Nuclear Magnetic Resonance Study of Fibrinogen-Like Peptides and Their Structure in Dimethyl Sulfoxide and Water†

P. H. Von Dreele, I. D. Rae, and H. A. Scheraga*

ABSTRACT: The ^1H nuclear magnetic resonance (NMR) spectra of four fibrinogen-like oligopeptides (H-Gly-Pro-Ala-NH_2, H-Arg-Gly-Pro-Ala-NH_2, H-Val-Arg-Gly-Pro-Ala-NH_2, and H-Gly-Val-Arg-Gly-Pro-Ala-NH_2) in dimethyl-d_6 sulfoxide (Me_2SO-d_6), and of the hexapeptide in water, and the ^{13}C NMR spectrum of H-Gly-Pro-Ala-NH_2 in Me_2SO-d_6, were recorded and interpreted in terms of preferred conformations in solution. Each peptide exists in Me_2SO-d_6 as a 30:70 mixture of cis and trans isomers about the Gly-Pro bond, and the hexapeptide in water is solely the trans isomer. For the trans isomers in Me_2SO-d_6, there is a hydrogen bond between the Gly CO group and one of the C-terminal primary amide hydrogens, and a β turn involving the Gly-Pro-Ala-NH_2 section of the molecules. A strong NOE between Pro $C^\alpha H$ and Ala NH for the trans isomer of the tripeptide in Me_2SO-d_6 completes the identification of this structural feature as a type II β turn.

Fibrinogen is cleaved by thrombin at four Arg-Gly peptide bonds, to give fibrinopeptides A and B and fibrin monomer which polymerizes to form a clot (Blombäck et al., 1976). The cleavage between Arg-16 and Gly-17 of the Aα chain of human fibrinogen produces the 16-residue peptide A, and the rate of this process parallels the rate of fibrin formation (Blombäck & Vestermark, 1958). We have previously examined the action of thrombin on oligopeptides whose sequence resembles that of the Arg-16/Gly-17 region of the Aα chain (Van Nispen et al., 1977; Scheraga, 1977; and papers cited therein) in order to determine the minimum requirements for activity at the level of the Aα chain or a portion thereof. We have also considered the possibility that some conformational feature of the Arg-16/Gly-17 region contributes to the specificity of thrombin action and now report that the NMR[1] spectra of four fibrinogen-like oligopeptides (H-Gly-Pro-Ala-NH_2, H-Arg-Gly-Pro-Ala-NH_2, H-Val-Arg-Gly-Pro-Ala-NH_2, and H-Gly-Val-Arg-Gly-Pro-Ala-NH_2) have been examined for evidence of preferred conformations in solution.

The stereochemistry of the Gly-Pro peptide bond was also of interest because most X-Pro residues in native proteins have the trans conformation (Huber & Steigemann, 1974), whereas a cis-trans equilibrium mixture is normally found in oligopeptides (Deslauriers & Smith, 1976; Grathwohl & Wüthrich, 1976). Brandts (Brandts et al., 1975; Brandts et al., 1977) has proposed that a contribution to slow protein folding and unfolding is the establishment of this equilibrium in denatured proteins by cis-trans isomerization of some of the prolines.

Experimental Procedure

Materials. The oligopeptides used in this study had been synthesized earlier (Liem et al., 1971). Each has a C-terminal primary amide group and, as a result of the final lyophilization, the amino group is present as the acetate salt, as is the Arg side chain. H-Pro-Ala-NH_2 acetate was synthesized as follows. Ala-NH_2 was reacted with Z-Pro-ONp in dimethylformamide and, after 2 days at room temperature, the unreacted active ester was precipitated by addition of water. The solution was then evaporated to dryness and the residue was partitioned between $CHCl_3$ and 5% aqueous Na_2CO_3. The $CHCl_3$ solution was adsorbed on a column of SiO_2 and the Z-Pro-Ala-NH_2 was eluted with $CHCl_3$:CH_3OH (9:1). This compound was deprotected by hydrogenation in CH_3OH over 10% Pd/C and converted to the acetate salt. Evaporation of the solvent gave H-Pro-Ala-NH_2 as a deliquescent gum which showed a single spot on thin-layer chromatography and a satisfactory amino acid analysis. Solvents were spectroscopic grades.

Instrumental Methods. The ^1H NMR spectra were obtained on a Varian HA-100 spectrometer, and on a 250-MHz spectrometer described elsewhere (Dadok & Sprecher, 1974) using an internal lock and frequency sweep mode. The sample concentrations were 0.05 to 0.10 M. All chemical shifts in Me_2SO-d_6 are downfield from the internal standard Me_4Si, and in aqueous solution from an external standard of Me_4Si in CCl_4. For the NOE experiment, a fresh solution of the tripeptide in 100% Me_2SO-d_6 was flushed with dry nitrogen for 10 min just before the experiment. Measurements of peak areas were recorded electronically for spectra arising from 40-100 scans in a correlation mode and were standardized against the areas of resonances from hydrogens not involved in the irradiation. In the reference scan, the irradiating frequency was moved some 30-50 Hz from the peak of interest. Changes in area also were visualized as difference spectra and by digital integration to confirm the observations.

The ^{13}C NMR spectrum of the tripeptide was recorded at 32 °C on a Varian CFT-20 instrument, for a 40-mg sample dissolved in 0.5 mL of Me_2SO-d_6. The pulse angle was 90° and the repetition time 3.0 s, and 8192 data points in a 5000-Hz scan were used.

Assignment of NMR Spectra. The NMR spectra of the four peptides in Me_2SO-d_6 and of the hexapeptide in water (at pH 5.3) are shown in Figure 1, and the characteristic shifts, etc., are shown in Tables I-III. The conformational information can be derived only after the peaks have been assigned, and our

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[1] Abbreviations used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Me_2SO-d_6, dimethyl-d_6 sulfoxide; Me_4Si, tetramethylsilane; Z, $C_6H_5CH_2OCO$-; ONp, *p*-nitrophenyl; Boc, *tert*-butoxycarbonyl.

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assignments are based, in the first instance, on the assumption that each amino acid residue is a separate spin system. Assignment commenced with the tripeptide and new signals were identified as residues were added in each of the succeeding peptides.

It is apparent from the spectrum of the tripeptide (Figure 1A) that two species are present in solution. Most of the resonances are doubled but the phenomenon is seen most clearly for the Ala-NH doublets near 8 ppm. In fact, doubling of peaks occurs in all of the Me_2SO-d_6 spectra (but not for H-Pro-Ala-NH_2), and we ascribe this to the existence of cis and trans isomers at the Gly-Pro peptide bond which interconvert slowly on the NMR time scale. The separation of the resonances is greatest for those hydrogens near the Pro residue and almost disappears after a few bonds, except for one of the primary amide hydrogens of Ala. Thus, in the tetrapeptide in Me_2SO-d_6 at 30 °C, the separations (in ppm) are: Pro $C^{\alpha}H$, −0.17; Ala peptide, NH, −0.32; Gly $C^{\alpha}H_2$, 0.06; Ala $C^{\alpha}H$, −0.06; $CONH_2$ syn, 0.00; $CONH_2$ anti, −0.32 ppm. The isomers may be identified as trans (major) and cis (minor) from the data in Table I and by comparison with literature values (Deber et al., 1970). The isomer ratio may be calculated from a number of pairs and, when this is done (Table III), there is obtained consistently a ratio of approximately 70:30 (trans:cis) for each of the peptides. In water at pH 5.3, the hexapeptide shows only one isomer, and titration from Me_2SO-d_6 to water established that it was the minor isomer that had disappeared; i.e., the conformation was all trans.

This isomerism about the Gly-Pro bond is also the cause of doubling of some peaks in the ^{13}C spectrum of the tripeptide Gly-Pro-Ala-NH_2 in Me_2SO-d_6 at 32 °C (Deslauriers & Smith, 1976). Single peaks were observed for Pro C^{α} (59.8 ppm), Pro C^{δ} (45.1 ppm), Ala C^{α} (48.0 ppm), and Ala C^{β} (17.8 ppm) and a single weak carbonyl was found at 171.9 ppm. Other carbons gave two peaks, for trans and cis isomers, respectively, with intensity ratio approximately 2:1, viz., Gly C^{α}, 42.5 and 42.9; Pro C^{β}, 29.1 and 31.6; Pro C^{γ}, 24.1 and 22.1 ppm. The assignment of Pro C^{β} and C^{γ} was made by reference to the work of Dorman & Bovey (1973).

In Me_2SO-d_6 solutions of all the peptides, the two hydrogens of the C-terminal primary amide group show separate resonances but some quadrupole broadening prevents observation of the coupling between them (Liler, 1971). The upfield peak, in each case, accidentally has the same chemical shift (6.94–6.97 ppm) in the two isomers arising from cis–trans isomerism at the Gly-Pro bond. The downfield primary amide hydrogen, however, has different chemical shifts in the trans and cis isomers, viz., 7.08–7.10 in the major (trans) isomer and 7.39–7.40 ppm in the minor (cis) isomer. This downfield hydrogen can be identified as the one which is anti to the primary amide carbonyl because the anti hydrogen is usually the more deshielded of the pair (Stewart & Siddall, 1970; Liler, 1971). When the temperature was raised to ~53 °C these signals collapsed to a singlet as exchange became fast on the NMR time scale.

This interplay of cis–trans isomerism about the Gly-Pro bond, and the internal chemical shift difference of the primary amide hydrogens, accounts for the doubling of some resonances and the appearance of three peaks with area ratio 0.3:0.7:1.0 representing the anti-cis, anti-trans, and syn (cis plus trans) protons of the primary amide group (Figure 1 and Table I).

Most of the other peaks were assigned by means of spin decoupling, in the conventional manner. Thus, irradiation of the strong methyl doublets at about 1.0 ppm (the Ala CH_3) served to identify the Ala $C^{\alpha}H$ signals, which in turn were connected to the Ala NH signals. For the hexapeptide in aqueous solution (Figure 1E), this involved irradiation under the water peak, a technique which has been discussed elsewhere (Dadok et al., 1972); only a few of the resonances were assigned in this solvent. The resonances of the N-terminal glycine residue in the hexapeptide were identified by observing their change of chemical shift when a small amount of acetic acid was added to the Me_2SO solution (CH_2, 3.16 moves to 3.28 ppm). The acid also affected the chemical shift of the adjacent Val-NH (8.01 to 8.16 ppm), but NH protons of the second residue in each of the three longer peptides were also, and more easily, identified by transfer of spin saturation from the water peak as described earlier (Von Dreele et al., 1972; Brewster & Hruby, 1973; Glickson et al., 1974). It is interesting to note that, while earlier workers described this technique for aqueous solutions, it also works for the small amount of water present in a Me_2SO-d_6 solution. Irradiation of the water peak reduced the intensity of peaks arising from rapidly exchanging, but slowly relaxing, protons such as those of the guanidino group and those activated by electron-withdrawing effects in the $H_3N^+CHRCONH$- system at the N terminus.

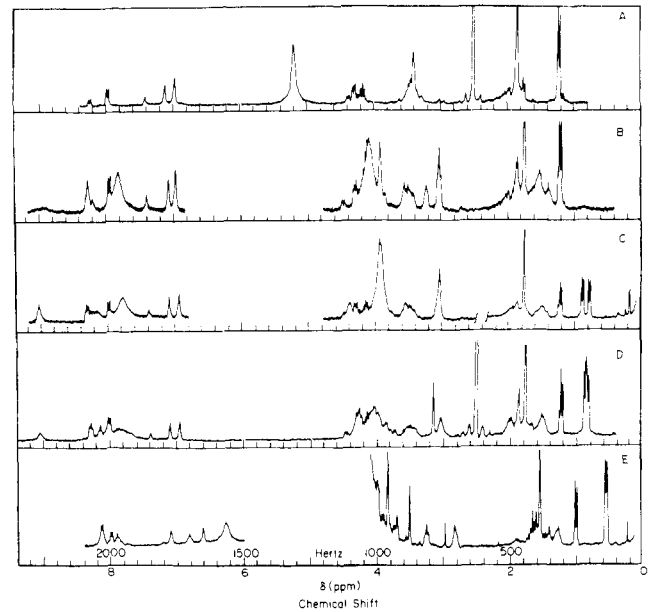

FIGURE 1: The 250-MHz NMR spectrum at 30 °C in Me_2SO-d_6 of the acetate salt of H-Gly-Pro-Ala-NH_2 (A), H-Arg-Gly-Pro-Ala-NH_2 (B), H-Val-Arg-Gly-Pro-Ala-NH_2 (C), and H-Gly-Val-Arg-Gly-Pro-Ala-NH_2 (D), and in H_2O at 30 °C at pH 5.3 of H-Gly-Val-Arg-Gly-Pro-Ala-NH_2 (E). The reference is the internal Me_4Si for Me_2SO-d_6 spectra and external Me_4Si/CCl_4 for the H_2O spectrum. In C, the amplitudes at $\delta < 2.4$ ppm are lower. In B and C, there were no peaks in the region 4.8 to 6.8 ppm.

The Gly-CH_2 resonances were complicated by small chemical shift differences, both within each methylene and between the two pairs, but it was possible to discern a J_{gem} for the internal glycine of approximately 17–17.5 Hz. The values of $J_{NH\text{-}C^{\alpha}H}$, and corresponding values of the dihedral angle ϕ calculated according to Bystrov (Bystrov et al., 1969; Bystrov, 1976), are shown in Table II for the hexapeptide. The same coupling constants were obtained for the other peptides in Me_2SO-d_6 (see Table I). Also in Table II are the temperature coefficients, $d\delta/dT$, for the NH chemical shifts.

Discussion

The chemical shift of the amide hydrogen which is anti to the carbonyl in the Ala-NH_2 residue is affected to a surprisingly large extent by the cis–trans isomerism of the Gly-Pro bond. We ascribe this to an interaction, possible only in the trans isomer, with some part of the molecule which must be no

TABLE I: ^{1}H NMR Spectra of the Fibrinogen-Like Oligopeptides.[a]

Proton	δ (ppm)				
	Tripeptide	Tetrapeptide	Pentapeptide	Hexapeptide	
Ala					
CH_3 major component	1.21	1.21	1.21	1.21	1.01
minor component	1.23	1.24	1.24	1.24	
$C^{\alpha}H$ major component	4.15	4.17	4.15	4.15	3.88
minor component	4.23	4.23	4.23	4.22	
NH major component	7.96	7.95	7.97	8.01	7.99
	(J = 7.2)[b]	(J = 7)	(J = 7.5)	(J = 7.5–8)	(J = 6.1)
minor component	8.23	8.27	8.27	8.27	
	(J = 7.5)				
Pro					
$C^{\delta}H_2$	3.4–3.5	3.4–3.6	3.4–3.5	3.4–3.6	
$C^{\gamma}H_2$		1.87	1.84	1.86	
$C^{\beta}H_2$		1.9–2.1	1.9	1.72	
$C^{\alpha}H$ major component	4.28	4.29	4.29	4.29	
minor component	4.37	4.46	4.46	4.46	
Gly[c]					
$C^{\alpha}H_2$ major component	3.99	3.92		3.80 3.86 3.97	3.72
minor component		3.86		3.73	
NH major component		8.26	8.29	8.12	7.90
		(ΣJ = 11)	(ΣJ = 11)	(ΣJ = 11)	(ΣJ = 10.6)
minor component		8.19			
Arg					
$(NH_2)_2^+$		7.84	7.76	7.82	6.28
NH		9.00	9.01	9.01	6.82
$C^{\delta}H_2$		3.04	3.04	3.04	
$C^{\gamma}H_2$		1.55	1.49	1.50	
$C^{\beta}H_2$		1.39	1.4–1.6	1.4–1.5	1.50
$C^{\alpha}H$		3.24	4.38	4.2–4.4	4.00
NH			8.17	8.27	8.13
				(J = 7–7.5)	(J = 7.0)
Val					
$C^{\gamma}H_3$			0.78	0.82	
$C^{\gamma}H_3$			0.88	0.86	
$C^{\beta}H$			1.99	1.99	
$C^{\alpha}H$			3.02	4.26	
NH				8.01	8.13
					($J \sim 7$)
Gly[d]					
$C^{\alpha}H_2$				3.16	
$CONH_2$ group					
syn-NH (both components)	6.96	6.97	6.94	6.95	6.61
anti-NH (major component)	7.10	7.08	7.09	7.10	
anti-NH (minor component)	7.40	7.40	7.39	7.39	7.10

[a] The spectra were recorded at 250 MHz at 30 °C in Me_2SO-d_6, except for the last column, which pertains to water at pH 5.3 at 30 °C. The chemical shifts are in ppm downfield from internal Me_4Si in Me_2SO-d_6, and from external standard Me_4Si/CCl_4 in water. [b] Coupling constants $J_{NH-C^{\alpha}H}$ in Hz. [c] This is the N-terminal Gly of the tripeptide and internal Gly of the other compounds. [d] This is the N-terminal Gly of the hexapeptide.

further away than the Gly residue since the effect is already noticed in the tripeptide. The differences in chemical shift for the two Ala-NH_2 hydrogens are 0.43–0.45 ppm in the minor, cis isomer, and this corresponds fairly well to differences observed in the model compound H-Pro-Ala-NH_2 (0.38 ppm) and in other primary amides: acetamide, 0.60 (cf. similar values for acetamide in several solvents, Liler, 1971); propionamide, 0.55; HCl-H-Leu-NH_2, 0.51; HCl-H-Gly-NH_2, 0.43; Z-Sar-NH_2, 0.33; Boc-Ala-NH_2, 0.31; Z-Gly-NH_2, 0.30 ppm. In the major, trans isomer these differences are only 0.11–0.15 ppm, and it is clear that the interaction mentioned above has caused an upfield shift of about 0.3 ppm for the amide hydrogen anti to the carbonyl. A hydrogen bond formed between this hydrogen and the Gly carbonyl would contribute to a downfield shift of this anti hydrogen, but would also place it in the shielding region of the pro-Ala peptide bond (Hooper & Kaiser, 1965; Karabatsos et al., 1967) where a counterbalancing upfield shift might be expected. Net shielding arising from two such interactions has been observed for intramolecularly hydrogen bonded peptide hydrogens of gramicidin S, 1.1 ppm (Stern et al., 1968); *c*-[Gly_2-Tyr]$_2$, 0.8, and *c*-[Gly_2-Leu]$_2$, 0.9 ppm (Kopple et al., 1972); and *c*-[Pro-Ser-Gly]$_2$, 0.4, and *c*-[Pro-Gly-Ser]$_2$, 0.5 ppm (Torchia et al., 1972). This effect seems to be a common consequence of bend formation

TABLE II: Amide Proton Chemical Shifts, Temperature Coefficients, $J_{NH-C^{\alpha}H}$ Coupling Constants, and the Backbone Dihedral Angles for the Hexapeptide in Me_2SO-d_6.

Proton	δ at 30 °C (ppm)	$d\delta/dT$ (ppb/°C)	$J_{NH-C^{\alpha}H}$ (Hz)	ϕ^b
Backbone NH				
Ala (major component)	8.01	−6.1	7.5 to 8	50°, 70°, −90°, −150°
Gly[a] (major component)	8.12	−6.1	Σ = 11	±60°; ±130°
Arg	8.27	−5.9	7 to 7.5	40°, 80°, −85°, −155°
$CONH_2$ group				
syn-NH	6.96	−5.4		
anti-NH (major component)	7.10	−5.2		
anti-NH (minor component)	7.39	−5.9		

[a] This is the internal Gly residue. [b] This is the dihedral angle for rotation about the N-C^{α} bond, in the standard IUPAC-IUB nomenclature [(1970) *Biochemistry 9*, 3471]. For the hexapeptide in water at pH 5.3 at 30 °C, the following values were obtained: Ala ($J_{NH-C^{\alpha}H}$ = 6.1 to 6.5 Hz for ϕ = 30°, 90°, −80°, −160°); Gly(internal) ($\Sigma J_{NH-C^{\alpha}H}$ = 10.6 Hz for ϕ = ±55°, ±135°); and Arg ($J_{NH-C^{\alpha}H}$ = 7 Hz for ϕ = 40°, 80°, −85°, −155°).

TABLE III: Areas of Selected Peaks Present in the NMR Spectra of the Tri-, Tetra-, Penta-, and Hexapeptide in Me_2SO-d_6.

	Tripeptide		Tetrapeptide		Pentapeptide		Hexapeptide	
Proton	δ (ppm)	No. of protons	δ (ppm)	No. of protons	δ (ppm)	No. of protons	δ (ppm)	No. of protons
$CONH_2$ *syn*-NH	6.96	1.00	6.97	1.02	6.94	1.00	6.96	1.00
$CONH_2$ *anti*-NH (major component)	7.10	0.66	7.08	0.73	7.09	0.67	7.10	0.68
anti-NH (minor component)	7.40	0.29	7.40	0.36	7.39	0.29	7.39	0.32
$C^{\alpha}H$ Pro (major component)	4.28		4.29	0.71				
Pro (minor component)	4.37	0.28	4.46	0.31				
NH Ala (major component)	7.96	0.64						
Ala (minor component)	8.23	0.23						
$C^{\alpha}H$ Arg			3.24	0.99				
$C^{\delta}H_2$ Arg			3.04	2.01				

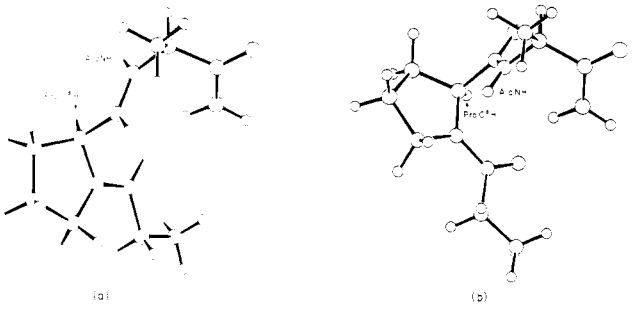

FIGURE 2: Drawings of the types II and I β turns, a and b, respectively, for H-Gly-Pro-Ala-NH_2.

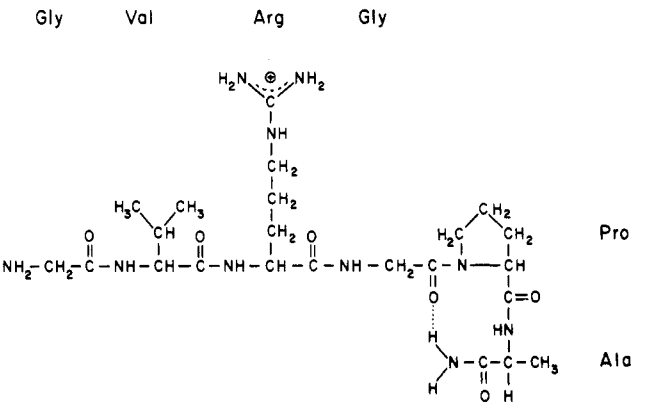

FIGURE 3: The primary structure of a fibrinogen-like hexapeptide showing a β turn.

of this type and has also been observed in the spectra of oxytocin (Urry et al., 1970) and lysine vasopressin (Von Dreele et al., 1971; Walter et al., 1972).

The temperature coefficients of Table II are mostly high (Kopple, 1971) but at least that for the proposed hydrogen bonded peptide hydrogen is the lowest for the molecule.

In summary, the peptides studied here appear to exist in two conformations with cis–trans isomerism about the Gly-Pro peptide bond. In *both* of these isomers, the NH proton which is syn to the C=O in the terminal $CONH_2$ group is in the same magnetic environment and hence exhibits the same chemical shift. However, the magnetic environment of the NH proton which is anti to the C=O differs for the two isomers. In the cis Gly-Pro isomer, this proton presumably is not involved in an internal hydrogen bond, but in the trans Gly-Pro isomer the formation of the i to i + 3 hydrogen bond (Figures 2 and 3) accounts for the upfield shift of its resonance.

The existence, then, of an i to i + 3 hydrogen bond suggests that the intervening residues form a bend conformation in which there are preferred values of ϕ_{Pro}, ψ_{Pro}, ϕ_{Ala}, and ψ_{Ala} (IUPAC-IUB Commission on Biochemical Nomenclature, 1970). We have assumed that all the peptide bonds are trans and planar and then sought values of these four dihedral angles which would be compatible with the NMR data. The following criteria were used to select discrete values of ϕ and ψ.

1. The values of ϕ_{Pro} considered were −50°, −60°, −70°, and −80°, which cover the range observed in x-ray structures of Pro residues (Momany et al., 1975).

2. Values of ψ_{Pro} were selected from the low-energy regions as calculated earlier (Gō & Scheraga, 1970): −80° to +15°, 65° to 180°; and −180° to −145°.

3. In accordance with the observed vicinal coupling constants (Bystrov et al., 1969; Bystrov, 1976), ϕ_{Ala} was taken to be ei-

ther 50°, 70°, −90°, or −150° and a range of ±15° was permitted around each value.

4. The conformational (ϕ, ψ) map for Ala (Lewis et al., 1973; Zimmerman et al., 1977) was used to obtain those values of ϕ_{Ala} and ψ_{Ala} which gave minimal conformational energies near the above values of ϕ. An additional region near (ϕ, ψ) = (−100°, 0°) was also included since this conformation occurs for several amino acids in lysozyme and myoglobin (Dickerson & Geis, 1969).

The values of ϕ were used in 10° steps, and ψ in 30° steps, to generate conformations for the C-terminal portion -Gly-Pro-Ala-NH_2, and those conformations were rejected which had (a) an Ala amide—N···O=C (Gly) distance outside the range of 2.5 to 3.2 Å, and/or (b) interatomic distances so short that repulsive energy of at least 10 kcal might be expected (Zimmerman et al., 1977). Those conformations that were retained fell into two separate categories—a type I or type II β turn (Venkatachalam, 1968; Zimmerman & Scheraga, 1977) as illustrated in Figure 2. In the type I structures, ψ_{Pro} = −20° and ψ_{Ala} had values of +40° to −20° (ϕ_{Ala} = −90°) giving rise to several structures with acceptable intramolecular hydrogen bonds. In the type II structures, $\psi_{Pro} \approx 130°$ with (ϕ, ψ) Ala = (60°, 30°).

We were able to select between these alternatives by means of NOE experiments on the tripeptide in Me_2SO-d_6 (Gibbons et al., 1975; Khaled & Urry, 1976; Leach et al., 1977). In the major, trans isomer, irradiation of the Pro $C^{\alpha}H$ signal produced a 10% increase in the area of the Pro-Ala peptide NH signal. The converse experiment produced a 9.8% increase in the area of the Pro $C^{\alpha}H$ signal. The irradiation of Ala NH in the type II β-turn structure (ψ_{Pro} = 130°) should produce a 22% increase in area of the Pro $C^{\alpha}H$ signal, taking into account that these two hydrogens are 2.16 Å apart, and one of the Pro C^{β} hydrogens contributes to the relaxation of the Pro C^{α} (see Appendix). By contrast, in the type I β turn (ψ_{Pro} = −20°) the Pro $C^{\alpha}H$ and Ala NH are 3.41 Å apart and only a 1.6% NOE is expected for this pair. Thus, the β turn cannot be type I. These calculations have been carried out with a computer program based on a treatment of the nuclear Overhauser effect in multispin systems (Noggle & Schirmer, 1971; Schirmer et al., 1970) which is described in the Appendix, and a listing for which is included in the supplementary material (see paragraph concerning supplementary material at the end of this paper). That the observed NOE is smaller than that calculated for the ideal type II β turn geometry suggests that ψ_{Pro} differs somewhat from the value of 130° given above.

The remainder of the chain in the hexapeptide is not well defined by these experiments, although the value of J_{gem} for Gly-4 in the hexapeptide indicates (Bystrov, 1976) that ψ = 0° or 180° for this residue. The latter value is consistent with a β-structure, and similar to the conformation of Gly in N-acetyl-Gly-Pro-N′-methylamide (Stimson et al., 1977) and in Boc-Gly-Pro-OH (Benedetti et al., 1976; Marsh et al., 1977).

The oligopeptides examined here are not the best substrates for the action of thrombin (the rates of hydrolysis are only 0.1% of those observed with fibrinogen), but we have identified a possible structure (Figure 3)—the type II β bend—close to the site of the Arg-Gly reaction site, which may help to account for the specificity of the enzyme–substrate interaction.

The free energy difference between cis and trans isomers about the Gly-Pro bond is obviously small, as evidenced by the 30:70 isomer ratio observed for these peptides in Me_2SO-d_6 solution. This is the normal situation for X-Pro peptides (Deslauriers & Smith, 1976; Grathwohl & Wüthrich, 1976; Stimson et al., 1977). Calculations show that small changes, e.g., in the dielectric constant of the solvent, can affect the free energy difference between the isomers and hence their relative abundance in solution (Zimmerman & Scheraga, 1976). The existence of a few percent of the cis form of the hexapeptide in water would not be detected in our experiments.

The change in the relative free energies of the isomers brought about, e.g., by change of solvent, is often enough to cause a substantial change in isomer ratio. For example, there is less of the cis isomer of *N*-acetyl-Gly-Pro-*N*′-methylamide present in water than in Me_2SO-d_6 (Stimson et al., 1977) and this change is in the same sense as that observed for the hexapeptide in the present work. Sometimes there will be a single isomer in the crystalline state, but an equilibrium mixture in solution. This is the case for sarcosylsarcosine (Howard et al., 1973) which is wholly cis in the crystal but shows a mixture of cis and trans isomers when dissolved in D_2O, due to small but important differences in conformational entropy between the two isomers in solution. Similarly, L-Pro-L-4-Hyp is cis in the crystalline state but in D_2O solution shows a 24:76 cis:trans ratio (Roques et al., 1977).

A recent example of a cis Gly-Pro bond is found in the crystal structure of Z-Gly-Pro-Leu (Yamane et al., 1976). Two homologues of this peptide, *p*-Br-Z-Gly-Pro-Leu-Gly (Ueki et al., 1969) and *o*-Br-Z-Gly-Pro-Leu-Gly-Pro (Ueki et al., 1971), have trans Gly-Pro linkages, however, and they are stabilized in type I β turn conformations by Gly^1 CO/Gly^4 NH hydrogen bonds. Thus, the conformations in the crystalline states are similar (in the sense of having a β turn) to those proposed for the fibrinogen-like peptides in this work.

The preferred structure of the peptides reported here, with the i to i + 3 hydrogen bond and the type II β turn, is possible only when the Gly-Pro bond is trans, and must be lost in the cis isomer when isomerization occurs about the peptide bond. When we consider this in the light of Brandts' proposal (Brandts et al., 1975, 1977), we note that establishment of a mixture of cis and trans isomers might well be accompanied by the loss of a preferred structure in the one isomer, but not necessarily in the other.

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Supplementary Material Available

The computer program for relating NOE to conformation of peptides (5 pages). Ordering information is given on any current masthead page.

Appendix: Use of Nuclear Overhauser Effect in Conformational Studies of Oligopeptides

The intramolecular nuclear Overhauser effect (NOE) is often employed to elucidate the stereochemistry of organic molecules in solution (Noggle & Schirmer, 1971; Bell & Saunders, 1973), and we have followed the usual procedure involving dilute solutions in deuterated solvents containing only small amounts of tetramethylsilane and removal of dissolved oxygen by flushing with nitrogen gas.

Most of the reported applications are qualitative in the sense that the observed NOE is not converted to an internuclear distance, but rather used to infer the proximity of two nuclei and thus permit a choice between alternative stereochemical proposals. In a more quantitative approach (Schirmer et al., 1970), the ratios of observed NOE's were found to correlate

quite well with ratios of internuclear distances, and a plot of observed NOE against the sixth power of internuclear separation was found to be linear (Saunders & Bell, 1970).

It was some time before this relationship between NOE and internuclear distance was applied to problems of peptide conformation (Gibbons et al., 1975; Khaled & Urry, 1976), and the wider applicability of such applications was realized (Leach et al., 1977). Some questions must arise concerning the constant of proportionality between NOE and the inverse sixth power of the internuclear distance which was derived by Saunders & Bell (1970) for similar compounds in $CDCl_3$ (and in one case, in C_6D_6) solutions. Indeed, these authors note (Bell & Saunders, 1973): ". . . the approximation of considering G a constant and comparing it with the two-spin system is a gross one with little theoretical justification . . . [however] . . . despite being suspect theoretically, it appears to be a good approximation experimentally." In our earlier work (Leach et al., 1977; Rae et al., 1977), we have used the same "constant" for oligopeptides in Me_2SO-d_6.

For the present work we have followed the treatment for multispin systems given by Noggle & Schirmer (Schirmer et al., 1970), using standard geometry (Momany et al., 1975) and appropriate dihedral angles for internal rotation, and combining these in a single computer program. The program requires the specification of the dihedral angles ($\phi, \psi, \omega, \chi^1$, etc.), and then uses the generator of the ECEPP program (Quantum Chemistry Program Exchange, Chemistry Department, Indiana University, Bloomington, Ind., Order No. ACPE 286) to construct the peptide model. The hydrogen whose signal is to be *observed* in the NOE experiments is nominated, and the program then lists all hydrogens within a chosen distance of it. We have found 4.0 Å to be a suitable distance, but this parameter is adjustable. Then the equation for calculating the expected effect for irradiation of each of the nearby hydrogens in this conformation (Schirmer et al., 1970; eq 6) is used, with the assumption that relaxation of the nominated hydrogen is achieved solely through dipole–dipole interactions with its neighbors.

This gives a somewhat more accurate estimate for the NOE expected in the $C^{\alpha}H_{i+1}/NH_{i+2}$ system than did the approximate treatment described in our earlier work (Leach et al., 1977; Rae et al., 1977).

One final cause for concern is the molecular correlation time of the solute and its effect on observed NOE. Negative NOE's in simple spin systems, i.e., not arising from three-spin effects, have been observed for gramicidin S (Gibbons et al., 1975; Rae et al., 1977), valinomycin (Glickson et al., 1976) and vancomycin (Williams & Kalman, 1977), and their nature has been explained in terms of slow molecular tumbling, i.e., long correlation time, of the solute (Glickson et al., 1976; Williams & Kalman, 1977). For small molecules such as the tripeptide reported in this work, and the the tetra-, penta-, and hexapeptides of Khaled & Urry (1976), this is not a problem, but for a larger molecule the NOE is strongly dependent on the molecular correlation time and negative "enhancements" may be observed. Thus, there is a small range of correlation times where the NOE changes sign. The critical correlation times are smaller for higher magnetic field strengths (Glickson et al., 1976) and precisely this combination of circumstances is commonly found with the larger molecules where high-field spectrometers are used to give adequate dispersion in the spectrum. If the correlation time is known, the observed NOE values may be corrected for the effects of slow molecular tumbling, and approximate values for internuclear distances may be derived. We have done this for gramicidin S (Rae et al., 1977), but the combination of circumstances required for the success of this procedure suggests that it will be of limited applicability.

We have also considered the possibility that, at the very high field strengths normally used for these experiments, hydrogen relaxation may not be exclusively dipole–dipole. This is certainly the case for ^{13}C nuclei (Lyerla & Levy, 1974) but, at least up to 100 MHz, ^1H relaxation is thought to be exclusively dipole–dipole (Berry et al., 1977).

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Effects of Shearing on Chromatin Structure†

Rose A. Maciewicz* and Hsueh Jei Li

ABSTRACT: The effects of mechanical shearing on chromatin structure were investigated by using thermal denaturation and circular dichroism (CD) spectroscopy. Under ordinary conditions of mechanical shearing used for preparation of soluble chromatin, we observed only minor changes (less than 10%) of chromatin properties with respect to (a) absorption melting curves, (b) CD spectra, (c) CD melting curves and (d) histone transfer from chromatin to exogenous DNA. Such small perturbation of structural properties could be due to the generation of free ends when a large chromatin was cut into smaller fragments and by weakening the binding of histones to DNA near these free ends. In addition to mechanical shearing, sonication was used to shear some samples of chromatin. The effect of sonication on chromatin structure was investigated by the same physical methods used for mechanically sheared chromatin. The results indicate that sonication only slightly changes the chromatin properties with respect to CD spectra, similar to the results obtained by mechanical shearing, but sonication at high settings has a greater effect on the thermal denaturation property of chromatin as contrasted to our results from mechanically sheared chromatin.

Mechanical shearing of chromatin has been used as a method for isolating soluble chromatin for structural studies (Bonner et al., 1968) as well as fractionation of chromatin into components for transcriptional studies (Frenster et al., 1963; Chalkley & Jensen, 1968; McCarthy et al., 1973; Gottesfeld et al., 1974; Simpson, 1974). Noll et al. (1975) reported disruption of chromatin structure after mechanical shearing as judged by nuclease digestion patterns. Circular dichroism (CD) spectroscopy (Nicolini et al., 1976) as well as CD and thermal denaturation properties (Miller et al., 1976) of chro-

† From the Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14260. *Received March 18, 1977; revised manuscript received November 16, 1977.* Supported by National Science Foundation Grant PCM 76-03268. The late H.J.L. was a recipient of a Career Development Award from the National Institutes of Health, GM 00262.

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