), *J. Chem. Soc. Perkin Trans. 2,* 601.

Gibbons, W. A., Crepaux, D., Delayre, J., Dunund, J. J., Hajdukovic, G., and Wyssbrod, H. R. (1975), in Peptides: Chemistry, Structure, and Biology, Walter, R., and Meienhofer, J., Ed., Ann Arbor, Michigan, Ann Arbor Science, p 127.

Ivanov, V. T., Filatova, M. P., Reissman, Z., Reutova, T. O., Efremov, E. S., Pashkov, V. S., Galaktinov, S. G., Grigoryan, G. L., and Ovchinnikov, V. A. (1975), in Peptides: Chemistry, Structure, and Biology, Walter, R., and Meienhofer, J., Ed., Ann Arbor, Michigan, Ann Arbor Science, p 151.

Jones, C. R., Garsky, V., and Gibbons, W. A. (1976), *Nature (London) 262,* 779.

Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970), *Anal. Biochem. 34,* 595.

König, W., and Geiger, R. (1973), *Chem. Ber. 106,* 3626.

Kopple, K. D., Dickinson, H. R., Nakagawa, S. H., and Flouret, G. (1976), *Biochemistry 15,* 2945.

Kopple, K. D., Wiley, G. R., and Tauke, R. (1973), *Biopolymers 12,* 627.

Kotelchuck, D., Scheraga, H. A., and Walter, R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3629.

Lowe, J. P. (1968), *Prog. Phys. Org. Chem. 6,* 1.

Manning, M. (1968), *J. Am. Chem. Soc. 90,* 1348.

Manning, M., Balaspiri, L., Acosta, M., and Sawyer, W. H. (1973), *J. Med. Chem. 16,* 975.

Meraldi, J. P., Yamoto, D., Hruby, V. J., and Brewster, A. I. R. (1975), in Peptides: Chemistry, Structure, and Biology, Walter, R., and Meienhofer, J., Ed., Ann Arbor, Michigan, Ann Arbor Science, p 803.

Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85,* 2149.

Merrifield, R. B. (1964), *J. Am. Chem. Soc. 86,* 304.

Pachler, K. G. R. (1964), *Spectrochim. Acta 20,* 581.

Stanley, E. H., Asher, I. M., Rothschild, K. J., Phillies, G. D. J., Carew, E. B., Bansil, R., and Michaels, I. A. (1975), in Peptides: Chemistry, Structure, and Biology, Walter, R., and Meienhofer, J., Ed., Ann Arbor, Michigan, Ann Arbor Science, p 227.

Sugeta, H., Go, A., and Miyazawa, T. (1973), *Bull. Chem. Soc. Jpn. 46,* 3407.

Urry, D. W., Long, M. M., Mitchell, L. W., and Okamoto, K. (1975), in Peptides: Chemistry, Structure, and Biology, Walter, R., and Meienhofer, J., Ed., Ann Arbor, Michigan, Ann Arbor Science, p 113.

Walter, R., Ballardin, A., Schwartz, I. L., Gibbons, W. A., and Wyssbrod, H. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4528.

Westall, F. C., Scotchler, J., and Robinson, A. B. (1972), *J. Org. Chem. 37,* 3363.

Wyssbrod, H. R., and Gibbons, W. A. (1973), *Surv. Prog. Chem. 6,* 209.

Yamashiro, D., and Li, C. H. (1973), *J. Am. Chem. Soc. 95,* 1310.