REVERSE TURNS IN PEPTIDES AND PROTEINS

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I. INTRODUCTION

Reverse turns are structural features of peptides and proteins, involving three (i.e., a γ-turn)[1,2] or four (i.e., a β-turn)[3] consecutive residues, hallmarked by the folding back on itself of the peptide chain and by the presence of an intramolecular hydrogen bond. Since their occurrence in globular proteins accounts for about one third of the residues in these molecules,[4,5] as well as a substantial proportion of the surface residues,[6] it is likely that certain reverse turns provide recognition sites for the initiation of complex immunological, endocrinological, or metabolic reactions. The identification of reverse turn conformations in various peptide hormones is also compatible with this suggestion.

Further, globular protein chains, themselves linear polymers, are known to fold into low energy conformations.[7] In order to achieve these conformations, which result in the formation of compact globular proteins, the chain frequently changes direction during the process of folding. The regions in which this change of direction occurs are reverse turns. Further, it has been proposed that reverse turns are the nucleation sites of protein folding,[8] and, as discussed in Section V, the conservation of the location of reverse turns, although not of amino acid sequence, among homologous proteins may be the result of such a crucial role of reverse turns in governing the initiation of protein folding.[9]

The presence of a β-turn structure was initially proposed to explain the C_2 symmetry of the cyclic decapeptide, gramicidin S, determined by X-ray diffraction,[10] as well as the ease of cyclization of this peptide during synthesis.[11] Additional proof for the existence of β-turn structures determined by various experimental techniques was as follows:

1. Infrared spectroscopy detected the concentration dependence of several blocked, linear tetrapeptides in nonaqueous solution, which indicated the localization of an intramolecular hydrogen bond in these peptides.[12,13]
2. X-ray diffraction patterns of Chysopa silk provided the first atomic coordinates for a β-turn structure.[14]

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3. Nuclear magnetic resonance (NMR) indicated by various experimental approaches that certain cyclic oligopeptides contained β-turns.[3,15-17]

The initial theoretical analysis of β-turn conformations was completed using hard-sphere computation.[18] Subsequently, theoretical circular dichroism (CD) calculations were used to predict characteristic spectral shapes and magnitudes of CD spectra attributable to β-turns.[19]

Since the time of these signal advances in our understanding of the structure and function of reverse turns in peptides and proteins, a prodigious expansion of the literature dealing with this conformational feature has occurred. The goal of this review is to assess critically this literature. Since much more is known about β-turns than γ-turns, due emphasis will be placed on β-turns. It should be stated that our knowledge of reverse turns is not complete, and, although this review purports to give a summary of what is known, many details regarding the structural and functional roles of reverse turns remain to be learned.

II. DESCRIPTION AND NOMENCLATURE OF REVERSE TURNS

The authors are aware that the nomenclature in a given field should be formulated by a commission or a consensus of those individuals working in the field, and they do not wish to supplant such a consensus. Rather, the authors hope that the nomenclature proposed herein will provide a working terminology which may be found useful and acceptable.

Reverse turns are considered as two different intramolecularly hydrogen-bonded arrangements of a polypeptide chain: one in which hydrogen bonding occurs between the C=O of residue i (i.e., the first residue of a turn) and the N–H of residue i + 3 (i.e., the residue located three residues towards the carboxyl terminus), and another in which hydrogen bonding occurs between the C=O of residue i and the N–H of residue i + 2. The former will be referred to as a β-turn and the latter as a γ-turn. To aid readers in the identification of previous descriptions dealing with the β-turn conformation, the authors point out that β-turns have been called: 3_{10} (or 3-10) bend,[6,18] 4-1 intramolecularly hydrogen-bonded nonhelical conformation,[18,20] $N_4H_4\cdots O_1C_1$ hydrogen-bonded conformation,[18,21] folded β-conformation (or β-fold),[13] β-turn,[3] β-bend,[8,22] hairpin bend,[6,22] corner,[6] β-loop,[6] 1-4 bend,[1] chain reversal,[23] reverse turn,[5] U-bend,[22] 10-membered hydrogen-bonded ring,[24] 1,4 turn,[25] β-twist, [20] and tight turn.[26] Further, the authors' definition of a γ-turn is a modification of the initial description in which two intramolecular hydrogen bonds ($N_1H_1\cdots O_3C_3$ and $N_3H_3\cdots O_1C_1$ were proposed.[1] However, with regard to currently studied γ-turn-containing peptides, the requirement for a single intramolecular hydrogen bond is a more appropriate distinction.[27,28]

The requirement that a true reverse turn contain an intramolecular hydrogen bond leads to a more restricted definition than has been used previously.[5,23] The authors will use the term "open reverse turn" for those instances when a peptide chain changes direction by 180° without concomitant intramolecular hydrogen-bond formation.[5]

Various arrangements of a polypeptide chain into β- and γ-turns are shown diagrammatically in Figure 1. The authors will adhere to Venkatachalam's original nomenclature for classifying β-turns into conformational types: I, II, III and their mirror images, I′, II′, III′. These types are defined in terms of ϕ, ψ angles in Table 1.

Two additional nomenclatures for β-turn types have been introduced. Both were based on results of conformational energy calculations as opposed to hard sphere computations used by Venkatachalam.[18] Lewis et al.[23] retained the three β-turn conforma-

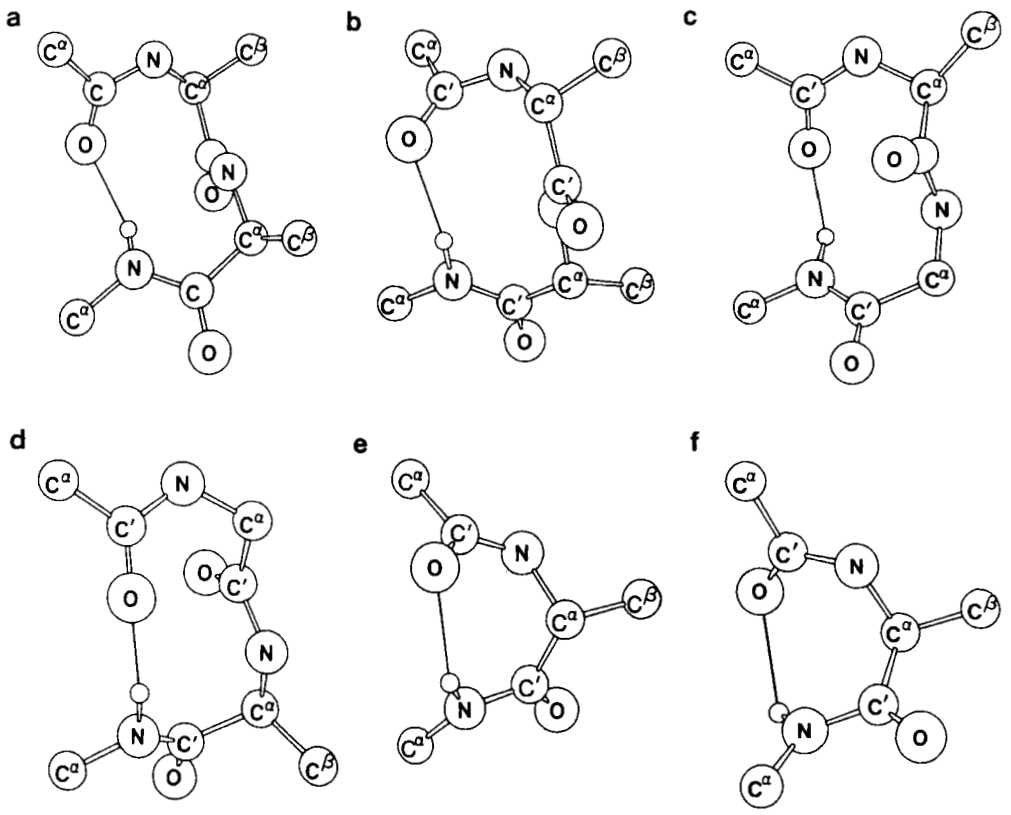

FIGURE 1. Diagrams of structures of various reverse turns. Only those hydrogens involved in hydrogen bonding are shown. Side chain (C^β) carbons are shown in those positions which are considered to be preferred for L-residues according to Venkatachlam[18] (β-turns). (a) Type I β-turn; (b) Type I′ β-turn; (c) Type II β-turn; (d) Type II′ β-turn; (e) γ-turn; (f) inverse-γ-turn. Types III and III′ β-turns, which are parts of 3_{10} helices, are not illustrated because they closely resemble types I and I′, respectively.

Table 1
DIHEDRAL ANGLES OF β-TURN TYPES

	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+1}	ψ_{i+2}
Type I	−60	−30	−90	0
(Type I′)	60	30	90	0
Type II	−60	120	80	0
(Type II′)	60	−120	−80	0
Type III*	−60	−30	−60	−30
(Type III′)	60	30	60	30

* Actually a 3_{10}-helix.

tional types I, II, and III, but they also posited four more types. Their expansion of "type" notation, based on an approximate range of dihedral angles, is an artificial

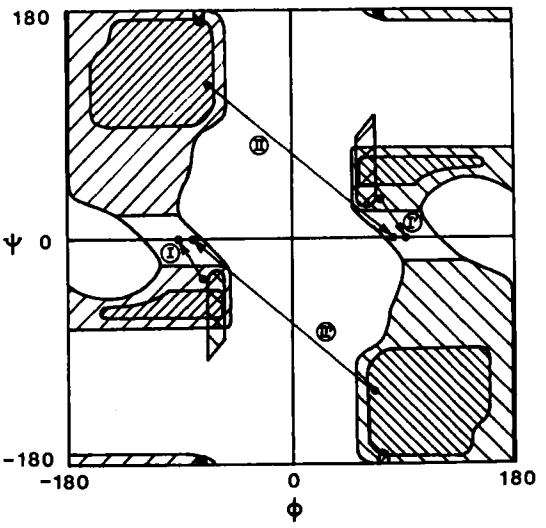

FIGURE 2. ϕ,ψ map showing β-turn conformations superimposed on sterically allowed regions for L- and D-residues[29] (see key below). Examples of specific β-turns are indicated by arrows originating at the ϕ,ψ angles of residue i + 1 and ending at the ϕ,ψ angles of residue i + 2. Note that, in general, Type I turns are L-L preferred; Type II, L-D; Type I′, D-D, and Type II′, D-L. Note that residues in both positions i + 1 and i + 2 of type III or III′ turns have the same ϕ,ψ values as i + 1th position for type I or I′ turns, respectively. Type III turns are therefore in general L-L preferred and type III′ D-D. ▨, Fully allowed for L-residue; ▧, fully allowed for D-residue; ▨, partially allowed for L-residue; ▧, partially allowed for D-residue.

(and somewhat confusing) distinction among reverse turn conformations. Chandrasekaran et al.[22] arrived at dihedral angles for reverse turns similar to those of Venkatachalam,[18] but they introduced a "region" notation, which is now rarely referred to in the literature. There is no reason to perpetuate either of these notations. However, to facilitate comparisons between the treatments of Venkatachalam[18] and Chandrasekaran et al.,[22] their correspondence is given below. The dihedral angles are presented as (ϕ_{i+1}, ψ_{i+1}, ϕ_{i+2}, ψ_{i+2}).

1. Type I ca. (−60, −30; −90, 0) corresponds to: Region Ia (−80 to −20, −90 to −10; −150 to −70, 10 to 80)
2. Type II ca. (−60, 120; 80, 0) corresponds to: Region Ib (−80 to −30, 80 to 140; 20 to 80, 10 to 70) and Region III (−90 to −30, 70 to 160; 30 to 170, −70 to 80)
3. Type I′ ca. (60, 30; 90, 0) corresponds to: Region IIa (20 to 80, 10 to 90; 70 to 150, −80 to −10)
4. Type II′ ca. (60, −120; −80, 0) corresponds to: Region IIb (30 to 80, −160 to −70; −80 to −20, −70 to −10) and Region IV (30 to 90, −160 to −70; −170 to −30, −80 to 70)

Because of the steric requirements of the individual types of β-turn, type I is generally considered as L-L preferred and type II as L-D preferred.[18,22] It follows then that the mirror imaged β-turn types I′ and II′ would be D-D and D-L preferred, respectively. A glycyl residue can be accommodated in any position of any β-turn. The basis for the preference for certain sequences of amino acids in turns can be conveniently dis-

Table 2
DIHEDRAL ANGLES OF THE i + 1th RESIDUE OF γ- AND INVERSE* γ-TURNS

γ-turn	70 to 85	−60 to −70
Inverse γ-turn	−70 to −85	60 to 70

* Approximate ranges from calculations[1] and experimental values.[2,27,28]

played on a Ramachandran plot. In Figure 2 are plotted the regions of dihedral angles allowed to an L- or D-amino acid (containing a C^{β} atom) in a peptide, according to Ramachandran and Sasisekharan.[29] Superimposed on these regions are the dihedral angles for residues i + 1 and i + 2 of the various β-turn types, which are connected by arrows. From Figure 2, it can be seen that an L-residue can occur in position i + 2 of a type II β-turn, although the region of conformational space available to it is smaller than for an L-residue in the i + 2 position of a type I β-turn. This is noted in order to emphasize that the generalities about the preferred sequence of β-turns are not exclusive of other sequence possibilities.

As for the γ-turn, there is one conformation, as well as its mirror image, which leads to i + 2 → i hydrogen bond formation. The dihedral angles for the i + 1 residue, the amino acid which determines the existence of an intramolecular hydrogen bond, are listed in Table 2. Furthermore, chain reversal requires that ψ_i and ϕ_{i+2} are approximately +120°, −120°, respectively, for the γ-turn or −120°, +120°, respectively, for the inverse γ-turn. The two γ-turn types have been called γ-turn[1,2] and inverse γ-turn.[2] They have also been called axial and equatorial γ-turns.[30] The latter terms lead to confusion in discussions of turns with differing configurations of the i + 1 residue. Therefore, the authors suggest that the original terms offer a notation which is straightforward and consistent with the actual relationship between the turn geometries (i.e., inverse symmetry), and the terms γ-turn and inverse γ-turn will be used throughout this review. In Figure 3 the dihedral angles for a γ-turn are plotted on a Ramachandran plot. Note that the γ-turn dihedral angles fall into a large region allowed for a D-residue. Analogously, the inverse γ-turn dihedral angles are in a large region of conformational space permitted for an L-residue.

III. REVERSE TURNS IN PEPTIDES

As the number of well-documented examples of reverse turns — notably β-turns — is still increasing rapidly, a three-fold purpose can be served by reviewing critically the methods of analysis and the specific experimental findings (e.g., unusual amino acid sequences or conformational parameters) reported in studies of reverse turn-containing compounds. First, the strategy and tactics for identifying and characterizing reverse turns, which occur in peptides and proteins, will emerge. Second, general conclusions can be drawn about the likelihood of occurrence of particular turns under different experimental conditions or with different amino acid sequences. Third, as more examples of reverse turns are established, it should become possible to hypothesize on the functional roles of reverse turns (see Section V).

The authors have organized this section by first treating examples of reverse turns in peptides, subdivided according to the method of analysis used to examine the particular peptides. Indeed, many of the peptides in which turns have been identified are of interest in terms of their biological functions. For example, hormones, antibiotics, and

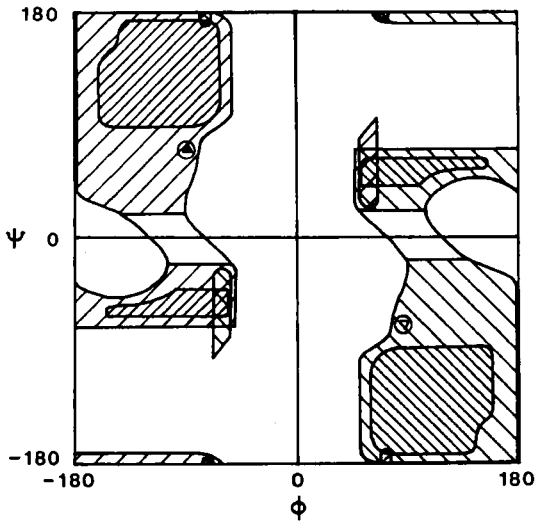

FIGURE 3. ϕ,ψ map showing conformations of residue i + 1 in γ-(▽) and inverse γ-(▲) turns superimposed on sterically allowed regions for L- and D-residues.[29] (See Figure 2 Legend). Note that, in general, γ-turns are D preferred, and inverse γ-turns are L preferred.

neural messengers count among the peptides which have been attributed reverse turn-containing conformations. By necessity, special reference will be made to synthetic model peptides which contain reverse turns, since such model peptides serve as reverse-turn standards for the various physical methods and provide clear-cut conformational parameters necessary for the interpretation of reverse-turn conformations in naturally occurring peptides (or proteins). In particular, cyclic peptides will be discussed since the majority of the unequivocal structural information currently available regarding the occurrence of reverse turn-containing peptides as assessed by various physical methods has resulted from experimental studies involving cyclic peptides. The use of conformational energy calculations to predict the occurrence of reverse turn conformations is also discussed.

A. X-ray Diffraction Evidence for Reverse Turns in Peptides

X-ray diffraction analysis of peptide crystal structures provides the only unequivocal determination of peptide conformation. The state-of-the-art of X-ray crystallography of reverse turn-containing peptides has been reviewed by Karle[28] and by Benedetti.[31] Particularly important are the methods of "direct phase" X-ray analysis[32] (i.e., determination of phases directly from X-ray intensities) which have allowed the determination of peptide structure without the necessity for the preparation of heavy atom derivatives or the requirement for additional structural information. Since the first successful application of this technique to a reverse turn-containing peptide, cyclo-$(Gly)_6$,[32] many other reverse turn-containing peptides have been identified, although the largest peptide studied, valinomycin,[32,34] contains only 156 atoms.

It should be emphasized that the structure (or structures, in some cases[32]) observed in a crystal is (are) of lowest energy only in the given crystal environment and that this environment may be dominated by intermolecular interactions. Only rarely will the crystal conformation of a peptide be identical to its solution conformation, as observed by other physical techniques. Nonetheless, the molecular conformation(s) observed for a peptide in a crystal is (are) likely to be among the possible low energy structures for

the particular peptide in other environments (e.g., membrane receptors). It is for this important reason that due emphasis is placed on the X-ray diffraction data contained in Table 3.

Tabulated in Table 3 are (1) the amino acid sequence of the i + 1 and i + 2 residues of each β-turn (or the i + 1 residue of each γ-turn), (2) the dihedral angles (ϕ,ψ) of each residue, (3) the intramolecular hydrogen bond length of each reverse turn-containing peptide, and (4) the torsional angle (ω) of the peptide bond between the i + 1 and i + 2 residues of each γ-turn. The data are grouped according to the type of reverse turn, as defined by Venkatachalam[18] and Matthews.[2]

Three conclusions can be drawn from the data in Table 3. First, the dihedral angles determined experimentally by X-ray diffraction for β-turn-containing peptides agree closely with the predicted values. The mean values and standard deviations for the dihedral angles of the two most frequently occurring β-turn types, types I and II, as determined from X-ray diffraction are listed below along with the dihedral angles, predicted by Venkatachalam.[18]

		Dihedral Angles (degrees)			
Type I		ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
	X-ray data (9 examples)	−63 ± 8	−27 ± 10	−96 ± 12	+4 ± 10
	Predicted[18]	−60	−30	−90	0
Type II					
	X-ray data (10 examples)	−61 ± 5	+128 ± 6	+85 ± 10	+2 ± 9
	Predicted[18]	−60	+120	+80	0

Further, the mirror-image β-turn types, I′ and II′, have dihedral angles with equal magnitude and opposite sign to types I and II, respectively. Second, the amino acid sequences and configurations occurring in each β-turn type are those expected based only on the areas of conformational space accessible to successive residues of individual turn types (*vide supra* and see Section III.E.): L-L in type I, D-D in type I′ (with one exception[43]), L-D in type II, and D-L in type II′. Furthermore, glycyl residues are located in various β-turn positions, although there are no examples of glycyl residues occurring at the i + 1^{th} of a type II β-turn or at the i + 2^{th} of a type II′ β-turn. Finally, amino acids of the D-configuration or Me_2 Gly (methyl groups replacing the two glycyl α protons) occur only in γ-turns, while amino acids of the L-configuration occur in inverse γ-turns. In addition, the deviation from planarity (i.e., ω = 180° for the *trans* peptide bond) of the peptide bond between the i + 1^{th} and the i + 2^{th} is probably characteristic of the γ-turn conformation.

B. Nuclear Magnetic Resonance (NMR) Evidence for Reverse Turns in Peptides

Foremost among the methods which have been used to determine the conformations of peptides in solution is NMR, in particular ^{1}H-NMR and ^{13}C-NMR. The amount of information available from NMR spectra is great, and seems to be increasing as additional theoretical and experimental correlations relating peptide conformation to various NMR parameters are established (e.g., nuclear Overhauser enhancement [NOE] and heteronuclear coupling constant geometric dependences). In the following discussion the aspects of NMR spectroscopy of particular importance to the elucidation of reverse turns are emphasized. The reader is referred to any of the general texts dealing with the NMR of peptides for additional information.[57-60]

1. Determination of Solvent Exposure of Amide (N-H) Protons

The most important role served by ^{1}H-NMR to date in the analysis of reverse turn-containing peptides has been the identification of peptide backbone N-H protons

Table 3
REVERSE TURNS IN PEPTIDES DETERMINED FROM X-RAY DIFFRACTION[a]

A. β-Turn-Containing Peptides

β-turn Type	Peptide	Sequence		Dihedral angles (degrees)[b]				NH···OC (Å)[c]	Ref.
		i + 1	i + 2	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}		
1. Type I	Cyclo-(Gly)$_6$	Gly	Gly	−69	−30	−94	+11	2.96	32
	S-benzyl-L-Cys-L-Pro-L-Leu-Gly-NH_2	L-Pro	L-Leu	−66	−29	−115	+13	—	35
	p-BrCbz-Gly-L-Pro-L-Leu-Gly-OH	L-Pro	L-Leu	−58	−27	−104	+8	2.96	36, 37
	o-BrCbz-Gly-L-Pro-L-Leu-Gly-L-Pro-OH	L-Pro	L-Leu	−65	−27	−105	+8	3.00	38
	N-Ac-L-Pro-L-Lac-$NHCH_3$	L-Pro	L-Lac	−55	−22	−81	−11	2.89	39
	Cyclo-(Gly$_4$-D-Ala$_2$)	Gly	Gly	−70	−15	−117	+16	3.04	40
	Li-antamanide	L-Ala	L-Phe	−69	−13	−84	−6	3.06	41
		L-Phe	L-Phe	−79	−13	−90	+8	3.00	
	Boc-L-Pro-Aib-L-Ala-Aib-OBz[d]	L-Pro	Aib	−56	−35	−52[d]	−38[d]	2.97	42
		Aib	L-Ala	−52	−38	−93	−13	3.09	
	Cyclo-(L-Ala$_2$-Gly$_2$-L-Ala-Gly)	L-Ala	L-Ala	−53	−43	−84	0	2.92	43
	Cyclo-(L-Ala$_2$-Gly-L-Ala-Gly$_2$)	L-Ala	L-Ala	−62	−33	−95	+14	3.10	43
2. Type I′	Cyclo-(Gly)$_6$	Gly	Gly	+69	+33	+92	−7	2.96	32
	Cyclo-(Gly$_4$-D-Ala$_2$)	D-Ala	D-Ala	+66	+15	+131	−31	3.16	40
	Cyclo-(L-Ala$_2$-Gly-L-Ala-Gly$_2$)	L-Ala	Gly	+54	+38	+91	−6	2.99	43
	[Leu^5]-enkephalin	Gly	Gly	+59	+25	+97	−7	2.99	44
3. Type II	Ferrichrome A	L-Ser	Gly	−57	+132	+82	−1	2.98	45
	H-L-Pro-L-Leu-Gly-NH_2	L-Leu	Gly	−61	+128	+72	—	3.04	46
	N-Ac-L-Pro-D-Lac-$NHCH_3$	L-Pro	D-Lac	−62	+140	+91	−8	2.97	47
	Valinomycin	L-Val	D-Hylv	−63	+129	+96	−3	3.07	42
		L-Val	D-Hylv	−67	+130	+82	+3	2.90	
	Valinomycin-K^+	L-Val	D-Hylv	−58	+132	+79	+3	2.93	34
	Cyclo-(L-Ala-L-Pro-D-Phe)$_3$	L-Pro	D-Phe	−60	+122	+78	+9	3.20	48
	Cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro)	L-Pro	Gly	−52	+126	+74	+12	2.87	49, 50
	Cyclo-(Gly-L-Pro-D-Ala)$_2$	L-Pro	D-Ala	−54	+125	+94	−5	3.04	51
		L-Pro	D-Ala	−70	+116	+79	+19	3.42	
	Cyclo-(L-Val-D-Pro-D-Val-L-Pro)$_3$	L-Val	D-Pro	−64	+131	+83	+6	3.0	52
	Cyclo-(Gly-L-Pro-D-Phe)$_2$	L-Pro	D-Phe	−64	+132	+106	−14	3.52	53

4. Type II′	Valinomycin	D-Val	L-Lac	+63	−134	−74	−6	2.86	42
		D-Val	L-Lac	+60	−135	−98	+14	2.99	
	Valinomycin-K[a]	D-Val	L-Lac	+58	−131	−72	+18	2.93	34
	Cyclo-(L-Val-D-Pro-D-Val-L-Pro)$_3$	D-Val	L-Pro	+58	−130	−78	−5	3.0	52
	Cyclo-(L-Ala$_2$-Gly$_2$-L-Ala-Gly)	Gly	L-Ala	+84	−113	−106	−9	3.35	43
	Cyclo -(Gly-L-Pro-L-Ser-D-Ala-L-Pro)	Gly	L-Pro	+58	−128	−75	−20	3.04	54
	Cyclo-(D-Phe-L-Pro-L-Val)$_2$	D-Phe	L-Pro	+60	−134	−86	−15	3.40	55
	Cyclosporin	MeGly	MeLeu	+56	−137	−112	+53	3.21	56

B. γ- and Inverse γ-Turn-Containing Peptides

Turn type	Peptide	i + 1[d] Residue	Dihedral angles (degrees) ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	NH···OC (Å)[c]	Ref.
1. γ-turn	dihydrochlamydocin	D-Pro	+83	−73	+156	2.94	28
		Me$_2$Gly	+72	−64	+162	2.82	
2. Inverse γ-turn	cyclosporin	L-Ala	−92	+64	+180	3.01	56
	cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro)	L-Pro	−86	+70	−160	2.92	49, 50

[a] Abbreviations: Ac, acetyl; Aib, α-aminoisobutyric acid; BrCbz, bromocarbobenzoxy; Hylv, hydroxyisovaleric acid; Lac, lactic acid; MeGly, *N*-methylglycine (sarcosine); MeLeu, *N*-methyl leucine; Me$_2$Gly, dimethylglycine.

[b] Designation of bonds and definition of the principal torsion angles is according to the IUPAC-IUB Commision on Biochemical Nomenclature;[41] ϕ denotes the rotation about N-C$^\alpha$, ψ denotes the rotation about C$^\alpha$-C, and ω denotes the rotation about C-N. i denotes the first residue of a reverse turn with successive numbering from the amino-terminal to the carboxyl-terminal end of the chain (e.g., i + 1, i + 2, etc.).

[c] NH···OC: hydrogen bond length (i.e., N to O distance) — i + 3 → i in β-turns and i + 2 → i in γ-turns (or inverse γ-turns). Dashes indicate that no value was reported.

[d] Actually a 3_{10} helix (i.e., a type III β-turn), since i + 2[th] residue of the first β-turn is also the i + 1[th] residue of the next turn. This leads to a large discrepancy of the (ϕ_{i+2}, ψ_{i+2}) of the first turn from expected type I dihedral angles.

which participate in intramolecular hydrogen bonding. The interpretations of ^{1}H-NMR N-H proton data have been based on a number of experiments designed to assess the accessibility of these protons to interact with bulk solvent or other peptide molecules intermolecularly. The premise then routinely invoked is that any N-Hs not exposed to these intermolecular interactions are likely to be involved in intramolecular hydrogen bonding.

A list of possible experiments which are variations on this theme include: (1) temperature dependence of N-H chemical shifts ($\Delta\delta/\Delta T$); (2) solvent dependence of N-H chemical shifts; (3) concentration dependence of N-H chemical shifts in nonpolar solvents; (4) deuterium exchange kinetics ($\tau_{1/2}[^1H-^2H]$); and (5) sensitivity of N-H chemical shift or line-width to addition of reagents which act as perturbants. Each of these approaches suffers to varying degrees from the underlying problem that any alteration of an experimental variable (e.g., temperature, solvent, etc.) required for the assessment of N-H exposure may appreciably alter the conformation or the distribution among conformations, if more than one conformation co-exists, of the peptide.

The first approach, temperature dependence of N-H chemical shift, has been the most frequently employed and, provided certain limitations are acknowledged, can be an extremely straightforward and powerful indicator of the participation of N-Hs in intramolecular interactions. In general, use of a solvent which acts as a hydrogen bond acceptor (most commonly, dimethyl sulfoxide-d_6 [Me_2SO-d_6] or water) facilitates the interpretation of observed shifts. The premise of such an experiment is that the existence of intermolecular hydrogen bonding is signaled by a large temperature dependence of the resonance of the proton involved in the hydrogen bond. This dependence reflects the perturbation of the equilibrium between a free proton and its hydrogen-bonded states with increasing temperature. The N-H resonance is shifted to a high-field position with increasing temperature as a consequence of the low-field position of a hydrogen bonded N-H resonance. This discussion applies both to peptide-peptide and peptide-solvent intermolecular hydrogen bonding. A small temperature dependence suggests either intramolecular hydrogen bonding or a "buried" (i.e., unexposed to solvent) N-H, which does not participate in intramolecular hydrogen bonding. In either case, little or no temperature dependence would be expected since the N-H in question is not involved in an equilibrium between bound and free states. In solvents which are weak hydrogen bond acceptors, the same arguments can be presented, although interactions of peptide molecules with one another must be occurring in order to cause exposed N-Hs to undergo temperature-dependent chemical shift changes, since any intermolecular hydrogen bonds between peptide molecules and solvent are weak if present. Hence, it is advisable to ensure that the peptide concentration is high enough to lead to peptide-peptide interactions before attempting to interpret $\Delta\delta/\Delta T$ values in nonpolar or weakly interacting solvents (*vide infra*). Representative data for $\Delta\delta/\Delta T$ of N-Hs of a few well-studied peptides in various solvents are presented in Table 4.

There are several intrinsic problems involving the interpretation of the experimental data from this type of experiment:[61]

1. It is difficult to differentiate whether a low $\Delta\delta/\Delta T$ is due to the involvement of an N-H proton in an intramolecular hydrogen bond or to the inaccessibility of the N-H proton to bulk solvent (see alumichrome data in Table 4[72]).
2. There are no absolute thresholds of $\Delta\delta/\Delta T$ values below which hydrogen bonding or inaccessibility of a N-H proton is unequivocal.
3. There is no available experimental method for determining the effect of reorientation of magnetically anisotropic groups on chemical shift and hence on $\Delta\delta/\Delta T$.
4. Conformational averaging can cause misleading results.[73]

Table 4
DETERMINATION OF INTRAMOLECULAR HYDROGEN BONDING IN REVERSE-TURN-CONTAINING PEPTIDES[a,b,c]

Peptide	Δδ/ΔT (×10, ppm/deg)	δ(solvent) (ppm)	$\tau_{1/2}(^1H-^2H)$	Δδ/Δ conc (ppm)	$\Delta\nu_{1/2}$/Δ nitroxide (Hz)	Ref.
N-methylacetamide	−5.9[d]	1.3[f]				3,16,61
	−10.9[e]					
Gramicidin S						
D-Phe	−7.7[e]	9.15,[d] 8.9,[e] 7.8[g]	5—10 min[e]			3,15,62,63
L-Val	−1.8[e]	7.2,[d] 7.7,[e] 7.9[g]	15 d[e]			
L-Orn	−7.3[e]	8.7,[d] 8.7,[e] 7.6[g]	30 min[e]			
L-Leu	−3.2[e]	8.35,[e] 8.8,[e] 8.9[g]	15 d[e]			
Cyclo-(D-Phe-L-Pro-L-Ala)$_2$						
D-Phe	−6.8[d]	8.42,[d] 7.34[h]				64
L-Ala	−1.7[d]	6.96,[d] 6.96[h]				
Cyclo-(L-Ala-L-Pro-Gly)$_2$						
L-Ala	−0.8[d]					65
Gly	−6.2[d]					
Cyclo-(Gly-L-Pro-L-Ala)$_2$						
Gly	−0.1[d]	7.45,[d] 7.81[h]				65
L-ala	−7.5[d]	8.28,[d] 7.92[h]				
Valinomycin-K[+]						
L-Val	−2.5[h]	8.3,[e] 8.28[h]				3,66
D-Val	−2.5[h]	8.4,[e] 8.4[h]				
Cyclo-(Gly^1-L-Pro-Gly^3-D-Ala-L-Pro)						
Gly^1	0,[d] −1.6[h]	7.38,[d] 7.80[h]		0.1[i]		67,68,69
Gly^3	−4.5,[d] −13.9[h]	8.49,[d] 7.76[h]		1.3[i]		
D-Ala	−0.5,[d] −3.2[h]	7.63,[d] 7.89[h]		0.2[i]		
Boc-Val^1-L-Pro-Gly^3-L-Val^4-Gly^5-OMe						
L-Val^1	−6.6,[d] −8.6[e]	8.44,[d,j] 8.01[g,j]				70
Gly^3	−4.7,[d] −7.5[e]	8.32,[d,j] 7.99[g,j]				
L-Val^4	−4.7,[d] −4.7[e]	7.74,[d,j] 7.89[g,j]				
Gly^5	−6.6,[d] −7.9[e]	8.60,[d,j] 8.71[g,j]				
Cbz-Gly^1-L-Pro-D-Ala-Gly^4-NHNHBoc						
Gly^1		−7.00,[e] 5.97[h]		0.15[h]	∼5[k]	71

Table 4 (continued)
DETERMINATION OF INTRAMOLECULAR HYDROGEN BONDING IN REVERSE-TURN-CONTAINING PEPTIDES[a,b,c]

Peptide	Δδ/ΔT (×10³ ppm/deg)	δ(solvent) (ppm)	$\tau_{1/2}$(^{1}H−^{2}H)	Δδ/Δ conc (ppm)	$\Delta\nu_{1/2}$/Δ nitroxide (Hz)	Ref.
D-Ala		8.76,[e] 6.47[h]		1.0[k]	>35[l]	
Gly[d]		8.27,[e] 7.98[h]		∼0[k]	∼5[l]	
Alumichrome C[m]						
Gly[1]	−5.4,[d] −5.2,[h] −5.3[g]	9.00,[d] 8.37,[h] 8.06[g]				72
L-Ala	−4.7,[d] −8.6,[h] −8.4[g]	8.65,[d] 7.47,[h] 7.34[g]				
Gly[3] [n]	−1.1,[d] −2.6,[h] −1.7[g]	6.89,[d] 7.16,[h] 7.20[g]				
L-Orn[1] [n]	+0.6,[d] +1.4,[h] +0.9[g]	6.44,[d] 6.60,[h] 6.81[g]				
L-*Orn*[2]	−1.7,[d] −1.9,[h] −1.6[g]	10.05,[d] 10.38,[h] 10.56[g]				
L-*Orn*[3]	−1.7,[d] −3.9,[h] −2.9[g]	7.96,[d] 8.36,[h] 8.57[g]				

[a] The magnitudes of the experimentally derived parameters in this table depend upon concentration and temperature used, as well as the compound itself (e.g., its susceptibility to aggregation). The original references should be consulted before making any comparisons using the numerical data expressed herein.

[b] Abbreviations used: δ, chemical shift downfield from tetramethylsilane; T, temperature; ppm, parts per million; deg, degrees centigrade; $\tau_{1/2}$ (^{1}H-^{2}H), half-life for deuterium exchange; $\nu_{1/2}$, line-width at ½ peak height; nitroxide, 3-oxyl-2,2,4,4-tetramethyloxazolidine; Boc, *t*-butoxycarbonyl; Me, methyl; Cbz, carbobenzoxy.

[c] The underlined residues are proposed to have intramolecularly hydrogen bonded amide protons.

[d] In dimethyl sulfoxide-d_6 (Me_2SO-d_6).

[e] In methanol.

[f] The value given is $\delta(Me_2SO\text{-}d_6)$-δ(Hexafluoro-2-propanol).

[g] In trifluoroethanol.

[h] In chloroform-d ($CDCl_3$).

[i] Δδ = δ(0.26 *M*) − (0.07 *M*) in $CDCl_3$.

[j] 0° C (extrapolated).

[k] Δδ = δ(∼60 mg/mℓ) − δ(∼2 mg/mℓ).

[l] $\Delta\nu_{1/2}$ = $\nu_{1/2}$(0.5% nitroxide, v/v) − $\nu_{1/2}$ (0% nitroxide) in $CDCl_3$.

[m] The enumeration is consistent with that used previously.[72]

[n] The amide proton of this residue is postulated to be "buried" but not intramolecularly hydrogen bonded.

The extent of perturbation of N-H resonances during a solvent titration or simply comparisons of chemical shifts in different solvents can be informative, provided that conformational changes occurring concomitantly are negligible. In order to establish that a conformational change has not occurred as the solvent is varied, a rule-of-thumb is that the chemical shifts of the upfield protons and their coupling constants should be relatively unchanged — by <0.2 ppm and <0.5 Hz, respectively. Representative data for various reverse turn-containing peptides are shown in Table 4.

As mentioned above, it is expected that peptide-peptide intermolecular hydrogen bonding would be favored at higher peptide concentrations in solvents which interact weakly with the peptide. This can be exploited to distinguish exposed and sequestered N-Hs by examining the concentration dependences of their chemical shifts. A buried or intramolecularly hydrogen-bonded N-H would display little or no change in chemical shift throughout a range of peptide concentration which goes high enough for aggregation to be favored. The exact concentration range in which aggregation will occur varies considerably from peptide to peptide. Again, it is necessary to emphasize that conformational changes can lead to spurious results and erroneous interpretations. Examples of the successful application of this approach to reverse turn-containing peptides are given in Table 4. In all cases, as expected, the N-Hs which are exposed undergo larger changes in chemical shifts as the peptide concentration is varied than those N-Hs which are shielded.

Deuterium-exchange kinetics of the N-^{1}H to N-^{2}H are measured by the rate of decrease in area of the N-H peak (usually reported as half-life for exchange, $\tau_{1/2}$), hydrogen bonds being assigned to the slowly exchanging N-H protons. Although the isotopic exchange method had been used on gramicidin S[15] (see Table 4) and in other applications,[16,74,75] the approach is now seldom used because of inherent problems in the interpretation of experimental results.[61] In light of several cases of conflict between temperature dependence and ^{2}H-exchange data, criticisms were raised against the latter method, citing the fact that it measures kinetics of solvent and peptide reaching a transition state, which may be only indirectly related to the equilibrium conformation of the peptide.[61] In addition, the general weaknesses of most of the methods described here are also present:

1. The method cannot determine whether a slowly exchanging N-H proton is hydrogen-bonded or solvent-shielded.
2. The approach cannot discriminate whether rapidly exchanging N-H protons are nonhydrogen-bonded or are N-H protons in rapid equilibrium between two different conformations.
3. There is no absolute criterion for establishing the half-life for the exchange which may be correlated with N-H hydrogen bonding.

The final experimental approach relies on detecting differences in the line-broadening of N-H proton resonances (i.e., increase of peak width at ½ peak height, $\nu_{1/2}$) that are produced by the addition of small amounts (<3%, v/v) of a stable hydrogen-bond-accepting free radical — for example, a nitroxide, 3-oxyl-2,2,4,4-tetramethyloxazolidine (see Table 4).[61,71,73] Because concentrations of free radical as small as 0.2% can be used, the likelihood that the free radical is perturbing the conformation of a peptide is minimized. Since the free radicals used are good hydrogen-bond acceptors, N-H line-width differences in a weakly hydrogen-bonding solvent (e.g., chloroform) will occur only for N-H protons which are exposed (not buried or intramolecularly hydrogen-bonded). The method has also been used in fairly strongly hydrogen-bonding solvents, where presumably a competition occurs between nitroxide and solvent for available hydrogen-bonding sites of the peptides. As pointed out by Kopple et al.,[71] the

major limitation of the approach is that spectral overlaps produced by free-radical-induced line-broadening allow only a qualitative determination of intramolecular hydrogen bonding. As is generally the case, conformational averaging must be considered as a possible complication.[73]

The possibility of conformational averaging is a general problem encountered during an evaluation of the accessibility of N-H protons. It is likely that most linear peptides have multiple conformations among which they interconvert. Unless these conformations are separated by energy barriers greater than approximately 10 kcal/mol (for chemical shift differences of approximately 50 Hz), the observed NMR spectrum will be a weighted average of the spectra of the multiple-conformer population. Urry and Ohnishi[76] have suggested that for a given N-H in a peptide, the mole fractions of the solvent-exposed and solvent-shielded protons may be calculated from the experimentally measured temperature and solvent dependences of N-H chemical shift. Such calculations rely on reference values acquired from model peptides which have well-defined populations of shielded or exposed protons (e.g., gramicidin S or valinomycin). In addition, Urry and Long[70] considered that the interactions of carbonyl groups with solvent might alter appreciably the observed solvent or temperature dependences of the amide proton of the same peptide bond. This idea has been discussed in detail by Llinás and Klein.[72] However, this approach, as noted by Urry and Long, is a qualitative one, since it is impossible to verify that the required reference values are applicable to a peptide with a different sequence, conformation, or distribution of magnetically anisotropic groups.

Since the conformational space available to cyclic peptides is considerably more restricted than that available to linear peptides, it is usually assumed that the problem of conformational averaging with cyclic peptide is less important. Indeed, the probability of more than two or three discrete conformers existing simultaneously in significant proportions and interconverting rapidly on the NMR timescale is quite small. However, examples of the occurrence of conformational averaging in cyclic peptides, leading to an apparent conflict between N-H accessibility data obtained by two different experimental approaches, are known.[74] In addition, the temperature coefficients ($\Delta\delta/\Delta T$) of the N-H chemical shifts of the cyclic hexapeptide cyclo-(Gly-D-Xxx-L-Yyy)$_2$ proved unreliable when they were compared with the data obtained from the hydrogen-bond-accepting free radical approach for assessing N-H exposure.[73] In both cases, the equilibria among the averaging conformations is perturbed to differing extents by the different approaches utilized. In all likelihood, the addition of a polar nitroxide (<3%, v/v) to a solution of peptide is a more gentle approach than altering the temperature by 40 to 60°, although whether the former approach results in a more accurate determination of N-H accessibility remains to be established.

In conclusion, the determination of N-H solvent accessibility by NMR is best done by the use of several corroborative techniques. Hydrogen-bonding nitroxide radicals as line-broadening agents appear to offer the most reliable data, although the technique has not been utilized extensively. Nonetheless, careful use of temperature, concentration, and solvent dependence data can be highly informative. In instances where any discrepancy is found among results from different experimental approaches, the peptide system must be analyzed in terms of possible conformational averaging and/or conformational alterations, resulting from the experimental approach used. Throughout such investigations repeated validation of a lack of change in the chemical shifts and coupling constants must be made.

2. Conformational Dependence of Coupling Constants

As originally formulated by Karplus[77] in his widely used semiempirical relationship between dihedral angles and vicinal ^1H-^1H coupling constants ($^3J_{1H\text{-}1H}$), geometric in-

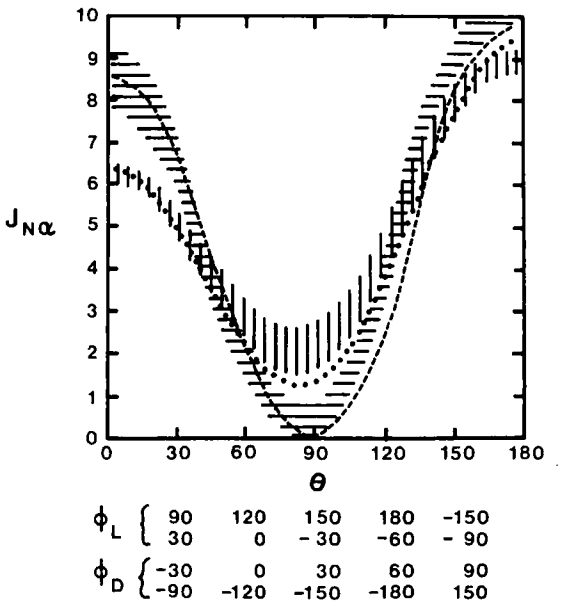

FIGURE 4. Plots of the $J_{N\alpha}$ coupling constant in Hz as a function of the dihedral angle θ according to various Karplus-type correlations. The equivalent ϕ angles for L- or D-residues are given below the corresponding θ values. $J_{N\alpha} = 8.5\cos^2\theta - 0.28$, $0° \leq \theta \leq 90°$, $J_{N\alpha} = 9.5\cos^2\theta - 0.28$, $90° \leq \theta \leq 180°$ (---);[77] $J_{N\alpha} = 9.4\cos^2\theta - 1.1\cos\theta + 0.4$ (mean permissible values) (—);[78] $J_{N\alpha} = 7.9\cos^2\theta - 0.55\cos\theta + 0.35\sin^2\theta$ (...);[79] $J_{N\alpha} = (5.41 \pm 0.02)\cos^2\theta - (1.27 \pm 0.15)\cos\theta + (2.17 \pm 0.54)$ (|||).[80]

formation can be derived from ^{1}H-NMR spectra. Of particular interest to investigators dealing with peptide conformation are $^3J_{NH\text{-}H\alpha}$ values ($J_{N\alpha}$), which can be related to the ϕ angles, as well as $^3J_{HiHi}$ for protons in the side chains, which can be related to the χ angles. Several correlations have been introduced for the former, based on the same basic premises as Karplus' initial work, but specifically adapted to peptides by incorporating experimental data from well-studied peptides (see Figure 4).[78-82] These correlations vary somewhat among themselves, with regard to their minimal and maximal values of $J_{N\alpha}$ for $\theta = 0$, 90, or 180° (θ defined in Figure 4) and to the mathematical expressions describing the relationship between vicinal coupling constants and dihedral angles (see Figure 4). However, from a practical standpoint, it is less important to choose a particular form of the $J_{N\alpha}$ vs. θ relation than to recognize the inherent limitations associated with all these relations.

1. The actual nature of the coupling interaction, which is a function of both the distribution of electrons and the geometric arrangement of molecular orbitals, may vary among compounds and their conformers. The relations in Figure 4 are generalizations which may be applied to any molecule so long as a range of θ (directly related to ϕ) values is associated with a particular $J_{N\alpha}$.
2. There is an ambiguity in these mathematical functions, since more than one value for θ may be associated with a given value of $J_{N\alpha}$, except for the extremely small or large values of $J_{N\alpha}$. In addition, two values of ϕ are possible for each value of θ, except for $\theta = 0$ or 180°.

Table 5
HETERONUCLEAR COUPLING RELATIONS POTENTIALLY USEFUL IN THE ANALYSIS OF PEPTIDE CONFORMATION

	Possible range of J values* (Hz)	Angular dependence	Ref.
$^{3}J_{^{1}H_i - C_i^{\alpha} - C_i' - ^{14,15}N_{i+1}}$	^{14}N: 0.8 — 5.5 ^{15}N: 1.8 — 5.1	ψ	84, 88
$^{3}J_{^{13}C_i - N_{i+1} - C_{i+1}^{\alpha} - ^{1}H_{i+1}}$	<0.5 — 3.4	ϕ	84
$^{3}J_{^{1}H_i - N_i - C_i^{\alpha} - ^{13}C_i^{\beta}}$	1.0 — 3.0	ϕ	84

* From experimental data from several compounds, not all peptides; absolute values given.

3. Conformational averaging, which is rapid on the NMR timescale, will lead to the observation of averaged coupling constants.
4. In many instances, accurate $J_{N\alpha}$ values may not be obtained directly from a spectrum, and there is a requirement for a simulation of non-first order coupling patterns.

For information regarding the use of coupling constants to determine χ angles, the interested reader is referred to Demarco et al.[83] and the references therein.

Recently, additional spin couplings, both homonuclear and heteronuclear,[84] have been correlated with peptide geometry. These correlations offer promise that more structural information may be extracted from NMR spectra,[85] but these are not yet routinely applied. For example, the geminal H-H coupling ($^{2}J_{H\alpha-H\alpha}$) in glycine residues has been related to ϕ,ψ angles.[86] This relation has also been applied to [Pro^{3},Gly^{4}]-oxytocin.[87] Heteronuclear coupling constants have been used to supplement the information derived from proton-proton couplings. A recent study of valinomycin by Bystrov and co-workers[84] exemplifies the application of these couplings to the analysis of conformation. The various geometric relationships which have been evaluated from these heteronuclear coupling constant measurements are listed in Table 5. It should be noted that these couplings generally require isotopically enriched material, since both ^{13}C and ^{15}N are "rare" nuclei with natural abundances of 1.1% and 0.015%, respectively, and that the coupling constants are often small.[89] These drawbacks, together with the fact that these geometric dependences are only now being established, have severely curtailed the application of heteronuclear couplings.

3. Nuclear Overhauser Enhancement (NOE)

A change in intensity of the NMR signal of a nucleus may arise when another nucleus is irradiated with a radiofrequency corresponding to the latter's resonant frequency. The change in intensity is termed the nuclear Overhauser enhancement or effect (NOE). Theoretical details and experimental applications of this effect have been presented by Noggle and Shirmer.[90]

The usefulness of NOEs for conformational analysis originates from the fact that the relaxation phenomenon from which the NOE arises occurs primarily via the dipole-dipole mechanism, which has a dependence on distance (r) of $1/r^{6}$. With certain limitations, it is theoretically possible to measure the increase in signal area of any of the hydrogens in a molecule as each of the other protons is irradiated and to relate the observed values of NOE to intramolecular distances.[91] Since the magnitude of the NOE generally diminishes rapidly with increasing distance between protons, only those pro-

tons in close proximity to one another will exhibit NOEs. NOEs between protons have a maximum value of 50% at 100 MHz. The actual value of an observed NOE depends on what fraction of the relaxation of the observed nucleus occurs via the dipole mechanism with the irradiated protons. This fraction in turn is related to the distance between these protons and to the availability of other nearby protons or other mechanisms of relaxation.

The authors' purpose in emphasizing the physical origin of measured NOEs is to provide a framework for critical interpretation and application of NOE experiments. The method should extend markedly the information derivable from ^{1}H-NMR spectra. Nonetheless, limitations inherent in the method are several.

1. The contributions to relaxation of the observed nucleus from protons other than the one irradiated, and mechanisms other than dipolar are not, in general, known.
2. Conformational averaging complicates interpretation of NOEs.[92]
3. In large molecules spin diffusion may become important.[90]
4. Molecules which tumble anisotropically must be analyzed in terms of the relationship of the internuclear vector to the axes of tumbling of the molecule.[90]

Despite these complications, it is possible to use NOEs profitably in conformational analysis.[93,94] Approaches are available which circumvent the theoretical difficulties.

1. A triangulation technique, which entails measuring NOEs among three types of protons where the distance between two is constant and known,[95] allows for quantitative conclusions about the other interproton distance.
2. If conclusions are drawn on the basis only of large NOEs ($\geq$10%), qualitative conclusions are possible: namely, that a relatively short distance and a rigid relationship between the nuclei obtains.
3. A semiquantitative relation between the internuclear distance and observed NOE, first suggested by Bell and Saunders,[96] can be successfully applied in certain instances. The limitations on its appropriateness are described in detail by Noggle and Schirmer.[90] Leach et al.[97] have applied the direct distance/NOE correlation to peptides, proposing that observed NOEs can be directly related to ψ angles, but because of the limitations enumerated by Noggle and Schirmer,[90] the generality of their proposal must be closely considered for each application.

In spite of these provisos, the NOE method offers the means for determining the presence of reverse turns in peptides and for assigning a particular conformational type to the reverse turn. The distance relationships among the hydrogens in a β-turn backbone which will be most informative when examined by NOE measurements are shown in Figure 5.

Examples of studies wherein NOE data were used to analyze reverse-turn peptides serve to illustrate the potential utility of this method. Howard et al.[98] examined the spectra of blocked linear tetrapeptides, CH_3CO-[Gly, Thr, Asp, Lys]-$NHCH_3$, observing the methylamide group under conditions of irradiation of the acetyl groups. Significant NOEs were observed, leading to the conclusion that these termini were brought into proximity by a folding of the peptide. This evidence, together with analysis of N-H resonances and other data, led to a proposed reverse-turn conformation. Possible complications arise in this study because intermolecular effects are difficult to distinguish from the intramolecular effects of interest. Also, the observed NOEs were negative, an unusual result for a molecule of the size of the tetrapeptide. Negative NOEs normally occur only when the correlation time (τ_c) of a molecule becomes long

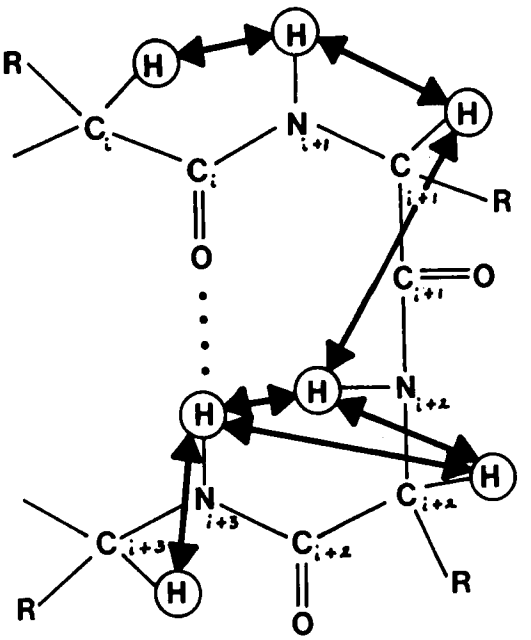

FIGURE 5. Examples of hydrogen-hydrogen distances (↔) in a β-turn which might be determinable from NOE measurements.

(τ_c ca. 10^{-9}s), depending on field strength.[99] Other studies have used similar approaches to Howard et al.[100,101]

Jones et al.[95] used the triangulation approach (i.e., analysis of a three-spin system) to refine the conformation of gramicidin S in the region of the D-Phe-L-Pro β-turns. Since the distance between the Pro $H^{\delta'}$s is fixed and the distances between the D-Phe N-H and each of the Pro $H^{\delta'}$s depends on $\psi_{D\text{-}Phe}$, a comparison of NOEs observed in this three-spin system can yield the distance from the D-Phe N-H to the Pro $H^{\delta'}$s and $\psi_{D\text{-}Phe}$ ψ is not directly obtainable from any of the usual NMR experiments.

Khaled and Urry[102] claimed to have demonstrated by NOE that a linear tetrapeptide adopted a type II β-turn conformation, as opposed to a type I, the other possible turn type. Their conclusion was based on a measurement of a 10% NOE between the Pro H^{α} (residue i + 1 of the proposed turn) and the Gly N-H (residue i + 2) (see Figure 5). As they pointed out, the distance between these protons would be markedly larger for a type I turn than for a type II turn. Hence, their measured value was interpreted as proof for a type II turn. A comparison was drawn with the value of 1.9% measured for valinomycin-K^+, which these authors mistakenly attributed to an NOE value characteristic of a type I turn. However, it has been established that the valinomycin-K^+ conformation in solution and crystal is comprised of alternating type II and II′ turns.[84]

4. ^{13}C NMR Chemical Shifts and Relaxation Times

^{13}C NMR spectroscopy has become feasible, and in fact routine, even at natural abundance, with the development of pulsed NMR methods.[57] Consequently, the spectra of many compounds, including peptides, have been measured and tabulated.[103] With a couple of exceptions, such chemical shift data have not been demonstrated to be very useful in peptide conformational analysis. However, in certain cases carbon NMR shifts have been used for the determination of reverse turns.

One correlation of ^{13}C chemical shifts with conformational aspects which has

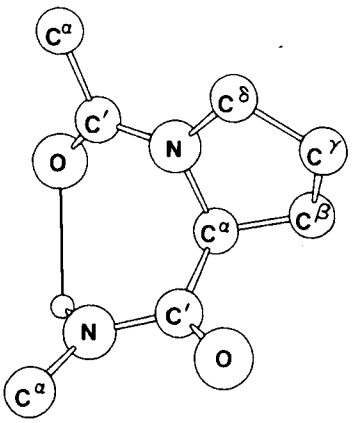

FIGURE 6. Diagram of an inverse γ-turn with an L-Pro in position i + 1. Note the eclipsed interaction between the C^{β} and C=O of the L-Pro.

emerged and has been observed in several types of molecules is the so-called steric compression effect.[104] First noted by Grant and co-workers in methyl cyclohexanes and methylbenzenes, this effect appears phenomenologically as an upfield shift of carbon resonances for groups which come into close steric contact with one another. The steric compression effect has been invoked in model cyclic peptides postulated to contain inverse γ-turns with prolines in the i + 1th position. Formation of the i + 2 → i hydrogen bond demands that the proline adopt a low *trans*′ ψ angle (circa 70°) with resulting eclipsing of the Pro $C^{\beta}H_2$ and C=O (see Figure 6). The Pro C^{β} resonance in these situations occurred at an unusually high field position.[68,105-107] This datum was taken in conjunction with results from other methods (^{1}H-NMR, CD, and/or conformational energy calculations) to be indicative of the presence of an inverse γ-turn.

Siemion et al.[108] suggested that a generalization of the above effect was possible. They plotted the difference in chemical shift between the Pro C^{β} and C^{γ} resonances (the shift of the C^{γ} is generally rather invariant) for several peptides as a function of the torsional angle between C^{β} and C=O (related directly to ψ). The validity of this correlation is suggested by the linearity of their plot, but data were sparse and not precisely known.

Urry et al.[109] followed the solvent dependence of peptide carbonyl chemical shifts using an approach which parallels that normally used with N-H resonances. In this way, the involvement of carbonyls in intra- or intermolecular hydrogen bonding could be analyzed. In linear tetrapeptides and repeat tetrapeptides of elastin, they found that one C=O was solvent-shielded. This result was consistent with their proposal, supported by other methods, that β-turns occurred involving the solvent-shielded C=O as an intramolecular hydrogen bond acceptor. The inherent difficulties in assignment of carbonyl resonances were circumvented by these authors through specific enrichment and chemical modification, methods unfortunately not always available. A similar study was carried out on alumichrome by Llinás et al.,[110] who assigned carbonyl resonances by a ^{13}C- $\{^{15}$N$\}$ double resonance method involving the use of ^{15}N-enriched peptide. Their findings suggested that the carbonyl resonances were very sensitive to perturbations (from hydrogen bonding) of the peptide-bond electron density, either through its acceptor (C=O · · · H) or donor (N-H · · · O) roles.

Spin-lattice relaxation times (T_1's) of carbon nuclei can be obtained routinely with present-day spectrometers, though certain experimental precautions (e.g., degassing,

accurate measurement of flip angle, and adequate delays between pulses) must be taken. The efficiency of dipolar relaxation (the usually predominant mechanism) of carbons depends in general on the number of bound protons and the frequency of the motion, relative to the Larmor frequency, of the relaxing nucleus. For most small peptides, molecular motions are rapid enough so that the extreme narrowing condition is satisfied, and the statement can be made that faster molecular motion involving the relaxing nucleus will lead to a longer relaxation time. The molecular motion referred to can arise from overall tumbling or from segmental motion (in a flexible molecule). Although the description presented here is qualitative, it comprises the essential aspects of relaxation time applications to small peptide conformational analysis.

For example, the result that T_1's of all of the carbons of a linear peptide are similar has been interpreted to mean that a specific conformation obtains, so that the molecule tumbles as a unit without flexible parts. This idea lead Smith and co-workers[111] to suggest that the melanocyte-stimulating-hormone-release-inhibiting-factor, H-Pro-Leu-Gly-NH_2, adopted a β-turn conformation in dimethyl sulfoxide. Similarly, the relaxation time data for angiotensin were interpreted to reflect a favored, folded conformation.[112]

In a study of ^{13}C relaxation times of (Xxx-L-Pro-Yyy)$_2$ cyclic hexapeptides[113] which take up a conformation containing two β-turns, it was observed that relative values of proline carbon T_1's varied depending on whether the proline was in the i + 1^{th} position of a type II turn or the i + 2^{th} position of a type II′ turn. Without other examples, it is not safe to rely on a single finding as diagnostic of a structural feature, but such studies suggest that relaxation times may potentially yield detailed conformational information in reverse turn-containing peptides.

5. ^{15}N NMR

^{15}N NMR spectroscopy is a relatively recent innovation, and as yet infrequent use has been made of this method in analysis of reverse turn-containing peptides. However, ^{15}N studies of three examples, gramicidin S,[114] ferrichrome,[115] and the model cyclic pentapeptide, cyclo-(Gly-Pro-Gly-D-Ala-Pro)[116] have been reported. Although trends, particularly arising from involvement in intra- or intermolecular hydrogen bonding, were postulated, no clearcut conformational applications of ^{15}N NMR data have been established.

6. Summary

Equipped with the methods described for applications of NMR techniques to reverse turn-containing peptides, researchers have ascribed a conformation containing β- and/or γ-turns to a large number of peptides. A summary of those examples in which the sequence and type of the turn(s) are well-established is presented in Table 6. The list of peptides is not intended to be exhaustive, but instead to be illustrative of the frequent, fruitful applications of NMR in the elucidation of reverse turns in peptides. It is clear that many synthetic model turn compounds have been characterized in detail and that these are useful as reference compounds for turn geometries and spectral parameters. Moreover, they may serve as test compounds for correlation of amino acid sequence with preferred turn conformations. Further, several examples of reverse turn conformations have been described in studies of antibiotics, hormones, and neuropeptides. The biological implications of these findings is discussed more fully in Sections V.G , V.H , and V.J.

C. Circular Dichroism (CD) Evidence for Reverse Turns in Peptides

Circular dichroism is sensitive to peptide conformation and is used extensively as an

Table 6
REVERSE TURNS IN PEPTIDES ESTABLISHED BY NUCLEAR MAGNETIC RESONANCE[a]

A. β-Turn-Containing Peptides

Peptide	β-Turn type	Sequence				NMR evidence	Ref.
		i	i + 1	i + 2	i + 3		
1. Synthetic							
Boc-L-Val-L-Pro-Gly[3]-Gly[4]-OMe	II	L-Val	L-Pro	Gly[3]	Gly[4]	Δδ/ΔT,Δδ(solvent), J_{N_α}, NOE	70, 102, 117
Cbz-Gly[1]-L-Pro-D-Ala-Gly[4]-NHNHBoc	II	Gly[1]	L-Pro	D-Ala	Gly[4]	$\Delta\nu_{1/2}$(nitroxide), Δδ(conc), J_{N_α}	71
Cbz-Gly[1]-L-Pro-D-Val-Gly[4]-NHNHBoc	II	Gly[1]	L-Pro	D-Val	Gly[4]		
Cyclo-(Gly[1]-L-Pro-Gly[3])$_2$	II	Gly[1]	L-Pro	Gly[2]	Gly[1]	Δδ/ΔT,Δδ(solvent), J_{N_α}	118, 119
Cyclo-(L-Ala-L-Pro-D-Phe)$_2$	II	L-Ala	L-Pro	D-Phe	L-Ala	$\Delta\nu_{1/2}$(nitroxide), Δδ(solvent), J_{N_α}	120
Cyclo-(D-Phe-L-Pro-L-Ala)$_2$	II′	L-Ala	D-Phe	L-Pro	L-Ala	$\Delta\nu_{1/2}$(nitroxide), Δδ(solvent), J_{N_α}	64
Cyclo-(Gly-L-Pro-L-Ala)$_2$	I	Gly	L-Pro	L-Ala	Gly	Δδ/ΔT, Δδ(solvent), Δδ(benzene), J_{N_α}	65
Cyclo-(L-Pro-L-Phe-Gly-L-Phe-Gly)	I[b]	Gly	L-Pro	L-Phe	Gly	Δδ/ΔT,Δδ(solvent)	121
Cyclo-(L-Phe[1]-L-Phe[2]-L-Phe[3]-Gly[4]-Gly[5])	II	L-Phe[2]	L-Phe[3]	Gly[4]	Gly[5]	Δδ/ΔT,Δδ(solvent), J_{N_α}	122
Cyclo-(Gly[1]-L-Pro[2]-Gly[3]-D-Ala-L-Pro[5])	II	Gly[1]	L-Pro	Gly[3]	D-Ala	Δδ/ΔT,Δδ(solvent), Δδ(conc), J_{N_α}	67—69
Cyclo-(Gly-L-Pro[2]-L-Ser-D-Ala-L-Pro[5])	I	L-Gly	L-Pro[2]	L-Ser	D-Ala	Δδ/ΔT,Δδ(solvent), Δδ(conc), J_{N_α}	107, 412
Cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$[c]	II	L-Pro	L-Val	D-Pro	D-Val	Δδ/ΔT, J_{N_α}	123
	II′	D-Pro	D-Val	L-Pro	L-Val		
Cyclo-(L-Val-Gly[2]-Gly[3]-L-Pro)$_3$-Ba[++]	II	L-Pro	L-Val	Gly[2]	Gly[3]	Δδ/ΔT,Δδ(solvent), J_{N_α}	124
	II′	Gly[2]	Gly[3]	L-Pro	L-Val		
Cyclo-(L-Val-Gly[2]-Gly[3]-L-Pro)	I	Gly[3]	L-Pro	L-Val	Gly[2]	Δδ/ΔT,Δδ(solvent), J_{N_α}	124
	II	L-Pro	L-Val	Gly[2]	Gly[3]		

Table 6 (continued)
REVERSE TURNS IN PEPTIDES ESTABLISHED BY NUCLEAR MAGNETIC RESONANCE[a]

A. β-Turn-Containing Peptides

Peptide	β-Turn type	Sequence				NMR evidence	Ref.
2. Naturally-occurring							
Gramicidin S	II′	L-Leu D-Phe	D-Phe	L-Pro	L-Val	Δδ/ΔT,Δδ(solvent), $\tau_{1/2}$(^{1}H-^{2}H), NOE, $J_{N\alpha}$	3, 15, 62, 63, 95
Valinomycin[d]	II	L-Lac	L-Val	D-HyIv	D-Val	Δδ/ΔT,Δδ(solvent), J(hetero), $J_{N\alpha}$, NOE	3, 66, 84, 93, 125, 126
	II′	D-HyIv	D-Val	L-Lac	L-Val		
Valinomycin[e]	II	LLac	L-Val	D-HyIv	D-Val		
Ferrichrome C and alumichrome C[f]	II	Gly^3	L-Ala^2	Gly^1	L-Orn^3	Δδ/ΔT,Δδ(solvent), $J_{N\alpha}$, ^{15}N	72, 81
Ferrichrome and alumichrome[f]	II	Gly^3	Gly^2	Gly^1	L-Orn^3		115, 127
Antamanide-Na[g]	I	L-Pro^3	L-Ala	L-Phe^5	L-Phe^6	Δδ/ΔT,$\tau_{1/2}$(^{1}H-^{3}H), $J_{N\alpha}$, ^{13}C	128, 129, 130
	I	L-Pro^8	L-Phe^9	L-Phe^{10}	L-Val^1		
Oxytocin	—[e]	L-Tyr	L-Ile	L-Gln	L-Asn	Δδ/ΔT,$\tau_{1/2}$(^{1}H-^{2}H), $J_{N\alpha}$	131—134

B. Inverse γ-Turn Containing Peptides

Peptide	Sequence			NMR evidence	Ref.
	i	i + 1	i + 2		
1. Synthetic					
Cyclo-(L-Pro-Gly)$_3$	Gly	L-Pro	Gly	$J_{N\alpha}$, ^{13}C	105, 106
Cyclo-(L-Pro-Gly)$_4$	Gly	L-Pro	Gly	$J_{N\alpha}$, ^{13}C	106, 135
Cyclo-(L-Pro-L-Phe^2-Gly^3-L-Phe^4-Gly^5)	Gly^3	L-Phe^4	Gly^5	Δδ/ΔT,Δδ(solvent)	121
Cyclo-(L-Phe^1-L-Phe^2-L-Phe^3-Gly^4-Gly^5)	Gly^5	L-Phe^1	L-Phe^2	$J_{N\alpha}$, Δδ/ΔT,Δδ(solvent)	122
Cyclo-(Gly^1-L-Pro^2-Gly^3-D-Ala-L-Pro^5)	D-Ala	L-Pro^5	Gly^1	$J_{N\alpha}$, Δδ/ΔT,Δδ(solvent), Δδ(conc), ^{13}C	67—69

Cyclo-(Gly-L-Pro[2]-L-Ser-D-Ala-L-Pro[5])	D-Ala	L-Pro[5]	Gly	$J_{N\alpha}$, $\Delta\delta/\Delta T$, $\Delta\delta$(solvent), $\Delta\delta$(conc), ^{13}C	107, 412

- Abbreviations used: δ, chemical shift downfield from tetramethylsilane; $\Delta\delta/\Delta T$, change in chemical shift with temperature; $\Delta\delta$(solvent), change in chemical shift upon changing solvents; $J_{N\alpha}$, $^3J_{NH\text{-}N\alpha}$; NOE, nuclear Overhauser effect; $\Delta\nu_{1/2}$(nitroxide), change in line-width with added nitroxide; $\tau_{1/2}(^1H\text{-}^2H)$, half-life for deuterium exchange; J(hetero), heteronuclear coupling constant analysis; Boc, *t*-butyloxycarbonyl; OMe, methyl ester; Cbz, carbobenzoxy.
- [b] Although not specifically assigned by Demel and Kessler,[121] a type I turn is the most likely structure based on sequence considerations.
- [c] Turns listed are present in both the uncomplexed peptide and in its K^+ complex.
- [d] Turns listed are present in both the uncomplexed peptide and its K^+ complex in nonpolar solvents (e.g., chloroform).
- [e] Turn listed is present in solvents of medium polarity (e.g., methanol).
- [f] The enumeration is consistent with that used previously.[72]
- [g] Turn type is not definitively established.

experimental technique for studying the conformations of peptides and proteins.[136] A CD spectrum displays the sum of the individual spectral contributions donated by the various conformations present within a given peptide. Hence, the application of deconvolution techniques to the analysis of a CD spectrum can yield an estimate of the contribution of each individual conformation (e.g., α-helix, β-sheet, extended structure, or reverse turn) present in a peptide to the overall spectrum.

Circular dichroism is the difference in absorption of left and right circularly polarized light, and is defined as ε_l-ε_r, where ε_l and ε_r are the molecular extinction coefficients for the left and right components. The origin of CD lies in the rotatory strength, R, of an electronic absorption band, which is expressed as:

$$R = \text{Im}\,[\underset{\sim}{M} \cdot \underset{\sim}{\mu}] \qquad (1)$$

where M is the magnetic dipole moment of a transition whose electronic transition moment is μ. The term Im refers to the imaginary part (i.e., the part which contains i $= \sqrt{-1}$) of the dot or scalar product shown. This expression indicates that the rotatory strength depends on the magnitudes of the electronic and magnetic transition moments and the cosine of the angle between them.

The CD derives from relative orientations of the transition moments, and hence is very sensitive to molecular geometries (at least to relative geometries of chromophores). As summarized by Schellman,[137] three mechanisms can contribute to rotatory strength

1. The electric and magnetic moments in one chromophore couple because of the perturbing field of the remainder of the molecule (called the one-electron or Condon, Altar, and Eyring theory).[138]
2. Two groups with single electric transitions couple to produce a magnetic moment (Kuhn-Kirkwood mechanism).[139] Conditionally, if the excited states are degenerate, the exciton theory of Moffitt[140] can be used.
3. One group with an electric transition and another with a magnetic transition couple: the μ − m mechanism.[141]

Bayley et al.[142] derived a general matrix formulation for the rotatory strength of molecules containing two peptide groups, each assumed to possess only the two lowest excited electronic states: $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$. They calculated the resulting rotatory strengths, including contributions from the three mechanisms above, as a function of dihedral angles (ϕ,ψ). Their results demonstrated that the optical rotation arises from all three mechanisms and that the relative contribution of each depends on the conformation. Those calculations were extended to larger peptides, and the CD spectra for cyclo-(L-Pro-Gly)$_3$ as a function of molecular conformation were predicted.[143] Good correlation between these predicted and the experimentally determined spectra was found for this inverse γ-turn-containing peptide.[105] Theoretical CD spectra of isolated β-turns calculated in a similar manner by Woody[19] further suggested that the gramicidin S-A (i.e., gramicidin S with acetylated ornithine side chains) experimental CD spectrum[144] derives largely from its two type II′ β-turns with little contribution from the phenylalanyl chromophores.

Prior to these studies, the shape of CD curves was analyzed in terms of the relative contribution of α-helical, β-sheet, and random coil structures which would result in such a curve.[145] Thus, the contribution of β-turns to CD of peptides and proteins was

Table 7
CLASSIFICATION SCHEME FOR THEORETICAL CIRCULAR DICHROISM SPECTRA OF REVERSE TURNS

Class*	Spectral extrema: Maximum/minimum	Spectral extrema: Wavelength range (nm)
A	Min	210—220
	Max	195—200
	Min	<190
B	Min	>220
	Max	200—210
	Min	<190
C	Min	200—210
	Max	180—195
D	Min	>225
	Max	210—220
	Min	190—200
	Max	<190

* A′, B′, etc., are mirror images of A, B, . . .

Adapted from Woody, R. W., in *Peptides, Polypeptides and Proteins*, Blout, E. R., Bovey, F. A., Goodman, M., and Lotan, N., Eds., John Wiley & Sons, New York, 1974, 338. With Permission.

neglected (actually obscured in the other categories) until the work of Woody.[19] He asked two fundamental questions about the CD of β-turns:

1. Is there a characteristic CD pattern to be expected for reverse turns?
2. Are the different types of turns distinguishable from one another by CD?

Using his published methods,[146,147] he calculated the rotatory strengths and theoretical CD curves for various blocked-dipeptide backbone conformations with dihedral angles of various β-turns.[19] These calculations resulted in four "unique" classes of CD spectra, depending on the conformation of the β-turn (see Table 7), rather than one pattern for all β-turn types. One difficulty in interpretation of these findings is that an unequivocal assignment of a β-turn conformation to a peptide displaying one such spectral class cannot be made without corroborating information obtained from another physical technique (e.g., X-ray diffraction or NMR). Despite this complication, there is now adequate experimental evidence indicating that the occurrence of these four spectral classes is related to the conformation of a given β-turn, if one is indeed present in the peptide.

A few generalizations can be made about the tendency of different β-turns to yield a unique spectral class.[19] However, as shown in Table 8, a given β-turn can, and does, result in more than one spectral class, although certain spectra are more commonly associated with certain reverse turn conformations. For example, class B spectra are the most common class associated with any β-turn conformation. Thus, the experimental observation of a class B spectrum is highly suggestive of a favored β-turn conformation for a given peptide, although the specific type of β-turn is uncertain. Further, class A spectra occur only with a type I, not type II, β-turn conformation. Class D spectra are most likely to occur with an open reverse turn, and class C′ (the mirror image of class C) spectra are likely to be associated either with type II β-turns or open

Table 8
PREDICTED CD CLASS FOR REVERSE TURNS OF DIFFERENT CONFORMATIONS

Reverse turn type[a,b]	Average predicted spectral class (%)[c]							
	A	B	C	D	A′	B′	C′	D′
I	16	70	7	1		5	1	
II		81					19	
III	17	53	19	1	1	7		2
Open		77	4	8			11	

[a] Reverse turn type is according to the notation of Venkatachalam.[18]
[b] Types I, II, and III are hydrogen-bonded (i + 3→i).
[c] A′, B′, etc., are mirror images of A, B, etc.

Adapted from Woody, R. W., in *Peptides, Polypeptides and Proteins*, Blout, E. R., Bovey, F. A., Goodman, M., and Lotan, N., Eds., John Wiley & Sons, New York, 1974, 338. With permission.

reverse turns. Conversely, class C spectra can also be associated with type II′ β-turns (as in the case of gramicidin S).[19]

The application of these theoretical CD curves to the interpretation of experimental CD curves is limited by three experimental factors. First, the theoretical calculations were done for the *in vacuo* case. However, experimentally a solvent which is relatively polar is used in order to solubilize most peptides being studied. Such solvents would be expected to shift the position and possibly alter the rotatory strength of a transition. Hence, it is difficult to compare theoretical *in vacuo* spectra with solution spectra (regardless of how nonpolar the solvent) or to compare spectra run in different solvents. Second, the level of sensitivity for CD measurements is approximately 10%. Hence, if a peptide exists in several conformations at a given temperature and in a given solvent, and if a reverse turn conformation does not predominate, it is very difficult to ascertain whether a given peptide exists in any reverse turn conformation and in which one. Third, there are no peptides which are absolute spectral standards for the CD of the various reverse turn types, although progress toward this goal has been made.

1. β-Turns

Suggestive evidence has been published from CD experiments for the existence of β-turn conformations in various linear peptides:

1. *N*-acetyl-L-proline-glycine-*N*′-methylamide[148]
2. *N*-acetyl, *N*′-methylamides of L-Asp-L-Lys-L-Thr-Gly, L-Asp-L-Lys-Gly-L-Thr, and L-Lys-L-Thr-Gly-L-Asp[98]
3. Poly-[Gly-(L-Pro)$_{3\ or\ 4}$][149]
4. Angiotensin II[150]
5. Neurotoxins[151,152]
6. Cardiotoxin[152]

However, none of these peptides has been shown to possess a β-turn conformation as its predominant conformer when assessed by more definitive methods such as NMR or X-ray analysis.

Table 9 summarizes the CD spectral data for some of the model reverse turn-containing peptides which have been proposed to contain β- and/or γ-turn types, as assessed by complementary NMR, IR, or X-ray experiments and conformational energy calculations. The CD spectral data characteristic of a type I β-turn have been determined for two peptides, a linear and a cyclic. The linear peptide is Cbz-Gly-L-Ser(OBu^t)-L-Ser-Gly-*O*-stearyl ester.[153] Although the assignment of a type I β-turn conformation to this peptide is equivocal because it was based on correlative statistical methods[163] and infrared data,[153] neither of which yields sufficient structural information to allow the assignment of a specific turn type, the CD spectrum of this model peptide differs from the CD spectra of either type II or type II′ (*vide infra*) β-turn-containing peptides. The wavelength position of the spectral maxima of this linear model and type II β-turn-containing peptides are similar, although the mean residue ellipticity is considerably greater for the proposed type I β-turn model peptide than for the type II β-turn models, with the exception of poly-(L-Ala$_2$-Gly$_2$)$_n$.[155] The fact that these dissimilar β-turn conformations may result in qualitatively similar CD spectra is expected in light of the theoretical CD data of Woody, which indicate that such class B spectra (see Tables 7 and 8) are usually characteristic of both type I and II β-turns.[19] Such class B spectra are identified by a spectral minimum at approximately 221 nm and a spectral maximum at approximately 198 nm. Further, in the case of the type I β-turn CD spectral data determined experimentally, the magnitude of the mean residue ellipticities for these extrema were larger than those predicted theoretically (−7800 vs. −5000 and +71,900 vs. +30,000).[19] It is also important to note that the characteristic type I β-turn CD spectrum was observed only in a limited peptide concentration range (i.e., 0.056 to 0.28 mg/m*l* cyclohexane), and that above that concentration, the observed CD spectrum resembled that usually associated with a random coil conformation. Further, the CD spectrum of this type I model peptide is markedly dependent on the polarity of the solvent used. For example, the peptide in methanol, in contrast to cyclohexane, resulted in a CD spectrum usually attributed to a random coil conformation. This solvent dependency is clearly a serious limitation to a more general use of this peptide (and possibly all linear peptides) as a CD standard for β-turn-containing peptides. The development of additional linear model peptides suitable for the determination of the CD spectral parameters associated with type I β-turns in polar solvents is certainly required, as is the need for a precise definition of the existence of a type I β-turn by NMR or X-ray analysis in any such compound.

A series of cyclic hexapeptides of sequence (Xxx-L-Pro-Yyy)$_2$ has been synthesized and examined by ^{1}H- and ^{13}C-NMR, IR, and CD by Pease.[65] The preferred conformation of these compounds is usually a C_2-symmetric, β-structure, which consists of two β-turns (see Figure 7), although the β-turn type is variable depending on the configurations of Xxx and Yyy.[65]

The cyclic peptide cyclo-(Gly-L-Pro-L-Ala)$_2$, which has been demonstrated by ^{1}H-NMR to contain a type I β-turn,[65] displayed a class C spectrum in both water and trifluoroethanol (see Table 9). As shown in Table 8, such a class C spectrum can be associated with a type I β-turn conformation, although either class A or B spectra occur more frequently in Woody's calculations for type I β-turns.[19] Such a class C spectrum is identified by its characteristic extrema — a minimum (possibly two minima) (approximately 200 to 210 nm) and a maximum (approximately 180 to 195) (see Table 7). In the case of cyclo-(Gly-L-Pro-L-Ala)$_2$, the CD spectra displayed two minima with mean residue ellipticities of −6000 to −9200 (215 to 218 nm) and −6000 to −9000 (202 to 208 nm) and a maximum of 8,000 to 12,000 (188 to 190 nm), depending on the solvent.[65]

Six model peptides have been introduced as suitable CD standards for type II β-turns. Urry and Ohnishi[154] have proposed, on the basis of detailed NMR data, that

Table 9
CIRCULAR DICHROISM SPECTRAL PARAMETERS FOR VARIOUS REVERSE TURN-CONTAINING PEPTIDES[a]

A. β-Turn-Containing Peptides

B-Turn type	Peptide	CD spectral extrema[b] λ nm	[θ] × 10^{-3}	λ nm	[θ]× 10^{-3}	λ nm	[θ]× 10^{-3}	CD spectral class[c]	Solvent	Additional data in favor of a β-turn conformation	Ref.
I	Cbz-Gly-L-Ser-(OBu')-L-Ser-Gly-*O*stearyl ester	221	−7.8	198	71.9			B	Cyclohexane	IR	153
	Cyclo-(Gly-L-Pro-L-Ala)$_2$	215	−9.2	208	−9.6	188	12.0	C	H_2O	^{1}H-NMR	65
		218	−6.0	202	−6.0	190	8.0		TFE		
II	HCO-(L-Val-L-Pro-Gly-Gly)$_{40}$Val-OCH_3	223	−2.4	203	2.3		B		TFE	^{1}H-NMR	154
	Poly-(L-Ala$_2$-Gly$_2$)	227	−5.2	207.5	63.2			B	D_2O	EM	155
	Cyclo-(Gly-L-Pro-D-Phe)$_2$	227	−4.5	205	14.6			B	Hexafluoro-2-propanol	X-ray	156
	Cyclo-(L-Ala-L-Pro-D-Phe)$_2$	227	−5.7	202	13.7			B	Hexafluoro-2-propanol	X-ray	156
	Cyclo-(L-Ala-L-Pro-Gly)$_2$	222	−4.6	195	15.4			B	H_2O	^{1}H-NMR	65
		223	−5.2	196	24.4	188	−13.0		TFE		
	Cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro)[d]	230.5	−12.3	203	6.1			B	H_2O	^{1}H-NMR; X-ray	67, 69
		236	−13	210	9.5				CH_3CN		
		231.5	−16.9	209.5	13.8				TFE		
		234	−11.9	207.5	13.3				Methanol		
III'	Cyclo-(D-Phe-L-Pro-L-Orn)$_2$	222	−14.9	200	−25.0			C	Hexafluoro-2-propanol	X-ray	156
	Cyclo-(D-Phe-L-Pro-L-Val)$_2$	223	−8.7	201	−16.3			C	CH_3CN	^{1}H-NMR; X-ray	157
	Gramicidin S	222	−23.2	202	−29.4			C	H_2O	^{1}H-NMR; X-ray	158
	Gramicidin S-A	215	−45.0	206	−55.0			C	Methanol		144

B. γ-Turn-Containing Peptides

γ-Turn type	Peptide	CD spectral extrema[b] λ nm	[θ] × 10^{-3}	λ nm	[θ] × 10^{-3}	CD spectral class[c]	Solvent	Additional data in favor of a γ-turn conformation	Ref.
1. Inverse	Cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro)[d]	230.5	−12.3	203	6.1	B	H_2O	^{1}H-NMR; X-ray	67, 69
		236	−13	210	9.5		CH_3CN		
		231.5	−16.9	209.5	13.8		TFE		
		234	−11.9	207.5	13.3		Methanol		
	Cyclo-(L-Pro-Gly)$_3$	211	−20.0	ND	ND		H_2O	^{1}H-NMR; energy calc.	105
		230	−19	ND	ND		1,4-dioxane		
	N-Ac-L-Ala-$NHCH_3$	220	−13	ND	ND		DCE	IR	159
		222	−4	ND	ND		TFE		160
	N-Ac-L-Pro-$NHCH_3$	227	−8	ND	ND		90% 1,4-dioxane/H_2O	IR; energy calc; NMR	161
		227	−24	ND	ND		1,4-dioxane		162
		227	−42	192	−2		Cyclohexane		162

[a] Abbreviations used: Ac, acetyl; Bu^t, tert-butyl; Cbz, carbobenzoxy; DCE, dichloroethane; EM, electron microscopy; ND, not detected; TFE, trifluoroethanol.

[b] Mean residue ellipticity (units: degree cm^2 dmol^{-1}).

[c] These spectral class assignments correspond to those described in Reference 19.

[d] This peptide has been shown to contain both a type II β-turn and an inverse γ-turn.

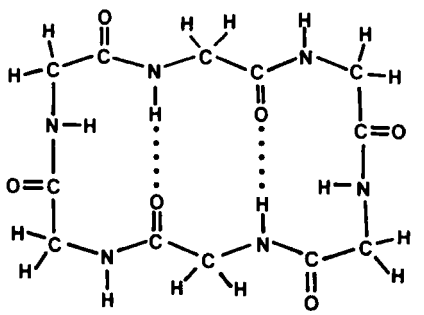

FIGURE 7. Diagram of the intramolecular hydrogen occurring in many cyclic hexapeptides. Shown here as an example is cyclohexaglycine. Note that this structure consists of two β-turns contained within a cyclic molecule.

the polytetrapeptide of elastin, HCO-(L-Val-L-Pro-Gly-Gly)$_{40}$-Val-OCH$_3$, is composed of repeating β-turns and that the CD spectrum of this polymer should be characteristic of a β-turn. The CD spectral parameters of their polymeric CD standard (see Table 9)[164] were compatible with their NMR data and the theoretical CD parameters predicted by Woody[19] for a class B spectrum (see Table 7), although which β-turn type gave rise to this spectrum remained equivocal until Khaled and Urry[102] proposed, on the basis of NMR nuclear Overhauser enhancement experiments, that the blocked tetrapeptide, HCO-L-Val-L-Pro-Gly-Gly-OCH$_3$, occurred as a type II β-turn in Me$_2$SO-d$_6$ and that the polytetrapeptide of elastin consisted of repeating type II β-turns. Urry et al.[164] have also observed a class B CD spectrum for the closely related tetrapeptide analog, Boc-L-Val-L-Pro-Gly-Gly-OCH$_3$, in methanol. The mean residue ellipticity value associated with the spectral minimum of the polytetrapeptide is approximately one half the magnitude of any of the other type II β-turn-containing model peptides, and the magnitude of the spectral maximum is also markedly reduced.

Recently Brahms et al.[155] proposed that another linear polymer, poly-(L-Ala$_2$-Gly$_2$), would yield a CD spectrum characteristic of type II β-turns. Their assignment of a repeating type II β-turn conformation to this polymer was based entirely on correlative predictive methods (see Section IV.C) and indirect electron microscopic evidence. Brahms et al.[155] observed a class B CD spectrum characteristic of type II β-turns, although their spectral extrema are slightly red-shifted from those detected by Urry et al.[164] for their polytetrapeptide. Such a red-shift was presumably due to the higher dielectric constant of the solvent used by Brahms et al.[155] (D$_2$O), as opposed to that used by Urry et al.[164] (trifluoroethanol). In addition, the mean residue ellipticities of the spectral extrema of poly-(L-Ala$_2$-Gly$_2$) differ from the ellipticities determined for the polytetrapeptide of elastin in that the positive extremum of the former has a mean residue ellipticity of 63,200, while the latter has an ellipticity of 2300. In agreement with these data for poly-(L-Ala$_2$-Gly$_2$), the CD spectra of *N*-acetyl-L-alanine-glycine-*N'*-methylamide at 22°C displayed a minimum of 225 nm and a maximum of 200 nm in trifluoroethanol, while the minimum was at 226 nm and the maximum at 204 nm in 92.5% 1,4-dioxane/water.[160] However, the ellipticities of the blocked dipeptide were markedly reduced in comparison to the polymer.[155] The ellipticities of poly-(L-Ala$_2$-Gly$_2$) resembled closely those values determined by Kawai and Fasman[153] for their type I β-turn model peptide. Since it is not known with certainty that the peptide of Brahms et al.[155] existed in a type II β-turn conformation, and since the mean residue ellipticity value CD maximum for this peptide differs markedly from the values for the other

type II β-turn model peptides, it may be that the class B CD spectrum attributable to this peptide resulted from a repeating type I β-turn conformation rather than the type II β-turn conformation. This possibility has not been disproven, and definitive NMR experiments are required.

Various cyclic penta- and hexapeptides in which type II β-turns have been established by X-ray crystallography and/or ^{1}H-NMR have also been used as model peptides to establish the CD spectral parameters of such β-turns (see Table 9). Pease[65] has shown that cyclo-(L-Ala-L-Pro-Gly)$_2$ in water or trifluoroethanol displays a class B CD spectrum and that the extrema are located at a slightly shorter wavelength than two other cyclic hexapeptides (containing type II β-turns),[156] poly-(L-Ala$_2$-Gly$_2$),[155] and the polytetrapeptide (minimum only).[164] The values of the mean residue ellipticities of the extrema of this peptide are remarkably similar to the other two cyclic hexapeptides, cyclo-(Gly-L-Pro-D-Phe)$_2$ and cyclo-(L-Ala-L-Pro-D-Phe)$_2$, as well as poly-(L-Ala$_2$-Gly$_2$) (minimum only). Bush et al.[156] have shown that cyclo-(Gly-L-Pro-D-Phe)$_2$ and cyclo-(L-Ala-L-Pro-D-Phe)$_2$ in hexafluoro-2-propanol both display a class B CD spectrum with extrema spectral positions and mean residue ellipticities that are very similar to each other. Although they also displayed minima that are similar to the repeating linear peptides of Urry et al.[164] and Brahms et al.[155] the values of the positive extrema of these peptides in comparison to the linear polymers differ appreciably. The origin of these differences is not readily explicable, but it is likely to result from contributions to the CD from conformations other than β-turns in the repeating linear polymers examined. Recently, the CD of cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro), which contains a type II β-turn, as well as an inverse γ-turn, has been examined.[67,69] Because of the coexistence of a β- and an inverse γ-turn, the unequivocal interpretation of the CD data for this compound cannot be made without resorting to theoretical CD calculations to elucidate the relative contribution of each of these structures to the CD spectrum. However, the magnitude of the mean residue ellipticities of the minima of this cyclic pentapeptide in the various solvents is considerably greater than the other type II β-turn-containing model peptides, but similar to the magnitude of the other inverse γ-turn-containing peptides. This suggests, but does not prove, that the inverse γ-turn moiety of the cyclic pentapeptide dominates the CD spectrum in the 220 to 230 nm spectral region. In contrast, the mean residue ellipticity of the CD spectral maximum resembles closely those values found for the other type II model compounds. Since a similar maximum was not detected for the other inverse γ-turn-containing model peptides, it seems likely that the maximum of the CD spectrum of the cyclic pentapeptide is dominated by the type II β-turn moiety. Overall, the CD of this pentapeptide appears to be a sum of the expected contributions from the two reverse turns present.

The CD spectra of four type II′ β-turn-containing peptides have been determined (see Table 9). The wavelength positions of the extrema and the mean residue ellipticities of cyclo-(D-Phe-L-Pro-L-Orn)$_2$,[156] cyclo-(D-Phe-L-Pro-L-Val)$_2$,[157] and gramicidin S[158] are in close agreement (see Table 9). Since the accepted conformation of gramicidin S consists of two anti-parallel β-chains connected by two type II′ D-Phe-L-Pro turns, the occurrence of two virtually identical turn sequences in the two cyclic hexapeptides should have mimicked closely the CD behavior of the antibiotic, as was observed. In another CD study, Laiken et al.[144] have reported the circular dichroism spectrum of gramicidin S-A (gramicidin S with acetylated ornithine residues) with the phenylalanyl aromatic side chains hydrogenated. As in the case of the other three type II′ model compounds, the CD spectrum in methanol of this fully hydrogenated cyclic decapeptide (i.e., containing only peptide backbone contributions to the CD) displayed two overlapping minima at approximately 206 nm and 215 nm with mean residue ellipticity values which are larger (i.e., two- to five-fold) than gramicidin S and the cyclic hexa-

peptides. Theoretical CD calculations of the spectra of isolated β-turns by Woody[19] strongly suggested that the CD spectrum of gramicidin S-A derived in large measure from the presence of β-turns. His predictions for the CD of a peptide which assumes a type II′ β-turn indicated a 50% probability (based on a sample of 81 conformations generated by successively varying Venkatachalam's[18] type II′ β-turn angles by 10°) of a spectrum like that observed for gramicidin S-A.

In some cases where β-turn-containing peptides have tyrosyl or phenylalanyl residues as their corner residues, the L_a transition,[165] associated with phenolic rings, may contribute significantly to the far UV (<250 nm) CD spectrum and obscure the spectral details attributable to the backbone conformation,[16] although this has not been observed for any of the phenylalanine-containing cyclic hexapeptides and gramicidin S as displayed in Table 9. The contribution of phenylalanyl and tyrosyl residues at the i + 1^{th} and i + 2^{th} residue of type I β-turns or the i + 1^{th} residue of a type II β-turn have recently been published.[166] Those results need to be thoroughly considered when interpreting the CD spectra of certain reverse turns containing phenylalanine or tyrosine.

2. γ-and Inverse γ-Turns

The origin of the CD of a γ- or inverse γ-turn, unlike a β-turn, can be rationalized qualitatively in terms of a one-electron mechanism using Schellman's CD quadrant rule for amides.[137] Madison and Schellman,[162,167] and later Cann et al.,[161] noted that model proline compounds (e.g., *N*-acetyl-L-proline-*N*′-methylamide; see Table 9) displayed a negative CD extremum at approximately 230 nm, which they attributed to an inverse γ-turn conformation containing a i + 2 → i hydrogen bond. As shown in Table 9, a similar observation has been made for *N*-acetyl-L-alanine-*N*′-methylamide in dichloroethane[159] and trifluoroethanol,[160] which displayed spectral minima at 220 nm and 225 nm, respectively. Since this compound contained an intramolecular hydrogen bond (as assessed by infrared spectroscopy in dichloroethane),[159] the CD minimum presumably resulted from a n → π* transition associated with the inverse γ-turn conformation of the peptide.

Madison has predicted CD spectra for cyclo-(L-Pro-Gly)$_3$ as functions of molecular conformation[143] and has found good correlation with the experimental spectra.[105] The input (unperturbed) wavelengths and band widths were those observed for monoamides. It was found using complementary information from CD, NMR, and theoretical calculations that in nonpolar solvents the cyclic hexapeptide adopted a C_3-symmetric all-*trans* conformation which was stabilized by three inverse γ-turns with intramolecular hydrogen bonds between the C=O of the Gly preceding Pro and the N-H of the Gly following Pro. According to the theoretical calculations, this latter structural feature, a nonlinear hydrogen bond, was responsible for a negative CD extremum at 230 nm (*vide supra*).

As discussed above (see Section III.C.1), the spectral minima of cyclo-(Gly-L-Pro-Gly-D-Ala-L-Pro) at 230.5 to 236 nm, depending on solvent used, may result from the presence of an inverse γ-turn, and the overall CD appears to be the sum of contributions from a type II β-turn and the inverse γ-turn.[67,69] A theoretical CD calculation is required to establish this rigorously.

D. Infrared, Raman, and Charge Transfer Evidence for Reverse Turns in Peptides

1. Infrared Spectroscopy (IR)

IR has been extensively employed in the conformational analysis of model amides and polypeptides.[168-171] In particular, the characteristic amide vibration frequencies have been found to be sensitive to hydrogen bonding. So well documented are the

hydrogen bonding shifts of IR bands that they have been incorporated into the working definition of this interaction as put forth by Pimentel and McClellan.[172] Hence, the absence of such shifts in an IR spectrum can be correlated either with the lack of any specific, strong hydrogen bonds or with anomalous band positions due to bond angle or bond length distortions.

Simple stretching vibrational bands involving the peptide bond hydrogen atom and their overtones occur between 3300 and 3500 cm.$^{-1}$ Although these bands are generally unaffected by peptide conformation per se, they are markedly affected by interactions of the hydrogen (NH) atom, for example, a hydrogen bond.

Shimanouchi and Mizushima[173] and Mizushima et al.[174] made the first assignments for the association of certain vibrational bands with hydrogen-bonded or nonhydrogen-bonded species. Tsuboi et al.[175] first observed that there were four characteristic NH stretching bands (3350, 3420, 3440, and 3460 cm^{-1}) in *N*-acetyl-*N'*-methylamides of single amino acids. Avignon et al.[176] synthesized three types of model compounds: (1) AcNHCHR^{1}CONHR^2; (2) AcNMeCHR^{1}CONHR^2; and (3) AcNHCHR^{1}CON(R^2)$_2$. Two types of intramolecular hydrogen bonds were observed and were attributed to different cyclized conformations. These conformations were called the C_5 and C_7 conformations, which contain five and seven atoms in their hydrogen bond-locked rings. In the case of the C_7 conformation there are theoretically two forms, an axial (i.e., a γ-turn) and an equatorial (i.e., an inverse γ-turn). (See Section II.) The inverse γ-turn is much more likely to occur for an L-residue in the i + 1th because of its lower conformational energy.[177,178] Using the above model compounds, Avignon et al.[176] ascribed the absorption bands located at 3340 and 3420 cm^{-1} to the N-H oscillators within the hydrogen bonds locking the C_5, γ-turn and inverse γ-turn conformations, and the other two bands were assigned to nonhydrogen-bonded NHs. Their assignments agree with those of Burgess and Scheraga,[179] but differ from those of Tsuboi et al.[175] Crippen and Yang[159] have also found evidence for the occurrence of a hydrogen-bonded inverse γ-turn conformation in 1,2-dichloroethane, but the addition of water or an increase in temperature resulted in less intramolecular hydrogen-bonding (the 3300 cm^{-1} peak decreased and the 3400 cm^{-1} peak increased) in *N*-acetyl-alanine-*N'*-methylamide. More recently, the same compound was trapped at 20 K in an argon matrix and examined by IR spectroscopy.[180] The C_5 and inverse γ-turn isomers were shown to occur, although the γ-turn isomer was not excluded or confirmed. This is essentially the same conclusion as that reached by Maurraud et al.[181] with carbon tetrachloride solutions of this peptide. Shields and McDowell[12] and Shields et al.[13] attributed the concentration-independent adsorption bands at 3330 and 3430 cm^{-1} to the presence of an intramolecular hydrogen bond in the peptides they were studying, and they interpreted the intramolecular hydrogen bonds as originating from a β-turn conformation in these linear peptides.

The observations of Deber[24] suggested that for some Boc dipeptides (Gly-L-Pro, L-Pro-Gly and L-Pro-D-Pro) there was a 30 cm^{-1} shift to a lower frequency of the urethane (carbamate) carbonyl band in the solid state. Although this was suggestive of the formation of a β-turn locked by a hydrogen bond, it is now clear from the X-ray structures of these compounds that intermolecular hydrogen bonds were the source of the carbonyl shift.[20] However, β-turn conformations have been identified in carbon tetrachloride or tetrachloroethylene solutions of dipeptides of the type RCO-L-Pro-Xxx-NHCH$_3$.[182] Similarly, evidence for the occurrence of intramolecular hydrogen bonds in linear tetrapeptides containing Pro has been reported by Kopple et al.[71] However, insolubility and structural complexity of these tetrapeptides limited the spectral detail achieved by Kopple et al.[71] in comparison to the detailed analysis made by Boussard et al.[182] In complex peptides, tetrapeptides or larger, the best estimate of intra-

molecular hydrogen bonding is the ratio of absorbance at the 3300 cm^{-1} maximum (attributable to hydrogen bonding) to that near 3430 cm^{-1} (attributable to nonhydrogen bonding), if two separate maxima are observed. The measurement of the 3300 to 3430 cm^{-1} ratio in a nonpolar solvent at various peptide concentrations yields an extrapolation to infinite dilution which can be interpreted in terms of intramolecular hydrogen bonding. If the absorbance ratio is greater than 0.4 and if the ratio is not strongly concentration dependent, intramolecular hydrogen-bonding is indicated.[13]

Pease[65] examined the IR spectra of nine cyclic hexapeptides, with the general formula, cyclo-(Xxx-L-Pro-Yyy)$_2$, in KBr discs and/or $CHCl_3$ or D_2O solutions. Although NMR and CD data for the same series of hexapeptides indicated the presence of reverse turn conformations in certain of these, there was no indication from IR that strong hydrogen bonding occurred within any of these molecules in chloroform or D_2O solution. In contrast, IR revealed significant intramolecular hydrogen bonding in the case of the cyclic pentapeptide studied by Pease and Watson.[67,69]

In addition, a method has been introduced for assigning either a hydrogen bonded or nonbonded state to each amide (N-H) in a given peptide by simultaneously obtaining the NMR and IR spectra during the time course of an ^1H-^2H exchange.[183] The NMR spectral changes can be used to determine the amino acid proton(s) which is (are) undergoing ^1H-^2H exchange. Using this information in combination with that determined from changes concomitantly occurring in the IR spectrum offers the potential of measuring free and intramolecularly hydrogen bonded N-Hs within reverse turn-containing peptides. This method has not been applied extensively, but holds promise for such studies.

In summary, IR can yield information about the presence of a hydrogen bond in a reverse turn only in those cases where a specific, strong hydrogen bond exists without additional anomalous band positions due to bond angle or bond length distortions. Hence, weak hydrogen bonding interactions such as i + 2 → i hydrogen bonds in γ-turns and inverse γ-turns or nonoptimal i + 3 → i hydrogen bonds in β-turns might not display significant shifts of IR vibrational bands, and the presence or absence of a hydrogen bond in a given reverse turn containing peptide cannot be determined unequivocally by IR.

2. *Raman Spectroscopy*

As discussed in the previous section, IR spectroscopy has been used to study peptides in a crystalline state, in nonpolar solvents, and in argon matrices. However, IR spectroscopy cannot be used in aqueous solutions since water is opaque to IR below about 1500 cm^{-1}. In contrast, Raman spectroscopy is ideally suited for aqueous solutions except for the important spectral regions near 3400 cm^{-1} and 1650 cm^{-1}.[184] However, by replacing H_2O with D_2O, spectra can be recorded in these regions. The disadvantage of Raman spectroscopy is its low-sensitivity which necessitates using peptide concentrations in excess of 1 m*M*.[184,185]

Raman spectroscopy is a type of vibrational spectroscopy which relies on a change in electrical polarizability during the vibration, even though there need be no change in the dipole moment (necessary for IR activity).[184] Thus, Raman spectroscopy is a complementary technique to IR spectroscopy in the study of the conformation of peptides. Although Raman spectroscopy does not yield direct information about backbone conformation since geometric parameters cannot be obtained from such spectra, the method does yield the following structural information: (1) the number of conformers can be determined (e.g., C_5, γ-turn, inverse γ-turn, or β-turn), as well as changes in the proportions of these isomers at different temperatures, solvent polarities, and salt concentrations; (2) the frequency of an absorption band, which is proportional to the vibrational energy state and is related to its environment, can be interpreted in terms

of local structure (e.g., hydrogen bonding); and (3) the vibrational couplings yield direct information on the orientation of the coupled oscillators.[186,187]

The structure of simple amino acid derivatives, *N*-Ac-Gly-$NHCH_3$ and *N*-Ac-L-Ala-$NHCH_3$, have been examined, and Avignon et al.[186] have claimed that the preferred conformation of such molecules is an inverse γ-turn conformation stabilized by two additional intermolecular hydrogen bonds involving one water molecule. That finding is consistent with the results of Koyama and Shimanouchi.[187,188]

Although Raman spectroscopy has the potential to yield useful structural information about reverse turn conformations in peptides, it has not been used extensively. However, recently the peptide backbone conformation of oxytocin was examined by laser Raman spectroscopy.[189] Spectra were obtained in the solid phase, in water, and in deuterated water. The presence of the amide I band at 1666 cm^{-1} (solid phase) and at 1663 cm^{-1} (in aqueous or deuterated solutions), as well as a distinct amide III band at 1266 cm^{-1} (solid phase) or 1260 cm^{-1} (aqueous) which shifts to 994 cm^{-1} upon deuteration of the peptide hydrogens was interpreted as arising from a β-turn conformation in the molecule. In particular, the amide III band at 1260 cm^{-1} was attributed to an open β-turn conformation (i.e., lacking intramolecular hydrogen bonding).

3. Charge Transfer

Donzel et al.[190] prepared three synthetic analogs of luteinizing hormone-releasing hormone (LH-RH), [Nic^+ Orn^8] LH-RH·Cl^-, [L-Ala^6, Nic^+, Orn^8] LH-RH·Cl^-, and [D-Ala^6, Nic^+, Orn^8] LH-RH Cl^-, in which ornithine was substituted for Arg^8 and its δ-NH_2 group was quaternized to a nicotinamidium (Nic). This Nic moiety is known to give a specific charge transfer interaction with a nearby tryptophanyl indole ring,[191] and hence the effect of substituting a D-alanyl or L-alanyl residue for a glycyl residue at position 6 in LH-RH (i.e., the i + 2th residue of a proposed type II β-turn) could be assessed. As expected, there was a higher charge transfer intensity for the D-Ala^6 substitution in comparison to another analog containing L-Ala^6. This suggested, but did not prove, that the residues (4 to 7) of LH-RH are involved in a β-turn conformation. The application of the charge transfer approach to the elucidation of β-turn conformations in other peptides has not been attempted, perhaps due to the relative lack of detailed structural information derived from considerable synthetic effort and the requirement for a tryptophanyl (or other chromophoric) group in the peptide sequence.

E. Conformational Energy Calculation Evidence for Reverse Turns in Peptides

1. Semiempirical, Nonquantum Mechanical Energy Calculations

The underlying hypothesis of the semiempirical energy calculation method is that the conformations of a molecule which are generated with low calculated energies are probable conformations for the given molecule. A detailed summary of the methods used for such calculations is beyond the scope of this review, but these can be found in other reviews.[29,192-194]

As discussed by Ramachandran and Sasisekharan,[29] the total energy of a conformation can be expressed as the sum of various potential energy terms: (1) nonbonded interactions (van der Waals attractions and short-range repulsions), (2) electrostatic energies, (3) hydrogen bonding, (4) unfavorable energies associated with bond length or angle deformation, and (5) hydrophobic effects. The sum of these contributions is minimized as the conformational space available to the molecule is searched. Several potential problems should be kept in mind when examining the results of semiempirical energy calculations.

1. The various energy terms referred to above rely strongly on experimental determinations of the forms and quantitative contribution of the various potential energy functions, and this may have associated errors.

2. It is possible that important energy terms are neglected, either through ignorance of the correct form of the potential function or through the need to keep calculations at a manageable size (i.e., within the capacity of available computation facilities).
3. Other energy terms may be weighted incorrectly, leading to overprediction of conformations dominated by the accentuated energy term and to underprediction of conformations primarily influenced by other terms. For example, by overweighting the hydrogen bonding energy contribution within a given molecule, it is likely that the predicted conformation will contain intramolecular hydrogen bonds.
4. In the past, the effect of solvation was not included in energy calculations. The fact that solvation is usually not accounted for in energy calculations of peptides suspected to contain low energy reverse turn conformations lends credence to the suggestion that any experimental verification of the predictions made by energy calculations may only be attainable in solutions of nonpolar (i.e., weakly solvating) solvents. Although certain methods for dealing with solvation are available,[194] these methods have not been applied to most reverse turn-containing molecules.
5. In addition, it should be noted that in most of the calculations no relaxation of bond angles is allowed. Such a restriction may in some cases make acceptable conformations appear as if they have high energies.

However, the advantage of semiempirical energy calculations is that the predictions have real experimental relevance, in contrast to quantum mechanical predictions, which are not always constrained by realistic energies.

The conformational details of β-turns have been the subject of a number of semiempirical energy calculations. The results of the first of these were reported by Venkatachalam.[18] He specified the requirement of a i + 3 → i hydrogen bond and calculated sterically permissible arrangements of four linked amino acids. Important to a critical consideration of his results are the criteria that he employed to insure hydrogen bonding: N-O distance between 2.6 and 3.2 Å and the NH-N···O angle less than 30°. There are three broad conformational regions where i + 3 → i hydrogen-bonded conformers were found to occur; their mirror images are fully allowed as well. The type I β-turn had dihedral angles for the i + 1th and i + 2th residues in the turn of approximately (−60, −30; −90,0); type II had dihedral angles approximately (−60,120; 80,0); while type III, part of a 3_{10} helix, had dihedral angles approximately (−60,−30; −60,−30).

A more sophisticated approach to the problems of characterizing the probabilities of specific β-turn types for various sequences than the simple stereochemical criteria applied by Venkatachalam[18] is exemplified in the work of Chandrasekaran et al.[22] They included further refinements on the calculations of β-turn potential energies by summing nonbonded interactions and a hydrogen-bonding potential (V_{hb}) of the form:

$$V_{hb}\ (\text{kcal/mol}) = -4.5 + 25\,(R - 2.95)^2 + 0.001\theta^2 \qquad (2)$$

where R is the distance in Å between the N and O atoms in the NH···O hydrogen bond and θ is the angle in degrees between the N-H and N···O directions. In this potential function, a maximum energy for a hydrogen bond of −4.5 kcal was chosen, with penalties for angular deviation and linear deviation becoming important (>1 kcal) at θ>31°, and R-2.95>0.2 Å. These geometric parameters were based on a compilation of X-ray data. The main contribution of this study was to delineate quantitatively

those conformations leading to a particular geometry of the hydrogen bond and to a favorable sum of nonbonded and hydrogen bonded interactions. No important deviations from the general β-turn types put forth by Venkatachalam[18] were reported.

Semiempirical energy calculations have also been performed on γ-turn conformations. In the original study by Némethy and Printz,[1] the dihedral angles for a stable γ-turn structure involving an L-residue were proposed, and concurrently an example was found in the X-ray structure of thermolysin by Matthews.[2] Later studies by Ramachandran and Chandrasekaran,[195] as well as Lewis et al.,[178] have demonstrated that for non-glycine L-amino acids an inverse γ-turn is the favored i + 2 → i hydrogen-bonded conformation because of steric hindrance between the C^{β} of the i + 1^{th} residue and the carbonyl and the amide N of the i^{th} and i + 2^{th} residues in the alternative γ-turn.

Semiempirical energy calculations have been completed on various peptides which have been shown to contain β-turns by various physical methods.

Karle et al.[40] determined the structure of cyclo-(Gly_4-D-Ala_2) by X-ray diffraction as a conformation with two β-turns having Gly-Gly sequences for their i + 1^{th} and i + 2^{th} residues. The dihedral angles of this conformation are very close to one of the four structures seen in the unit cell of cyclo-$(Gly)_6$ hemihydrate crystals.[32] Using conformational energy calculations to generate low energy conformers which fitted their measured NMR coupling constants, Tonelli and Brewster[196] described 25 conformations for cyclo-(Gly_4-D-Ala_2). They pointed out that according to their calculations, the crystal structure is a very high energy one, approximately 14 kcal above their predicted conformers. This discrepancy is apparently compensated in the crystal by the inter- and intramolecular hydrogen bonds, although it is not clear why hydrogen-bonding to solvent could not play a similar role in solution. Nonetheless, the NMR data are not consistent with one conformer, and the authors conclude that an equilibrium among several of the low energy conformers that they generated is the most likely situation. None of the cyclic structures generated had two intramolecular hydrogen bonds, so that the conclusions of these workers supported only one hydrogen bond between the Gly N-H preceding the D-Ala and the second D-Ala C=O. Coincidently, the enantiomer of this compound was included among those peptides examined by Portnova et al.[17] and their conclusions, although including two intramolecular hydrogen bonds, were also consistent with several low energy conformers in equilibrium.

Because of the problem of multiple conformers separated by low energy barriers, the most simple cyclic hexapeptide, cyclo-$(Gly)_6$, cannot be analyzed in detail by NMR. However, in the unit cell of cyclo-$(Gly)_6$ hemihydrate four distinct conformations have been identified.[32] All of these are involved in intermolecular hydrogen bonding, and one, which recurred four times per asymmetric unit, contains an i + 3 → i intramolecular hydrogen bond. Calculations performed on cyclo-$(Gly)_6$, assuming C_2 symmetry and i + 3 → i intramolecular hydrogen bonding have been completed.[197,198] The more recent study[198] added electrostatic and hydrogen-bonding potentials to the nonbonded interactions to arrive at four low energy conformations containing β-turns with intramolecular hydrogen bonding. Gō and Scheraga[199,200] have also completed an extensive study of cyclo-$(Gly)_6$ conformations, including those with C_6, S_6, C_3, I, and C_2 symmetries. Among their C_2-symmetric structures, they found nine local minima, with all but one stabilized by intramolecular hydrogen bonds. In fact, they observed the occurrence of both i + 2 → i and i + 3 → i hydrogen bonds, in some cases simultaneously. This result was influenced primarily by the degree of directional dependence included in the hydrogen-bonding potential energy term employed by them.

The ambiguities in NMR analyses arising from rapidly interconverting conformers are largely resolved in the proline-containing cyclic hexapeptides. The most simple case is the C_2-symmetric peptide, cyclo-(Gly-L-Pro-$Gly)_2$. On the basis of ^{1}H-NMR studies, this peptide was ascribed as C_2-symmetry[201] and its conformation was elucidated by

examination of specifically deuterated and ^{13}C-enriched analogs.[118,119] Energy calculations by Madison[202] were also consistent with the presence of β-turns in the low energy conformation of this peptide.

After the initial studies with cyclo-(Gly-L-Pro-Gly)$_2$, NMR measurements were employed to determine the position of proline as a function of the sequence of cyclic hexapeptide. The first proline-containing cyclic hexapeptides examined in detail, cyclo-(L-Ser-L-Pro-Gly)$_2$ and cyclo-(Gly-L-Pro-L-Ser)$_2$, were both found to take up solution conformations with prolyl residues at positions i + 1 of the component β-turns, as judged by the extent of solvent-shielding of the N-Hs of the residue preceding proline.[203,204] Energy calculations performed by Tonelli[205] fully supported the experimentally derived results favoring the all-*trans* conformers of cyclo-(Gly-L-Pro-L-Ser)$_2$ and cyclo-(L-Ser-L-Pro-Gly)$_2$. Tonelli used the following assumptions in his calculations: (1) C_2-symmetry was assumed, based on the observation of single NMR resonances, (2) only *trans* Xxx-Pro bonds were considered, (3) unfavorable transannular interactions were excluded by inspection of models, (4) the actual value of potential energy was a summation of independent residue energies, and (5) ring closure was insured by requiring a $C^{\alpha} \cdots C^{\alpha}$ distance of 3.7 to 3.9 Å.

Momany et al.[206] used semiempirical energy calculations to derive a model for gramicidin S in which the β-sheet segments were joined by type III β-turns. This prediction agreed closely with the experimental NMR data of Stern et al.[15] Later, Ovchinnikov et al.[63] demonstrated by IR and additional NMR data that the preferred conformation has intramolecular hydrogen bonding of the Val and Leu N-Hs and turns involving the D-Phe-L-Pro sequences. These turns can now be categorized as type II′ β-turns. Subsequently, semiempirical energy calculations yielded results consistent with this observation.[22,207,208]

The conformation of cyclo-(L-Pro-Gly)$_3$ was originally deduced by NMR to contain three *cis* Pro C^{α}–C=O bonds and three *cis* Gly-Pro bonds.[209] However, a reevaluation of these NMR data, prompted by energy calculations,[143] has led to the currently accepted all-*trans* inverse γ-turn-containing conformation.[105]

Madison and Schellman[162] have also demonstrated, in their multifaceted study employing energy calculations, CD, and NMR, that *N*-acetyl-L-Pro-*N*′-methylamide existed as an inverse γ-turn conformation.

Among those compounds for which energy calculations have been done but for which experimental verification is necessary are the following examples.

Lewis et al.[23] have found the conformational energy minima of three *N*-acetyl,*N*′-methylamide tetrapeptide sequences from α-chymotrypsin reverse turn regions using five different starting conformations for the energy minimization procedure. They found that the final conformational energy minima were not independent of the starting conformation, that short range interactions between side chains and adjacent backbone stabilize turn conformations, and that reverse turn conformations have lower conformational energies than non-turn conformations. Howard et al.[98] reexamined the conformation of the α-chymotrypsin sequence (35 to 38) examined previously[23] and three of its sequence variants. They found evidence of reverse turn formation in three of the four tetrapeptides calculated by their energy minimization. Another sequence variant, *N*-acetyl-L-Thr-L-Asp-Gly-L-Lys-*N*′-methylamide, another permutation of the α-chymotrypsin sequence (35 to 38), has recently been predicted not to have a β-turn as its low energy conformation.[210] Hurwitz and Hopfinger[211] have also published a complete conformational analysis of the α-chymotrypsin region (35 to 38). They reached three interesting conclusions:

1. The conformation of region (35 to 38) was determined to be a reverse turn conformation, although it differed from that of native α-chymotrypsin.

2. If the energy of the tetrapeptides derived by adding residue 34 or 39 and removing residue 38 or 35, respectively, was minimized, a reverse turn involving region (34 to 37) was found to be more stable than the native (35 to 38) reverse turn.
3. A minimization of region (34 to 39) resulted in a structure similar to that found in the native protein. The latter finding suggests that the occurrence of a reverse turn in linear peptides may be enhanced by sequences longer than the di- and tetrapeptides usually examined.

Chandrasekaran et al.[22] have demonstrated by energy calculations that peptide chains containing alternating L- and D-amino acid residues (regardless of the order) tend to assume reverse turn conformations because of a favorable stabilization energy for this conformation. Chandrasekaran and Prasad[212] have thoroughly reviewed this subject.

Hiltner and Walton[213] explored theoretically the forces directing reverse turn formation in region (52 to 59) of hen egg-white lysozyme, although they found that reverse turn formation in aqueous solution (using a hydration shell model) was unfavorable by approximately 2 kcal/turn. They suggested, without proof, that the energy of formation of all reverse turns is likely to be positive (i.e., unfavorable) and that the driving force for their formation comes from more distant interactions within the folding chain.

Nishikawa et al.[214] have shown that the low energy conformer of *N*-acetyl-L-Ala-L-Ala-*N'*-methylamide resembles closely a type II β-turn. However, in another study, the lowest energy conformation for *N*-acetyl-(L-Ala)$_4$-*N'*-methylamide was predicted to be a repeating inverse γ-turn.[23]

Semiempirical energy calculations have also predicted the occurrence of β-turns in other peptides, for example, *N*-acetyl, *N'*-methylamides of L-Pro-Xxx,[215] L-Ala-Xxx and Xxx-L-Ala,[216] and Gly-Xxx and Xxx-Gly,[217] L-Ser-Xxx and Xxx-L-Ser,[218] and elastin peptides.[117,219,220]

The results of the energy calculations of various peptides, including melanotropin release-inhibiting factor,[221] TRF,[222,223] enkephalin,[224-227] contraceptive peptide,[228] antamanide,[229] and valinomycin[125] are discussed in Section V of this review. In each case no definite proof that the predicted reverse turn conformation is important for receptor binding has been attained.

2. Quantum-Mechanical Energy Calculations

A detailed discussion of the methods employed for the quantum-mechanical treatment of the conformation of peptides and polypeptides is beyond the scope of this review, although the interested reader should consult the review of Pullman and Pullman[230] and the references therein. Although quantum-mechanical calculations are not prejudiced by the appropriateness of empirical input, the results of these calculations are also not constrained by realistic energies. In general, the peptide length that can be easily accommodated in such calculations seldom exceeds four amino acids. Further, there are few examples of reverse turn-containing peptides which have been examined by both quantum-mechanical and semiempirical energy calculations. However, in the case of [Met^5]-enkephalin, the quantum-mechanical calculations of Loew and Burt[227] predicted β-turn conformations which were quantitatively similar to their semiempirical energy calculations, as well as to those of others.[224-226] In contrast, the recent quantum-mechanical calculations of thyrotropin-releasing factor by Flurry et al.[231] predicted an extended structure for the molecule in agreement with the semiempirical energy calculations of Burgess et al.,[223] but in marked contrast to the semiempirical prediction of Blagdon et al.,[222] which suggested a reverse turn within the molecule.

IV. REVERSE TURNS IN PROTEINS

A. X-ray Diffraction Evidence for Reverse Turns in Proteins

At present, the identification and characterization of reverse turns in proteins can be definitively accomplished only by the use of X-ray diffraction analysis. It is foreseeable that NMR and CD may yield results applicable to the determination of reverse turns in proteins, as these methods are refined and improved (*vide infra*), but to date all conclusions about the frequencies of occurrence, sequences, and conformations of turns in proteins have been based on available protein crystal structures. Although these structures are numerous, not all are available at high enough resolution to allow dihedral angles and hydrogen bond distances to be determined. A recent review by Matthews[232] provides a description of state-of-the-art methodology in protein X-ray analysis, extensive references on the method and the various determined structures, and a tabulation of structures which had been solved when the review was written.

Chou and Fasman[163] searched a set of 29 proteins for which X-ray data were available, and found examples of 459 β-turns, defined by the criterion that the $C^{\alpha}_{i} \cdots C^{\alpha}_{i+3}$ distance be ≤7 Å (excluding helical regions). They tabulated the types of turns (classified according to Lewis et al.[23]), the sequences, the dihedral angles (ϕ_{i+1}, ψ_{i+1}; ϕ_{i+2}, ψ_{i+2}), the $C^{\alpha}_{i} \cdots C^{\alpha}_{i+3}$ and $N_{i+3} \cdots O_{i}$ distances. This extensive and extremely useful compendium allowed these authors to predict, from a statistical analysis of the sequences of these β-turns, the occurrence of β-turns in other proteins. This predictive method and several alternative methods are described more fully in Section IV.C. Included there is Table 13, which summarizes the frequency of occurrence of amino acids in β-turns derived from the data sets analyzed by various authors.

Table 10 presents a subset of the data compiled by Chou and Fasman,[163] selected by using a more restricted definition of a β-turn. Only those β-turns which have an $N_{i+3} \cdots O_{i}$ distance ≤3.5 Å and which therefore can be considered to be hydrogen bonded are included. Rather than listing the turns according to protein, Table 10 lists them by β-turn type[18] in order to emphasize conformational trends. In addition, any β-turns with large angular deviations (>50°) from Venkatachalam's[18] predicted geometries for the various types were examined using molecular models to ensure that their inclusion as β-turns is justified (viz., hydrogen bonding possible and overall geometry resembling others of the same turn type). The resulting tabulation comprises 210 examples of β-turns.

Several observations can be made from an inspection of the data collected on the hydrogen-bonded β-turns in Table 10: (1) Type I turns are represented by the largest number of examples [94 (45%)], followed by III [46 (22%)] and II [38 (18%)]. The mirror image types are observed much less frequently: I′ [9 (4%)]; II′ [16 (8%)]; III′ [7 (3%)]. (2) Although there is significant scatter among the dihedral angles adopted by the various residues within a particular β-turn type, the means and standard deviations for these dihedral angles at each position in each β-turn type are similar to Venkatachalam's[18] original predictions. The calculated standard deviations indicate that these dihedral angles are within approximately 25° of the predicted angles (*vide infra*).

		Dihedral Angles (Degrees)			
		ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}
Type I					
	X-ray data (94 examples)	−58 ± 15	−34 ± 23	−93 ± 24	3 ± 18
	Predicted[18]	−60	−30	−90	0
Type II					
	X-ray data (38 examples)	−55 ± 18	125 ± 17	87 ± 25	3 ± 29
	Predicted[18]	−60	120	80	0
Type III					
	X-ray data (46 examples)	−52 ± 18	−36 ± 19	−60 ± 14	−30 ± 19
	Predicted[18]	−60	−30	−60	−30

Table 10
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

β-Turns[a]

β-Turn type	Protein	Sequence[b] i + 1	Sequence[b] i + 2	Dihedral angles (degrees)[c] φi + 1	ψi + 1	φi + 2	ψi + 2	NH···OC (Å)[d]
Type I	Carbonic anhydrase C (human)	Glu 13	His 14	−29	−34	−98	12	2.6
		Asn 100	Gly 101	−72	−23	−81	20	3.5
		Thr 124	Lys 125	−50	−33	−90	36	3.2
		Pro 153	Gly 154	(−60)[e]	−44	−67	5	2.9
		Pro 179	Arg 180	(−60)[e]	27	−98	−43	3.5
	Carboxypeptidase A (bovine)	Thr 4	Asn 5	−73	−34	−82	−17	3.3
		Asn 5	Thr 6	−82	−17	−99	13	3.3
		Pro 30	Gly 31	−59	−11	−117	23	3.2
		Tyr 42	Glu 43	−56	−36	−87	−15	2.9
		Arg 124	Leu 125	−67	−13	−95	9	3.0
		Ala 241	Asn 142	−44	−40	−81	24	2.7
		Pro 160	Cys 161	−62	−19	−85	−12	2.7
		Glu 163	Thr 164	−65	−44	−79	21	3.0
		Ile 243	Ile 244	−36	−62	−62	7	2.4
		Ala 283	Ser 284	−61	−41	−61	18	3.4
		Ser 284	Gln 285	−61	18	−128	2	2.6
	Chromatium high potential iron protein	Ala 4	Asn 5	−78	−18	−98	24	3.4
		Ala 9	Asp 10	−64	−33	−88	17	3.1
		Thr 24	Lys 25	−67	−13	−108	3	3.0
		Glu 39	Glu 40	−47	−10	−117	4	2.6
		Ala 44	Asp 45	−49	−36	−118	43	3.1
		Gln 47	Phe 48	−55	−44	−72	−4	3.1
		Gln 64	Leu 65	−48	−35	−85	−3	2.6
		Val 73	Asn 74	−53	−20	−106	18	2.7
		Ala 78	Ser 79	−53	−11	−95	8	3.0
	α-Chymotrypsin (bovine)	Thr 62	Ser 63	−60	−17	−87	−18	3.1
		Ser 92	Lys 93	−64	−33	−102	10	3.3
		Ser 96	Leu 97	−62	−8	−114	−33	2.8
		Asp 178	Ala 179	−48	−24	−93	−3	2.8
		Ser 218	Thr 219	−56	−36	−98	8	2.9
		Thr 232	Ala 233	−40	−45	−89	7	2.7
	Concanavalin A (jack bean)	Asp 16	Ile 17	−49	−15	−99	−53	3.3
		Val 57	Asp 58	−41	−68	−81	−32	3.3
		Thr 150	Asp 151	−36	−62	−131	26	3.1
		Ser 184	Ser 185	−42	−67	−100	21	3.3
		Ile 217	Asp 218	−57	−19	−89	−8	2.8
	Cytochrome b_5 (calf liver)	Pro 40	Gly 41	−71	−1	−97	−7	3.1
		Pro 81	Asp 82	−60	−11	−97	−30	2.7
	Cytochrome c (horse)	His 33	Gly 34	−111	−58	−94	24	2.9
	Elastase (porcine)	Arg 24	Asn 25	−52	−16	−112	−9	2.9
		Pro 28	Ser 29	−83	24	−115	−8	3.3
		Gln 49	Asn 50	−52	−30	−113	7	3.1
		Cys 58	Val 59	−61	−13	−115	23	3.5
		Leu 73	Asn 74	−56	−52	−78	1	3.1
		Pro 92	Tyr 93	−62	−18	−93	−1	2.8
		Thr 96	Asp 97	−42	−32	−73	−8	2.8
		Val 99	Ala 99A	−90	−31	−69	0	3.1
		Ala 99A	Ala 99B	−69	0	−95	−21	3.0
		Ser 116	Tyr 117	−50	−24	−91	−17	3.0
		Asn 178	Ser 179	−50	−9	−89	11	2.9
		Arg 217A	Leu 218	−66	−33	−82	1	2.9
		Ser 232	Ala 233	−56	−5	−111	5	2.9

Table 10 (continued)
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

β-Turns[a]

β-Turn type	Protein	Sequence[b]		Dihedral angles (degrees)[c]				NH···OC (A)[d]
		i + 1	i + 2	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}	
	Flaxodoxin (*Clostridium MP*)	Ser 36	Asp 37	−66	−29	−95	3	3.3
		Leu 44	Asn 45	−66	−12	−98	13	2.8
		Asp 122	Gly 123	9	−48	−94	−28	2.2
	α-Hemoglobin (horse)	Pro 44	His 45	−68	−10	−87	−28	3.5
	β-Hemoglobin (horse)	Lys 120	Asp 121	−59	−21	−79	−11	2.7
	Hemoglobin (lamprey)	Ser 28	Asn 29	−43	−54	−77	2	2.3
		Pro 53	Lys 54	−60	−82	−55	31	3.3
	Hemoglobin (midge larva/ erythro-cruorin)	Lys 17	Gly 18	−53	5	−92	−15	3.0
		Thr 39	Gln 40	−48	−15	−100	−26	2.6
		Pro 89	Arg 90	(−60)[e]	−22	−103	37	3.1
		Ala 116	Ala 117	−74	−9	−140	9	3.4
	Lactate dehydrogenase (dogfish)	Lys 129	His 130	−51	−38	−96	1	3.2
		Pro 132A	Asp 132B	(−60)[e]	−29	−89	27	3.3
		Trp 203	Ser 204	−80	4	−99	3	3.3
		Met 262	Lys 263	−52	−50	−95	28	2.9
	Lysozyme (hen egg-white)	Thr 40	Gln 41	−69	−17	−84	−3	3.4
		Thr 47	Asp 48	−52	−37	−81	−3	3.2
		Ile 55	Leu 56	−62	−31	−108	7	3.3
		Leu 75	Cys 76	−62	−23	−86	−15	2.9
	Myogen (carp muscle)	Ala 3	Gly 4	−32	−56	−139	30	2.8
		Gln 52	Asp 53	−45	−76	−74	−1	3.4
	Myoglobin (sperm whale)	Lys 77	Lys 78	−41	−20	−83	−5	2.4
	Papain (papaya)	Gln 9	Lys 10	−63	7	−104	−10	2.8
		Leu 202	Tyr 203	−36	−28	−102	7	2.9
	Staphylococcal nuclease	Lys 84	Tyr 85	−83	−13	−91	0	3.5
		Thr 120	His 121	−79	−17	−120	32	3.5
	Subtilisin BPN'	Ser 24	Asn 25	−51	−59	−87	22	3.2
		Ser 37	Ser 38	−90	−10	−99	18	3.3
		Pro 40	Asp 41	−44	−23	−92	−6	2.4
		Pro 52	Ser 53	−38	−43	−98	−6	3.0
		Ala 116	Asn 117	−54	−24	−117	34	2.9
		Pro 172	Ser 173	−53	−10	−95	9	3.2
		Ser 182	Ser 183	−69	−9	−107	9	3.1
		Ser 188	Phe 189	−46	−38	−91	11	2.9
		Pro 194	Glu 195	−69	−26	−100	3	3.5
		Thr 220	Ser 221	−59	−10	−101	4	3.0
		Pro 239	Asn 240	−57	−1	−101	−21	2.7
	Thermolysin	Val 13	Leu 14	−59	−36	−87	7	3.4
		Ser 134	Gly 135	−72	−21	−84	2	2.8
		Pro 208	Ala 209	−54	−34	−70	−7	2.7
		Ser 218	Lys 219	−60	−14	−105	−18	2.6
		Pro 277	Thr 278	−58	−25	−116	5	3.5
Type I'	Carbonic anhydrase C (human)	Asp 109	Lys 110	52	49	85	−15	3.4
	α-Chymotrypsin (bovine)	Asn 204	Gly 205	45	31	85	−41	2.9
	Elastase (porcine)	Asn 204	Gly 205	64	38	75	15	2.9

Table 10 (continued)
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

β-Turns[a]

β-Turn type	Protein	Sequence[b]		Dihedral angles (degrees)[c]				NH···OC (Å)[d]
		i + 1	i + 2	φi + 1	ψi + 1	φi + 2	ψi + 2	
	Lactate dehydrogenase	Gly 221	Val 222	9	75	89	−26	2.3
	Lysozyme (hen egg-white)	Arg 21	Gly 22	52	16	92	13	3.1
	Myoglobin (sperm whale)	Lys 79	Gly 80	43	45	93	−16	3.0
	Papain (papaya)	Asn 184	Gly 185	64	29	101	−9	3.1
		Cys 200	Gly 201	51	17	81	9	2.8
	Staphylococcal nuclease	Asp 95	Gly 96	68	34	115	−23	3.3
Type II	Carboxypeptidase A	Phe 151	Gly 152	−56	132	83	16	3.2
		Ala 154	Gly 155	−53	120	136	26	3.4
		Ala 170	Asn 171	−80	107	95	16	3.3
	Chromatium high potential iron protein	Ala 54	Gly 55	−75	144	88	2	3.5
		Pro 67	Gly 68	−48	137	88	−21	3.0
	α-Chymotrypsin (bovine)	Pro 24	Gly 25	−60	134	72	9	2.8
		Asn 100	Asn 101	−64	150	57	47	3.5
		Ala 132	Gly 133	−61	152	86	−12	3.3
		Met 192	Gly 193	−69	133	110	−27	3.1
		Ser 195	Gly 196	−53	144	89	−11	2.9
	Concanavalin A (jack bean)	Asp 44	Gly 45	−60	133	76	6	3.0
		Pro 68	Asn 69	−66	127	83	−33	3.2
		Ser 223	Gly 224	−74	120	66	42	3.5
	Cytochrome b_5 (calf liver)	Ala 50	Gly 51	−57	128	83	34	3.2
	Cytochrome c (horse)	Lys 22	Gly 23	−13	86	105	−11	2.6
		Phe 36	Gly 37	−41	120	104	−24	3.2
		Pro 76	Gly 77	−54	112	132	−17	2.5
	Cytochrome c_2 (*Rhodos rubrum*)	Gln 22	Gly 23	−41	110	110	−11	2.6
		Ala 57	Lys 58	−88	106	108	−46	3.0
	Elastase (porcine)	Ala 126	Gly 127	−58	144	84	−22	3.3
		Asn 132	Asn 133	−72	144	76	21	3.3
		Thr 147	Asn 148	−78	129	61	41	2.9
		Gln 192	Gly 193	−53	139	105	−25	3.0
		Ser 195	Gly 196	−44	145	65	4	3.1
		Thr 222	Arg 223	−65	135	58	15	2.9
	Flavodoxin (*Clostridium* MP)	Ser 78	Gly 79	−69	145	84	−26	3.3
	Hemoglobin (glycera)	Gly 19	Asn 20	−21	109	84	42	2.7
	α-Hemoglobin (horse)	Pro 77	Gly 78	−64	94	144	−52	3.4
	Lactate dehydrogenase (dogfish)	Met 60	His 158	−60	113	137	8	3.4
		Gly 164	Cys 165	−8	96	105	−40	3.2
		Asp 197	Ser 198	−51	115	106	11	2.4
		Asp 295	Gly 296	−26	87	36	49	2.8

Table 10 (continued)
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

β-Turns[a]

β-Turn type	Protein	Sequence[b]		Dihedral angles (degrees)[c]				NH···OC (Å)[d]
		i + 1	i + 2	ϕ_{i+1}	ψ_{i+1}	ϕ_{i+2}	ψ_{i+2}	
	Lysozyme (hen egg-white)	Asp 18	Asn 19	−64	123	60	15	3.2
		Tyr 20	Arg 21	−63	130	52	16	2.9
		Lys 116	Gly 117	−38	117	76	35	3.3
		Arg 125	Gly 126	−67	141	96	−28	3.5
	Myogen (carp muscle)	Ala 21	Asp 22	−36	121	68	54	3.1
	Papain (papaya)	Val 199	Cys 200	−57	137	51	17	3.1
Type II′	Carboxypeptidase A (bovine)	Gly 278	Phe 279	51	−122	−70	−24	2.6
	α-Chymotrypsin (bovine)	Gly 173	Thr 174	62	−132	−39	−32	3.2
	Concanavalin A (jack bean)	Gly 144	Asp 145	49	−107	−106	14	3.4
		Gly 227	Arg 228	74	−137	−81	−9	3.4
	Elastase (porcine)	Gly 368	Ser 369	38	−96	−61	−35	3.3
		Gly 173	Ser 174	64	−153	−68	−8	3.5
	α-Hemoglobin (horse)	Gly 18	Gly 19	32	−111	−92	26	2.8
	Insulin (porcine A and B chains)	Gly B8	Ser B9	84	−107	−92	−24	3.0
	Lactate dehydrogenase	Ala 31	Val 32	0	−120	−73	−3	3.1
		Ala 209	Lys 210	91	−103	180	29	3.2
	Lysozyme (hen egg-white)	Gly 104	Met 105	57	−148	−74	7	3.1
	Papain (papaya)	Gly 20	Ser 21	65	−127	−100	21	3.4
	Staphylococcal nuclease	Gly 20	Asp 21	−4	−83	−86	32	2.5
	Subtilisin BPN′	Gly 160	Ser 161	46	−110	−136	−6	3.4
		Gly 264	Lys 265	70	−138	−67	−21	3.1
	Thermolysin	Gly 36	Asp 37	37	−119	−79	7	2.3
Type III	Carbonic anhydrase C (human)	Gly 81	Pro 82	−35	−56	(−60)[e]	10	2.5
	Carboxypeptidase A (bovine)	Ser 70	Arg 71	−25	−63	−33	−51	2.9
	Chromatium high potential iron protein	Ala 23	Thr 24	−48	−29	−67	−13	2.8
		Pro 38	Gly 39	−55	−48	−47	−10	2.9
		Cys 43	Ala 44	−45	−36	−49	−36	3.1
	α-Chymotrypsin (bovine)	Pro 28	Trp 29	−56	−34	−75	−22	3.0
		Ala 56	His 57	−77	−36	−65	−6	3.3
		His 57	Cys 58	−65	−6	−72	−17	3.1
		Thr 174	Lys 175	−39	−32	−66	−26	2.9
		Val 231	Thr 232	−59	−45	−40	−45	3.1
	Concanavalin A	Thr 15	Asp 16	−44	−44	−49	−15	2.9
	Cytochrome b_5 (calf liver)	Ser 18	Lys 19	−70	−8	−78	−53	3.2
		Thr 65	Asp 66	−75	−18	−66	−51	3.5

Table 10 (continued)
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

β-Turns[a]

β-Turn type	Protein	Sequence[b] i + 1	Sequence[b] i + 2	Dihedral angles (degrees)[c] φi + 1	ψi + 1	φi + 2	ψi + 2	NH···OC (A)[d]
	Elastase (porcine)	Ala 56	His 57	−34	−45	−41	−52	3.2
		His 57	Cys 58	−41	−52	−61	−13	3.0
		Ser 170A	Ser 170B	−72	−25	−77	−23	2.9
	Flavodoxin (*Clostridium* MP)	Val 35	Ser 36	−53	−33	−66	−29	2.6
		Ile 40	Asp 41	−63	−55	−17	−50	3.1
	α-Hemoglobin (horse)	Pro 114	Asn 115	−67	−37	−74	−36	3.3
	β-Hemoglobin (horse)	Asp 43	Ser 44	−69	7	−54	−21	3.2
		Lys 82	Gly 83	−23	−29	−79	−63	2.6
	Hemoglobin (midge larva/erythrocruorin)	Pro 32	Ser 33	(−60)[e]	−19	−55	−59	3.3
	Lactate dehydrogenase (dogfish)	Val 87	Ser 88	−64	−19	−72	−28	3.0
		Ser 184	Cys 185	2	−58	−40	−74	2.9
		Cys 185	Leu 186	−40	−74	−65	−55	3.5
		Lys 213	Asp 214	−57	−32	−44	−60	2.5
		Ser 238	Asn 239	−24	−75	−42	−38	3.4
		Pro 309	Asp 310	(−60)[e]	−35	−58	−36	2.9
	Lysozyme (hen egg-white)	Pro 70	Gly 71	−53	−40	−66	−30	3.3
		Val 99	Ser 100	−62	−25	−76	−23	3.3
		Met 105	Asn 106	−74	7	−72	−16	3.4
		Asn 106	Ala 107	−72	−16	−66	−16	3.1
	Myogen (carp muscle)	Thr 36	Ser 37	−34	−68	−44	−36	2.6
		Ala 72	Asp 73	−36	−37	−76	−18	2.6
		Ser 91	Asp 92	−40	−66	−49	−11	2.8
	Myoglobin (sperm whale)	Asp 44	Arg 45	−61	−42	−67	−9	3.2
		Lys 47	His 48	−38	−53	−41	−35	2.5
	Papain (papaya)	Arg 58	Arg 59	−92	−14	−68	−20	3.4
		Arg 98	Glu 99	−47	−50	−65	−36	2.9
		Ser 196	Tyr 197	−62	−38	−62	−53	3.1
	Ribonuclease S (bovine)	Lys 66	Asn 67	−35	−42	−59	6	2.5
	Subtilisin BPN′	Gly 169	Lys 170	−64	−44	−66	−18	3.3
		Ser 260	Phe 261	−30	−43	−78	−19	3.2
	Thermolysin	Ser 65	Tyr 66	−54	−25	−71	−23	2.8
		Gly 160	Ser 161	−67	−44	−59	−38	3.1
		Ala 209	Lys 210	−70	−7	−55	−35	3.3
Type III′	Concanavalin A (jack bean)	Leu 230	Gly 231	20	22	53	36	2.9
	Cytochrome b_5 (calf liver)	His 26	Tyr 27	35	57	59	4	2.3
	Flavodoxin (*Clostridium* MP)	Gly 57	Asp 58	−1	71	66	63	2.4
	Lysozyme (hen egg-white)	Asn 37	Phe 38	57	39	59	19	3.1
	Thermolysin	Lys 45	Tyr 46	26	35	74	29	2.9
		Asp 226	Asn 227	34	58	44	52	3.4
		Asn 227	Gly 228	44	52	83	44	2.3

Table 10 (continued)
REVERSE TURNS IN PROTEINS DETERMINED FROM X-RAY DIFFRACTION

γ-Turns[f]

Protein	Sequence	Dihedral angles (degrees)		NH···OC (Å)[g]
		[c]i + 1	[e]i + 1	
Thermolysin	Thr 26	86	−57	1.6

[a] The data presented were taken from Table 2 of Chou and Fasman.[163]
[b] All nonglycyl residues are of the L-configuration.
[c] Designation of bonds and definition of the principal torsion angles is according to the IUPAC-IUB Commission on Biochemical Nomenclature;[411] ϕ denotes the rotation about N-C$^\alpha$, ψ denotes the rotation about C$^\alpha$-C, and ω denotes the rotation about C-N. i denotes the first residue of a reverse turn with successive numbering from the amino-terminal to the carboxyl-terminal end of the chain (e.g., i + 1, i + 2, etc.).
[d] NH···OC, hydrogen bond length, given in terms of the N···O distance, i + 3 → i.
[e] The Pro ϕ was not reported by Chou and Fasman,[163] and is here indicated to be near to −60° due to the constraints of the pyrrolidine ring.
[f] From Matthews.[2]
[g] NH···OC, hydrogen bond length, given in terms of the H···O distance, i + 2 → i.

Table 11
FREQUENCY OF OCCURRENCE OF AMINO ACIDS IN β-TURNS IN PROTEINS ACCORDING TO β-TURN TYPE AND POSITION IN TURN[a]

	Residue position in β-turn													
	i + 1 β-Turn type[b]							i + 2 β-Turn type[b]							
Amino acid	I	I′	II	II′	III	III′	Sub-total	I	I′	II	II′	III	III′	Sub-total	Total occurrence
Ala	10	0	8	2	5	0	25	7	0	0	0	2	0	9	34
Arg	3	1	1	0	2	0	7	2	0	2	1	3	0	8	15
Asn	3	3	2	0	1	2	11	12	0	7	0	4	1	24	35
Asp	3	2	4	0	2	1	12	13	0	1	3	7	1	25	37
Cys	1	1	0	0	2	0	4	2	0	2	0	3	0	7	11
Gln	5	0	1	0	0	0	6	3	0	0	0	0	0	3	9
Glu	3	1	1	0	1	0	6	5	0	0	0	2	0	7	13
Gly	0	0	2	14	2	1	19	7	7	23	1	2	2	42	61
His	1	0	0	0	2	1	4	4	0	1	0	3	0	8	12
Ile	3	0	0	0	1	0	4	2	0	0	0	0	0	2	6
Leu	4	0	0	0	0	1	5	6	0	0	0	1	0	7	12
Lys	5	1	2	0	4	1	12	8	1	1	2	4	0	16	28
Met	1	0	2	0	1	0	4	0	0	0	1	0	0	1	6
Phe	0	0	2	0	0	0	2	2	0	0	1	1	1	5	7
Pro	19	0	5	0	6	0	30	0	0	0	0	1	0	1	31
Ser	14	0	4	0	9	0	27	12	0	1	5	8	0	26	53
Thr	12	0	2	0	4	0	18	5	0	0	1	2	0	8	27
Trp	1	0	0	0	0	0	1	0	0	0	0	1	0	1	2
Tyr	1	0	1	0	0	0	2	3	0	0	0	2	2	7	9
Val	4	0	1	0	4	0	9	1	1	0	1	0	0	3	12

[a] The data presented here were drawn from Table 2 of Chou and Fasman,[163] as described in the text.
[b] β-Turn types are as defined by Venkatachalam.[18]

The mirror image types have average dihedral angles close to the expected values (i.e., equal in magnitude and opposite in sign to the types above). (3) An analysis of the frequency of occurrence of particular amino acids in positions i + 1 or i + 2 of the various β-turn types is shown in Table 11. It can readily be seen from the total number

Table 12
RECURRENT DIPEPTIDE SEQUENCES AT THE i + 1th AND i + 2th POSITIONS OF β-TURNS IN PROTEINS

Sequence and β-turn position		Total occurrence	Occurrence within various β-turn types*					
i + 6	i + 2		I	I′	II	II′	III	III′
Pro	Gly	7	2	0	4	0	1	0
Ala	Gly	6	1	0	5	0	0	0
Asn	Gly	5	1	3	0	0	0	1
Gly	Ser	5	0	0	0	5	0	0
Lys	Gly	5	1	1	2	0	1	0
Ser	Gly	5	1	0	4	0	0	0
Thr	Asp	5	3	0	0	0	2	0
Ala	Asn	4	3	0	1	0	0	0
Gly	Asp	4	0	0	0	3	0	1
Pro	Asp	4	3	0	0	0	1	0
Pro	Ser	4	3	0	0	0	1	0
Ser	Ser	4	3	0	0	0	1	0
Ala	Lys	3	0	0	1	1	1	0
Asp	Gly	3	0	1	2	0	0	0
Pro	Asn	3	1	0	1	0	1	0
Pro	Glu	3	2	0	0	0	1	0
Ser	Lys	3	2	0	0	0	1	0
Ser	Tyr	3	1	0	0	0	2	0
Ser	Asn	3	2	0	0	0	1	0
Thr	Lys	3	2	0	0	0	1	0
Thr	Ser	3	2	0	0	0	1	0
Val	Ser	3	0	0	0	0	3	0

* β-Turn types are as defined by Venkatachalam.[18]

of times each amino acid occurs that Gly (61 examples), Ser (53), Asp (37), Asn (35), Ala (34), Pro (31), Lys (28), and Thr (27) are frequent residues in β-turns. Many insights into this general finding are provided by the breakdown according to position and β-turn type in Table 11. For example,

1. The i + 1th position of the type II′ β-turns is occupied 14 out of 16 times by Gly and the remaining two by Ala.
2. Pro occurs 30 times in the i + 1th position and only once in the i + 2th position. In addition, Pro is seen most often in type I turns.
3. The i + 2th position of type II β-turns is occupied 23 out of 38 times by Gly.
4. Eight residues have high frequencies of occurrence in the 1 + 1th position: Pro (30), Ser (27), Ala (25), Gly (19), Thr (19), Lys (12), Asp (12), and Asn (11). Among these, Asn and Asp occur in all turn types except II′, Gly occurs frequently in type II′, and the other residues are predominatly found in types I, II, or III.
5. Five residues are particularly frequent in the i + 2th position: Gly (42 examples), Ser (26), Asp (25), Asn (24), and Lys (16). Among these residues the nonglycyl residues are encountered most often in type I and III, although Ser is seen five times in type II′ β-turns.

Table 12 presents the 22 dipeptide sequences which recur three or more times in the

set of 210 β-turns. Both their overall frequencies of occurrence and a compilation of the occurrences in the various β-turn types are shown. Only eight out of the 88 β-turns deviate from the expected β-turn types predicted from stereochemical consideration (see Section II), and three of these involve Asn-Gly sequences which occur in type I′ β-turns.

Multiple factors, including steric requirements, hydrophilicity, and conformation required for activity or recognition, contribute to the occurrence of the particular residues or sequences in the observed β-turns. Hence, it is difficult to speculate about rationales for the trends and frequencies presented. Nonetheless, it is tempting to ascribe to Gly the role of a "pseudo-D" residue, which may readily occur in the i + 1^{th} position of type II′ β-turns or the i + 2^{th} position of type II β-turns, due to its lack of a side chain. Beyond this suggestion, and the previously recognized prevalence of polar residues,[6] too many factors are intertwined to warrant definitive conclusions at this time.

Only one example of a protein γ-turn has been reported to date, namely, that described by Matthews[2] from his X-ray structure analysis of thermolysin (Ser[25]-Thr[26]-Tyr[27]: $\phi_{25} = 148°$, $\psi_{25} = 92°$; $\phi_{26} = 86°$, $\psi_{26} = -57°$; $\phi_{27} = -114°$, $\psi_{27} = 148°$). The lack of other reported examples may derive from the fact that appropriate searches have not been carried out, rather than from the rarity of γ-turns in proteins. However, a definitive search is necessary in order to establish the frequency of occurrence of γ-turns. Interestingly, the one turn reported does not follow the expected conformational preference for the i + 1^{th} residue of a γ-turn, since it is an example of an L-residue occurring in a γ-turn, and not in an inverse γ-turn (see Section II).

B. Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) Evidence for Reverse Turns in Proteins

Although few studies using CD[151,152] and none using NMR to assign reverse turn structures in proteins have been reported, both of these methods show promise of making significant contributions to this field in the future.

The approach to conformational analysis of proteins based on CD has been briefly mentioned in Section III.C. Such an analysis depends upon a breakdown of the observed CD spectrum for a protein into contributions from the various types of secondary structure (α-helix, β-structure, reverse turns, and "random coil").[145] Woody's[19] CD calculations for β-turns unfortunately reveal that there is not a unique CD which can be said to be characteristic of a β-turn structure (see Tables 7 and 8). Hence, it is difficult to deduce unequivocally the presence of β-turns in proteins from CD.

NMR analyses of protein conformation have become feasible due to new methods of spectral simplification and to improvements in instrumentation. No studies thus far have been specifically concerned with the elucidation of turns, but the following examples illustrate state-of-the-art techniques which provide details of protein structures in solution, and may be applied to turn regions: (1) high field, high resolution ^1H NMR of well-separated resonances;[233-237] (2) paramagnetic probes[238,239] or lanthanide shift reagents;[240] and (3) transient NOE analysis.[241]

C. Correlative Predictive Methods: Evidence for Reverse Turns in Proteins

Three reviews dealing exhaustively with the methods for empirical prediction of protein structure and their applications have been published.[242-244] Although these reviews have discussed the prediction of various types of protein secondary structure (e.g., α-helices, β-sheets, and reverse turns), only the application and success of these methods for the prediction of reverse turns will be discussed in this section.

In all the correlative methods, the first stage is the development of sets of frequencies

of occurrence or equilibrium constants, which describe the likelihood of adoption of specific secondary structures by the 20 common amino acids. Such a development rests on the statistical analysis of protein crystallographic data or on semiempirical physicochemical parameters associated with the amino acids. The predictive capacities of these methods are limited in their accuracy by (1) the extent of the input data (i.e., the number of proteins or the number of experimental measurements), (2) the correctness of conformational assignments, and (3) the appropriateness of the data set to the protein conformation being predicted. For example, the interpretation of protein crystallographic data suffers from potential distortions due to the subjective nature of the analysis. In addition, the criteria for the identification of the various structural features have differed among studies.

Early attempts to determine the conformational preferences of amino acids for protein β-turns relied on X-ray crystallographic data which identified β-turn regions by specifying a maximum distance between the C^{α} atoms belonging to the i^{th} and $i + 3^{rd}$ residues (e.g., ≤ 7 Å;[8,23,245,246] $\sim$4.8-4.9 Å;[6] and <5.7 Å[5]) or by Venkatachalam's[18] criteria.[247] Rose and Seltzer[248] suggested an objective method for finding turns from protein X-ray structures based on a geometric analysis which involves searching for local minima in the radius of curvature of the protein chain.

The second stage is the application of the frequencies or equilibrium constants using a specific mathematical approach to elucidate the secondary structure of a protein. In one type of analysis exemplified by the approach of Chou and Fasman,[249,250] the frequencies were analyzed statistically using groups of four contiguous residues as basic units. The premise of the Chou-Fasman method is that the predictive accuracy will be sufficient using tetrapeptide sequences. Initially, their data set was based on a statistical analysis of 15 proteins.[250] Later they added 14 more proteins to their set.[163] As was the case for the β-turn criteria proposed by Lewis et al.[8,23] Chou and Fasman[163] considered any four-residue sequence whose C^{α}_i to C^{α}_{i+3} distance was ≤ 7 Å and whose sequence was not included in an helical region to be a β-turn. Chou and Fasman[163] also observed that hydrophobic residues occur more frequently than hydrophilic residues in the regions immediately adjacent to β-turns and that these regions often consist of β-sheets and/or α-helices, as was also found by Nagano.[251] Using their algorithm, Chou and Fasman[163] predicted 78% of the β-turns in 29 proteins (i.e., the same proteins used to derive these frequencies initially) to within $\pm$ 2 residues in all conformations.

The Chou and Fasman[163,243,244,250] correlative method is the most widely employed procedure for predicting the occurrence of reverse turn conformations in proteins.[252] Its widespread use has resulted primarily from the easy application of its predictive rules and the advantage that little mathematical skill is required.

Analyses by other statistical methods have also been applied to X-ray crystallographic data from larger sets of proteins. Nagano[253] analyzed conformational data from 95 proteins belonging to 13 homologous families and correctly predicted the location of 64.4% of the β-turns in seven proteins not included in his original data set. His data also indicated that the localization of β-turns in proteins involves interactions other than short-range.[253] In fact, when interactions between contiguous α-helices and β-sheets were included in the statistical analysis, 71% of the β-turns were then correctly predicted.[251]

Burgess et al.[245] introduced a nonapeptide predictive algorithm, for which they considered the frequencies of occurrence of β-turn residues in a set of eight proteins, to predict the location of β-turns in 13 additional proteins. This method also indicated that residues adjacent to β-turns contributed to the localization of these structures.

Levitt and Greer[254] introduced an automated procedure which assigns secondary

structure within a protein on the basis of local conformation and the pattern of hydrogen bonding. In a subsequent paper, Levitt[255] analyzed these assignments to yield frequencies of occurrence of the twenty naturally occurring amino acids within reverse turns. In contrast to the results of Chou and Fasman,[250,256] where a given amino acid preferred more than one secondary structure (e.g., Tyr in reverse turns and β-sheets), the Levitt analysis allows the convenient compartmentalization of each individual residue into a unique conformational preference. Furthermore, Gln, Lys, Tyr, Thr, Gly, and Arg were found to be indifferent to their location in a reverse turn conformation; while Ala, Leu, Met, His, Val, Ile, Phe, Cys, and Arg were reverse turn-breaking amino acids.[255]

An alternative approach to the mathematical analysis of frequencies or equilibrium constants of the individual amino acids relies on statistical mechanical methods such as those applied by Tanaka and Scheraga.[257] They have derived a four-state (i.e., α-helix, coil, β-turn, and extended structure), one-dimensional, short-range (Ising) model to predict the reverse turn conformations in proteins. Conformational sequence probabilities for finding a given residue and for observing two successive residues in a reverse turn conformation were calculated for 23 proteins. Their results predicted correctly 219 reverse turn regions out of 372 turns observed by X-ray analysis of the same set of proteins. Since they did not present data to indicate their degree of overprediction, it is not straightforward to compare their results to the other correlative methods. Various other approaches to the prediction of turns differing in some aspect(s) from those described above have been proposed but not extensively applied.

Kuntz[6] first observed that hydrophilic residues occur frequently within β-turns and suggested that this finding might be used to predict the occurrence of these structures, using a simple compositional rule. Subsequently, Rose and Wetlaufer[258] demonstrated an approximate linear relationship between the number of β-turns in a globular protein and its molecular weight, and they also suggested that local juxtapositioned sequences of amino acids are the major determinant of β-turn formation. This idea was extended by Rose[259] in his prediction method based on hydrophobicity. A similar notion was applied to the nucleation of protein folding as initiated by hydrophobic contacts.[260]

An information theory approach, based on that introduced by Robson and Pain,[261] was utilized in a β-turn predicting scheme by Maxfield and Scheraga.[262] Denisov[263] used another method involving pattern recognition to predict the occurrence, but not the location, of turns.

Finkelstein and Ptitsyn[264] coupled a statistical mechanical analysis with a thermodynamic semiempirical calculation of the equilibrium constants for amino acids in various types of secondary structure, including β-turns.

Outgrowths of the various predictive methods are the compilations of the frequencies of occurrence of amino acids in β -turns.

As shown in Table 13, the amino acid residues that occur most frequently, as determined by those statistical analytical methods, are those with short polar side chains (i.e., Asp, Asn, Ser), with a cyclic side chain (Pro), or without a side chain (Gly). There is also an increased occurrence of Tyr, as determined by four of the eight analyses.

In addition, Chou and Fasman[256] have shown that certain amino acid residues occur preferentially within β-turns, as follows: at the i^{th} position: Asn, Asp, and Cys; at the $i + 1^{th}$ position: Pro, Ser, Lys; at the $i + 2^{th}$ position: Asn, Asp, and Gly; and at the $i + 3^{th}$ position: Trp, Gly, and Tyr. These observations serve to illustrate that additional amino acids (e.g., Cys, Lys, and Trp) do occur frequently at specific positions within β-turns, but that the effect of including all the residues at the other positions in the β-turns has tended to mask the position-specific frequency of some residues. The

Table 13
AMINO ACIDS WITH FREQUENT OCCURRENCE IN PROTEIN β-TURNS AS DETERMINED BY STATISTICAL ANALYSIS

Amino acids frequently occurring in β-turns[a]	Number of proteins used for analysis	Ref.
Gly, Pro, Asp, Asn, Ser, Thr, Tyr, Trp	3	8
Gly, Pro, Ser	6	246
Gly, Pro, Asn, Ser, Tyr, Arg	95[b]	251
Gly, Asp, Asn, Ser, Thr, Ala	8	23
Gly, Pro, Asn, Tyr	7	5
Gly, Pro, Asp, Asn, Tyr, Arg, Lys	8	245
Gly, Pro, Asp, Asn, Ser	29	163
Gly, Pro, Asp, Asn, Ser	>50	255

[a] The order is arbitrary but consistent between entries for ease of comparison. Data specifically detailing the frequency of occurrence of each amino acid are available only in References 246 and 256.
[b] This is an artificially high value due to the inclusion of homologous proteins in the data set.

data of Table 13 may be compared with the data in Table 11 (see Section IV.A for further discussion).

V. MULTI-FACETED FUNCTIONAL ROLE OF REVERSE TURNS

The surface localization of β-turns in proteins and the predominance in turns of amino acids containing potentially reactive functional groups in their side chains (e.g., Asn, Ser, Pro, Asp and Lys) lend credence to the suggestion that at least some β-turns act as recognition sites for the initiation of complex immunological, metabolic, hematological, and endocrinological reactions. The extent to which β-turns have been definitely implicated in biological mechanisms is presently limited to isolated examples. However, taken as a whole, these examples indicate that the functional role of β-turns in nature is extensive. Hence, this element of protein structure must be thoroughly understood by every biologist and chemist who is concerned with proteins and peptides.

A. Immunological Recognition

1. Antigenic Sites in Proteins

Antigenic sites of proteins generally occupy a surface location and, because β-turns share this tendency, it is likely that at least certain β-turn regions will function as antigenic sites. Although certain turns in sperm whale myoglobin, hen egg-white lysozyme, and staphylococcal nuclease have been demonstrated to be coincident with antigenic sites in these proteins, it is not clear whether this finding related to an inherent recognition of such structures by the immune system or whether it is related only to the

frequency of occurrence of β-turn structural elements among the other structures monitored by immune surveillance. However, by examining the location of antigenic sites in relation to the position of β-turns in those various well-characterized protein antigens, several definite conclusions can be drawn.

Atassi and co-workers have elucidated the antigenicity of sperm whale myoglobin.[265,266] This protein contained five continuous antigenic sites.[267,268] As shown in Table 14, two of these sites contain reverse turn conformations as assessed by X-ray crystallography.[163] Although not shown in Table 14, other myoglobin reverse turns exist (see Section IV.A), although they have not been implicated as myoglobin antigenic sites. Thus, in some cases a reverse turn structural element is a sufficient but not a necessary condition for an antigenic site.

Atassi and co-workers[269,270,271] have also identified three discontinuous antigenic sites in hen egg-white lysozyme (see Table 14). As shown in Table 14, site 1 consisted of residues 6, 7, 10, 13, 14, 126, and 128. Only residue 126, which is part of the type II β-turn (124-127),[23,163] was present in this antigenic site. Their antigenic site 2, consisting of residues 62, 87, 89, 93, 96, and 97, had only one residue, residue 62, contributed by a β-turn. Interestingly, residue 62 occupied the i + 3^{rd} position of a type III β-turn (59 to 62), as well as the i + 2^{nd} position of a type I β-turn (60 to 63). Site 3 consisted of residues 20, 21, 23, 33, 34, 113, 114, and 116. This site had four residues contributed by β-turns. Residue 20 occupied the i + 3^{rd} position in a type II β-turn, the i + 1^{st} position in an adjacent type II β-turn, and the i^{th} residue in another adjacent type I′ β-turn. Residue 21 occupied the i + 2^{nd} position of the type II β-turn, as well as the i + 1^{st} position of a type I′ β-turn. Residue 23 occupied only the i + 3^{rd} position of a type I′ β-turn. Residue 116 occupied the i + 1^{st} position of a type II β-turn.

The fact that 6 out of the 22 residues implicated in these antigenic sites were residues contributed by β-turns is consistent with the observation that approximately one third of the residues of globular proteins are in a reverse turn conformation[4,5] and in fact argues against β-turns being more antigenic than other protein surface structural elements.

However, the antigenicity of protein regions containing an isolated series of three adjacent and overlapping β-turns, in particular those turns containing residues 20, 21, and 23, may be a real phenomenon, since a continuous antigenic site in staphylococcal nuclease region (46 to 52), containing an isolated series of three adjacent and overlapping β-turns, has also been observed[160] (see Table 14). Certainly, one effect of three adjacent and overlapping β-turns is that they form a compact loop which extends from the surface of a protein, possibly in such a way that it enhanced the interaction of the antigen with an antibody. However, it should not be generalized that such regions will always be antigenic because, at least in the case of lysozyme, its residues (103 to 106), (104 to 107) and (105 to 108) are indeed overlapping, adjacent β-turns without apparent antigenicity. Whether this is due to a loss of the compactness of such a structure, invoked by the proximity of two additional adjacent and themselves overlapping β-turns (98 to 101) and (99 to 102), is not known. Although only the conformational aspects of various antigenic sites have been considered here, antigenicity is certainly also a function of the sequence and side chain orientations within a given site. Until the detailed antigenic structures of various other proteins have been elucidated unequivocally, the exact relationship between antigenicity and the occurrence of β-turns in proteins will remain a mystery.

2. Antigenicity of the Lysozyme "Loop" Region

Arnon and Sela[272] have attributed an antigenic site to the loop formed by the disulfide 64-80. However, it has now been unambiguously demonstrated that no such con-

Table 14
PROTEIN ANTIGENIC SITES CONTAINING RESIDUES LOCATED IN β-TURNS

Protein	Type of antigenic site	Sequence and location of antigenic site[a]	Sequence and location of X-ray crystallographically defined β-turn(s)[b]	β-Turn type(s)[b]	Ref.
Sperm whale myoglobin	Continuous[c]	Lys^{16}-Val-Gly-Ala-Asp-Val^{21}	Glu^{18}-Ala-Asp-Val^{21}	Open	265, 266
	Continuous	Ala^{94}-Thr-Lys-His-Lys-Ile^{99}	Thr^{95}-Lys-His-Lys^{98}	III	265, 266
Staphylococcal nuclease	Continuous	Lys^{24}-Leu-Met-Tyr-Lys-Gly-Gln-Pro^{31}	Met^{26}-Tyr-Lys-Gly^{29}	II	160
			Tyr^{27}-Lys-Gly-Gln^{30}	III′	
	Continuous	His^{46}-Pro-Lys-Lys-Gly-Val-Glu^{52}	Pro^{47}-Lys-Lys-Gly^{50}	Open	160
			Lys^{49}-Gly-Val-Glu^{52}	Open	
			Glu^{52}-Lys-Tyr-Gly^{55}	II	
Hen egg-white lysozyme	Discontinuous[d]	(Cys)^{6}-Gly^{7}---Ala^{10}-Lys^{13}-Arg^{14} and Gly^{126}---Arg^{128}	Ile^{124}-Arg-Gly-Cys^{127}	II	269
	Discontinuous	Trp^{62} and Leu^{65} and Asp^{87}---Thr^{89} and Asn^{93}---Lys^{96}-Lys^{97}	Asn^{59}-Ser-Arg-Trp^{62}	III	269
			Ser^{60}-Arg-Trp-Trp^{63}	I	
	Discontinuous	Tyr^{20}-Arg^{21}---Tyr^{23} and Lys^{33}-Phe^{34} and Asn^{113}-Arg^{114}---Lys^{116}	Leu^{17}-Asp-Asn-Tyr^{20}	II	269
			Asn^{19}-Tyr-Arg-Gly^{22}	II	
			Tyr^{20}-Arg-Gly-Tyr^{23}	I′	
			Cys^{115}-Lys-Gly-Thr^{118}	II	

[a] The notation (---) indicates that the intervening residues are not involved in the delineated antigenic site. The superscripted numbers refer to the residue number. ( ) Indicates that the involvement of this residue in the antigenic site is uncertain.
[b] Data from Reference 163.
[c] The term continuous was originally introduced in Reference 267 and rigorously defined in Reference 268.
[d] The term discontinuous was originally introduced in References 270 and 271, and then rigorously defined in Reference 268.

tinuous antigenic sites occur in native lysozyme[269] (see Section V.A.1), so that the antigenicity being measured by Arnon and Sela[272] and Maron et al.[273] represented the contribution of a portion of a discontinuous antigenic site and hence was determined with less efficiency than might be achieved with a complete antigenic site.[269]

Despite these limitations, Arnon et al.[274] have correlated changes in the binding capacity of synthetic loop analogs having various residues replaced by alanine to antiloop antibodies with the relative probability of occurrence of β-turns in these synthetic analogs. By substituting one amino acid residue at a time, they were able to establish a relationship between differences in antigenicity and differences in the theoretically predicted probability for β-turn formation. Since no direct measurements of the changes in β-turn conformation in the various synthetic analogs were made, and since the antigenicity of any antigenic site depends on its sequence, as well as its conformation, this correlation must be viewed with caution.

An alternative experimental approach correlating immunological measurements of the degree of cross-reaction between proteins from different species in which only amino acid substitutions occurred (i.e., no additional inversions, additions, or deletions) with the probability of β-turn formation was also carried out,[275] and for the reasons above no direct interpretation is possible. However, an interesting, albeit incidental finding of this study was that there are quantitative differences in the antiloop antibodies raised in rabbits as compared to goats. Whether this represented differences in the specific immunological recognition of β-turn conformations between species has not been satisfactorily determined, but the resolution of this question remains as a worthwhile challenge to immunochemists.

3. Tuftsin

Tuftsin is a basic tetrapeptide, H-L-Thr-L-Lys-L-Pro-L-Arg-OH isolated from a specific cytophilic γ-globulin, leucokinin.[276,277] This peptide has been shown to increase the phagocytic[276,277] and bactericidal[278] activities of macrophages and polymorphonuclear neutrophil leukocytes at concentrations as low as 1×10^{-7} *M*.[279] Although the replacement of the *N*-terminal threonyl residue by L-Ser,[280,281] D-Ser,[280] L-Leu[282] or *p*-Glu[283] yielded active analogs, the retroenantio-tuftsin has been observed to have no biological activity.[284] An evaluation of various additional analogs revealed that only one basic group is required for biological activity since the replacement of either the lysyl or arginyl residue by an alanyl did not appreciably diminish the biological activity of the molecule.[280] However, when L-Ala was substitued for L-Pro, there was a marked activity loss.[280] Konopinska and co-workers[280] suggested that tuftsin might contain a β-turn like those found for the two D-Phe-L-Pro type II′ turns of gramicidin S. However, since tuftsin contains only L-amino acids, a type I β-turn conformation should be preferred (see Section II). Further, the CD of tuftsin in methanol, trifluoroethanol, and 80% dioxane-water, but not water, were also inconsistent with such a type II′ β-turn,[160] since those spectra resembled other class B spectra for model peptides containing either type I or II β-turns (see Table 9). Recently, Blumenstein and Najjar[285] have confirmed by ^{1}H-NMR the presence of a β-turn in DMSO-d_6, but not in H_2O. Energy calculations by Fitzwater et al.[286] also suggested that the peptide has a high probability of forming an open reverse turn.

As pointed out by Smith[160] and Tzehoval et al.,[287] Arg-Pro and Pro-Arg sequences often occur in biologically active peptides. Although the reason for this occurrence is unknown, it is well known that an Arg-Pro sequence is normally highly resistant to proteolytic degradation. Since Xxx-Pro and Pro-Xxx sequences are likely to occur in β-turn conformations,[257] it may be that such a conformational feature involving the Arg-Pro sequence protects it from enzyme cleavage. However, the sequence Gln-Arg-

Pro-Gly has been demonstrated to undergo rapid proteolytic digestion,[288] and this may have resulted from an altered conformation of this sequence, possibly involving a β-turn with L-Pro and Gly as the i + 1th and i + 2th residues, respectively. Further, it has not been shown that such a β-turn conformation, if present, was necessary for binding to certain membrane receptors,[278] although the selective advantage of being able to bind peptides containing Arg-Pro or Pro-Arg sequences without concomitant enzymic degradation by membrane-bound esterases may account for the biological potency of these peptides and, in particular, tuftsin.

B. Phosphorylation of Proteins

Protein kinase dependent phosphorylation of proteins is one of the mechanisms leading to post-translational modification[289] of protein structure.[290] Intracellular protein kinases have been demonstrated to transfer a phosphate from adenosine triphosphate (ATP) to certain seryl or threonyl residues (so-called phosphorylation sites) in various protein substrates, including membrane-bound sugar-transport proteins, basic (i.e., histones) and acidic nuclear proteins, ribosomal proteins, and mitochondrial proteins. Although aberrant membrane protein phosphorylation has been shown to be associated with various disease states, including muscular dystrophies,[292,293] hereditary stomatocytosis and spherocytosis,[294-296] and sickle cell disease,[294,297] the relationship between such abnormal phosphorylation and an alteration of membrane protein structure has not been elucidated.

However, Williams[298] has pointed out that the location of basic amino acid residues (i.e., Lys, Arg, or His) within the amino sequence adjacent to the phosphorylated hydroxy-amino acid residue may function as the recognition site for the phosphorylating kinase. He analyzed the amino acid sequences surrounding 25 phosphorylation sites in 11 proteins and concluded that a phosphorylated seryl and threonyl residue is generally separated from an adjacent basic amino acid residue by only one intervening residue. Although he emphasized the importance of the primary structure of a given site in determining whether or not phosphorylation would occur, he conceded that phosphorylation might also be governed by the array (in a three-dimensional sense) of amino acid residues surrounding a phosphorylation site or by a specific conformation associated with the site. However, only recently has a correlation between the occurrence of a specific conformational feature (i.e., β-turns) and phosphorylation sites been completed. Small et al.[299] demonstrated that 24 out of 30 phosphorylated residues found in their set of 14 proteins, including many of the phosphorylation sites examined by Williams,[298] existed within their predicted β-turn conformations. It must be emphasized that this location of phosphorylation sites primarily to β-turn-containing regions was based on a correlative method of prediction (see Section IV.C) and that these predictions have not been confirmed by X-ray diffraction. Among those phosphorylated residues predicted to occur with β-turn conformations, 69% were located at either the ith or i + 3th positions of the β-turns. However, phosphorylation sites were also observed that were not located within β-turn-containing regions. The validation of the correlation of the high frequency of occurrence of β-turn conformations found in association with phosphorylation sites must await detailed X-ray analysis of: (1) the location of all phosphorylated residues inside and outside β-turn conformations, as well as (2) the location of all potentially phosphorylated residues inside and outside β-turn conformations.

As shown in Table 15, if the data of Small et al.[299] and Williams[298] are examined in conjunction with one another, several interesting relationships between the location of the phosphorylated residue inside or outside a predicted β-turn and the location of basic amino acids with respect to the phosphorylated residue are evident.

Table 15
PROTEIN PHOSPHORYLATION SITES: OCCURRENCE OF β-TURNS AND DISTRIBUTION OF BASIC AMINO ACID RESIDUES[a]

Protein	Amino acid sequence surrounding the phosphorylation site[b,c]	Location of predicted β-turn in phosphorylation site[d]	Turn position of phosphorylated residue[d]	Basic residue(s) position(s) relative to phosphorylated residue[e]
Histone H1	Arg/Lys-Lys-Ser[160]-Pro-Lys-Lys	+	i	−2,−1,+2,+3
Histone H2A	*N*-Ac-Ser[1]-Gly-Arg-Gly	+	i	+2
Histone H4	*N*-Ac-Ser[1]-Gly-Arg-Gly-Lys	+	i	+2,+4
Histone H5	Ser[46]-Ser-Arg-Gln	+	i	+2
Phosphorylase b	Lys/Arg-Gln-Ile-Ser[14]-Val/Ile-Arg	+	i	+2
Protamine I-1A	Ser[6]-Ser-Ser-Arg	+	i	+3
Protamine I-1A	Arg-Ser[21]-Arg-Arg-Arg	+	i	−1,+1,+2,+3
Histone H1	Arg-Lys-Ala-Ser[38]-Gly-Pro	+	i + 1	−3,−2
Histone H2B	Arg-Ser[32]-Arg-Lys	+	i + 1	−1,+1,+2
Histone H2B	Arg-Lys-Gly-Ser[36]-Thr-Ser	+	i + 1	−4,−3
Myelin basic protein	Arg-Gly-Ser[55]-Gly-Lys	+	i + 1	−2,+2
Protamine I-1A	Arg-Ser-Ser[7]-Ser-Arg	+	i + 1	−2,+2
Myelin basic protein	Arg-His-Gly-Ser[12]-Lys-Tyr	+	i + 2	−3,−2,+1
Protamine I-1A	Arg-Ser-Ser-Ser[8]-Arg	+	i + 2	−3,+1
Protein phosphatase inhibitor	Arg-Pro-Thr-Pro	+	i + 2	−2
Histone H1A	Ala-Ser-Gly-Ser[105]-Phe-Lys	+	i + 3	+2
Histone H2A	Thr-Arg-Ser-Ser[19]-Arg-Ala	+	i + 3	−2,+1
Histone H2B	Lys-Lys-Gly-Ser[14]-Lys-Ala	+	i + 3	−3,−2,+1
Troponin-I	Val-Lys-Ser-Ser[114]-Lys-Glu	+	i + 3	−2,+1
Histone H2B	Pro-Ala-Lys(Ac)-Ser[6]-Ala-Pro-Lys-Lys(Ac)	−	−	+3
Myelin basic protein	Gly-Arg-Gly-Leu-Ser[110]-Leu-Ser-Arg	−	−	−3,+3
Phosphorylase kinase (α-subunit)	Arg-Leu-Ser[3]-Ile-Ser-Thr-Glu	−	−	−2
Troponin-I	Arg-Ala-Ile-Thr[36]-Ala-Arg-Arg	−	−	−3,+2,+3
Troponin-I	Val-Arg-Met-Ser[143]-Ala-Asp-Met	−	−	−2
Pyruvate kinase (pig)	Leu-Arg-Arg-Ala-Ser-Leu	−	−	−3,−2
Pyruvate kinase (rat)	Arg-Arg-Ala-Ala-Ser-Val-Ala	−	−	−4,−3

[a] The data were taken from Table 1 of Williams[298] and Table 2 of Small et al.[299]

[b] Superscripted number identifies the sequence location of the phosphorylated residue.

[c] Abbreviation: Ac = acetyl.

[d] Presence (+) or absence (−) of a β-turn containing a phosphorylated residue in a given sequence was predicted by Small et al.[299] Notation i, i + 1: i denotes the first residue of a reverse turn with successive numbering from the amino-terminal to the carboxyl-terminal end of the chain (e.g., i + 1, i + 2, etc.).

[e] The relationship between sites of phosphorylation and the location of basic amino acids was initially pointed out by Williams.[298] (+) indicates carboxyl terminal direction; (−) indicates amino terminal direction. The phosphorylated residue is assigned the zero position.

1. If the phosphorylated residue occupied the ithposition of a predicted β-turn (7 examples), the basic amino acid residue(s) occurred in six cases on the carboxyl terminal side of this residue and was (were) located at least one residue away where they occasionally occurred as a pair of basic residues.
2. If the phosphorylated residue occupied the i + 1th position of a predicted β-turn (5 examples), the basic amino acid residue on the carboxyl side of the phosphorylated residue never occurred more than one residue away. Further, there was a tendency (with one exception) for basic residues to be located one residue away from the phosphorylated residue in either or in both directions.
3. If the phosphorylated residue occupied the i + 2th position of a predicted β-turn (3 examples), the basic residues in two cases were located adjacent in the carboxyl terminal direction in combination with another basic residue located three residues toward the amino terminus. In one case a third basic residue two amino acids toward the amino terminus was also present, although in one other example a basic amino acid in this position without the presence of the other two residues was also observed.
4. If the phosphorylated residue occupied the i + 3th position of a predicted β-turn (4 examples) (with one exception), the basic residues were located adjacent to the phosphorylated residue in the carboxyl terminal direction and one residue away in the amino terminal direction (with one exception).
5. In phosphorylation sites which were not predicted to be β-turns (7 examples), the basic residues were located one or more residues away from the phosphorylated residue in either direction. In two cases a pair of basic residues was located one and two residues away in the amino terminal direction from the phosphorylated residue as was the case for i + 2th positioned sites in β-turns. In two other sequences, a basic residue occurred two residues away from the phosphorylated residue in both the amino and carboxyl terminal direction.

In summary, the data presented in Table 15 indicate that there may be a relationship between the occurrence of a β-turn conformation and the distribution of basic amino acid residues (with respect to the position of the phosphorylated residue) within a specific phosphorylation site. The intricacies of these relationships, as well as rigorous proof for the mandatory recognition of a β-turn conformation by a protein kinase remain to be achieved. However, the circumstantial evidence implicates both structural requirements, turns and basic residues, as necessary for the post-translational modification.

C. Glycosylation of Proteins

Two different modes of linkage between protein and carbohydrate have been identified: (1) the carbohydrate chain is linked *N*-glycosidically to an asparagine residue of the protein, or (2) the carbohydrate is linked *O*-glycosidically to a serine, threonine, hydroxylysine, or hydroxyproline residue of the protein.[300] In those glycoproteins which contain more than one carbohydrate chain per molecule, both types of glycosidic linkage may occur.[300]

1. N-Glycosidic Linkages

Although much remains to be determined about the conformational requirements of the glycosylation sites in proteins, it has been observed in glycoproteins containing carbohydrate linked *N*-glycosidically to an Asn residue that the associated sequence is invariably -Asn-Xxx-[$^{Thr}_{Ser}$]-.[301] When the sequences of numerous glycoproteins were carefully examined, it became apparent that this tripeptide sequence was a necessary

but not a sufficient requirement for glycosylation to occur.[301] Aubert et al.[302] realized that asparagine, serine, and threonine are among the most frequently occurring amino acids within β-turns (see Tables 11 and 13), and they analyzed the sequences around the glycosylated residues in various glycoproteins by the correlative methods of Chou and Fasman[249,250] in an effort to determine whether or not a β-turn conformation might be involved in addition to the invariant tripeptide sequence requirement. For their set of 28 glycosylated Asn residues from 14 different glycoproteins they found that in 19 of the 28 sites, the Asn residue was located within a predicted β-turn, usually at the i + 2th position of the β-turn. In those cases where Asn occupied the i + 2th position, the Thr (or Ser) residue would not be situated within the turn. However, a central position of the Asn within a turn, a position where the residue of the Asn side chain is furthest removed from the protein surface, might serve to facilitate the specific transferase catalyzed transfer of oligosaccharide from its lipid (i.e., dolichol pyrophosphate)-linked intermediate[303] to an acceptor protein.

As in the case of the factors controlling the site specificity of the phosphorylation of proteins (see Section V.B), the control of *N*-glycosylation, although linked in part to the presence of a unique receptor sequence and/or the occurrence of a β-turn conformation, remains to be determined. In particular, various experimental observations on non-native glycosylated substrates need to be considered.

For example, Pless and Lennarz[304] have found that RNase A and α-lactalbumin, both containing the sequence -Asn-Xxx-[$^{Thr}_{Ser}$]-, were converted from non-acceptors to acceptors after covalent modification of their cysteine residues under denaturing conditions. Additional fragmentation of reduced and alkylated α-lactalbumin by treatment with cyanogen bromide followed by tryptic or chymotryptic digestion has led also to the production of polypeptide acceptors of 7 to 123 residues in length, differing little in their rates of in vitro glycosylation.[305] Recently, seven more proteins which do not normally occur in a glycosylated form and which contain one or more of the necessary tripeptide sequences, were examined as potential acceptors for carbohydrate.[306] None of these proteins in their native state was found to undergo glycosylation. However, after reduction and alkylation, only two proteins, rabbit muscle triosephosphate isomerase and ovine prolactin, were converted to acceptors of oligosaccharide-chains.[306] In the native triosephosphate isomerase molecule the glycosylation site (195 to 197) occurs within an α-helical conformation,[254] and it may be that this structural feature protected the Asn^{195}-Val^{196}-Ser^{197} sequence from glycosylation until after the protein was denatured by reduction and carboxymethylation.[306] After fragmentation by cyanogen bromide cleavage, peptides from two other proteins, catalase and concanavalin A, were also found to serve as carbohydrate acceptors. Although the two potential glycosylation sites in concanavalin A, regions (118 to 120) and (162 to 164),[306] are known to occur in regions containing three juxtaposed and overlapped β-turns located between two β-sheets,[254] no glycosylation occurred until fragmentation was carried out. This observation suggested that the presence of a β-turn, even in a highly exposed position characteristic of repeating β-turns,[160] was insufficient for glycosylation. This indicated that there may be unique conformations (possibly β-turns) available only in the free peptide that were required for recognition of the tripeptide as a receptor. This suggestion might also explain the effective glycosylation of other linear peptides, including a heptapeptide from human thyroglobulin[307] and peptides of various lengths from α-lactalbumin.[305]

2. *O*-Glycosidic Linkages

A great deal remains to be learned about the structural requirements necessary for *O*-glycosylation of a protein. However, as in the case of *N*-glycosylation the recognition of a β-turn conformation by a glycosyl transferase has been implicated, although

this conformational requirement has not been rigorously defined. Aubert et al.[302] used the correlative methods of Chou and Fasman[249,250] to predict possible β-turn conformations in nine glycosylation sites in five glycoproteins. They also noted the presence of one or more prolyl residues within or adjacent to their predicted β-turns. In addition, although they did not point it out, their data indicated that one or more glycyl residues were also frequently located within the sequences of their predicted turns. Since Gly and Pro residues are known to have a high frequency of occurrence in β-turns, their findings were not surprising. However, what is surprising was the consistency of the occurrence of Pro and Gly residues which occurred in 89% of the glycosylation sites which they examined. In addition, in two thirds of their sites the glycosylated residue occupied a central residue (i.e., the i + 1th or i + 2th) of a predicted β-turn. Their observations were also consistent with the amino acid compositions of various proteoglycan fractions from ox and pig laryngeal cartilage,[308,309] which had an increased proportion of Ser, Gly, Glu, and Pro. Recently, Robinson et al.[310] have demonstrated that another proteoglycan, heparin, isolated from pronase-digested rat skin, consists of a polypeptide composed entirely of Ser and Gly residues. They have assumed, without experimental proof, that these residues occur in an alternating sequence. Since Ser-Gly or Gly-Ser-containing sequences have a high probability of forming β-turn conformations,[218] it may be that this conformational feature is necessary for the xylosidic linkage between the seryl hydroxyl and the polysaccharide in heparin. The exact structural features required for the *O*- glycosylation of heparin remain to be determined. In the case of heparin, a correlation between the solution conformation of Gly/Ser copolymers and their level of in vitro glycosylation may elucidate these features, as has been done for elastin and collagen hydroxylation[311] (see Section V.D). It may then be possible to establish the detailed structural requirements in other more complicated amino acid sequences which occur in glycosylation sites in other proteoglycans and glycoproteins.

D. Hydroxylation of Proline in Collagen and Elastin

A conformational specificity for the enzyme, proline hydroxylase (E.C. 1.14.11.2), which hydroxylates prolyl residues in procollagen has been suggested by Brahmachari and Ananthanarayanan.[312] They proposed that this conformational requirement for hydroxylation resides in the recognition by the enzyme of an Xxx-Pro-Gly-Yyy sequence, where Pro and Gly occupy the i + 1th and i + 2th positions of a β-turn, respectively. They also pointed out that the Yyy residue plays a major factor in determining whether or not a given sequence Xxx-Pro-Gly-Yyy will adopt a β-turn conformation. Bhatnagar et al.[311] have also found that the nature of the Xxx side chain may be equally important in the modulation of hydroxylation in collagen-like polymers. In addition, the sequence Xxx-Gly-Pro-Yyy has been demonstrated by energy calculations[215] and various physical methods (NMR, CD, and IR)[148] not to favor the formation of a β-turn, in comparison to the sequence Xxx-Pro-Gly-Yyy. Whether or not all procollagen Xxx-Pro-Gly-Yyy sequences are equally well hydroxylated has not been demonstrated satisfactorily at the present time. Indeed, since hydroxyproline formation in collagen has been associated with enhanced stability of the collagen triple helix,[313,314] presumably due to hydrogen bonding between the Hyp O$^{\gamma}$H and the backbone carbonyl group,[315-317] it may be that certain restraints imposed by such hydrogen bonding on conformational freedom of either a β-turn region or a region juxtaposed to a β-turn may alter the rate or site of hydroxylation. However, there are collagen-like synthetic peptides of the sequence (Pro-Pro-Gly)$_n$ which, although they cannot occupy a β-turn conformation,[215] are nevertheless optimally hydroxylated.[311] Further, the mere presence of a defined β-turn conformation within an Xxx-Pro-Gly-Yyy peptide (e.g., (Val-Pro-Gly-Gly)$_n$[154]) is not sufficient to lead to enhanced levels of hydroxylation.[311]

As reviewed by Urry and Long,[70] much of the proline in elastin occurs in Xxx-Pro-Gly-Yyy sequences. Taking advantage of the five polypeptide models of elastin developed by Urry and co-workers,[154,318-321] Bhatnagar et al.[311] examined the ability of each of these model polypeptides to inhibit the hydroxylation of collagen by chick embryo prolyl hydroxylase. Two of the polypeptides, $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ and $(Ala\text{-}Pro\text{-}Gly\text{-}Gly)_n$, resulted in significant inhibition of collagen hydroxylation, although the amount of endogenous hydroxylation of each of these polypeptides was not significantly different from the non-inhibiting peptides.[311] This suggests that the structural requirements for collagen and elastin are not equivalent and that additional work is necessary in order to elucidate the structural requirements which are mandatory for the proline hydroxylase-induced post-translational modification of the prolyl residues in collagen and elastin.

In summary, proline hydroxylation in the connective tissue proteins elastin and collagen has been shown to be dependent in part on the recognition of (Pro-Gly)-containing sequences which occupy β-turn conformations. The subtleties of this recognition and the resultant post-translational modification of protein structure on subsequent hydroxylation are unclear.

E. Elastin

Urry and Long[70] recently reviewed the prodigious amount of synthetic and spectroscopic studies carried out in the Urry laboratory on three repeating sequences found in tropoelastin:[322] a tetrapeptide $Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4$, a pentapeptide, $Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5$, and a hexapeptide, $Ala^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5\text{-}Val^6$. This group of peptides represents one of the most thoroughly studied series of reverse turn-containing peptides in the literature and should be thoroughly reviewed by the interested reader. Additional papers dealing primarily with coacervation[319] and cross-linkage[320] of these model elastin peptides have also appeared, but the details of these papers are beyond the scope of this review.

F. Antifreeze Peptides and Proteins

Approximately one third of the freezing temperature depression activity, located in the sera of certain extensively studied polar fishes, has been accounted for by a group of eight glycoproteins, so-called antifreeze glycoproteins (AFGP).[323] These glycoproteins differed from one another by charge and molecular weight.[323] AFGP-1 to -5 were shown to have molecular weights in the range of 10,500 to 30,000 and were found to depress the freezing point of water as effectively as NaCl, when they were compared on a weight basis. The structure of the AFGP-1 to -5 was determined to be a repeating glycotripeptide with the following sequence: -Ala-Ala-Thr- with a disaccharide linked *O*-glycosidically to each threonyl residue.[324] AFGP-6 to -8 differed from AFGP-1 to -5 in that they have a lower molecular weight, have little[325,326] or no[323,324,327] antifreeze activity when tested alone, (although when combined with AFGP-1 to -5 they extensively potentiated the antifreeze effect of the larger glycoproteins),[328] and have additional proline residues inserted in their sequence after some of the threonine residues.[324] The mechanism by which these AFGPs function remained obscure until DeVries and Lin[329] determined the sequence of an antifreeze peptide, not a glycopeptide, whose sequence revealed the presence of clusters of polar amino acids, usually threonine and aspartic acid, separated from one another by multiple alanine residues, which spaced the polar clusters by approximately 4.5 Å.

Since this is the distance known to separate the oxygen atoms of water in an ice crystal lattice, the authors suggested that the polar side chains of their antifreeze peptide might occupy positions in an ice lattice normally occupied by hydrogen bonds

between water molecules. The effect would be to diminish the freezing point of water. Using the empirical predictive methods of Chou and Fasman,[256] Loucheux-LeFebvre[330] predicted that every polar amino acid cluster in the antifreeze peptide of DeVries and Lin[329] had a relatively high probability of occurring in a β-turn conformation. Since the CD spectrum of the antifreeze peptide indicated that the major conformational feature of the peptide was a α-helix,[331] its structure was predicted to be made up of helical regions of repeating alanyl residues, interspersed among β-turns containing polar amino acid clusters.

A Thr-Pro-Ala-Thr sequence found in AFGP-8[324,332] has also been predicted to occur in a β-turn conformation.[330] In the case of this glycopeptide, with little or no detectable antifreeze activity of its own,[328] it remains to be established whether this predicted β-turn conformation has any function in the cooperative potentiation of the antifreeze activity of AFGP-1 to -5.

In summary, it appears likely that β-turns interspersed among non-polar regions in peptides may play a role in the adsorption of certain antifreeze peptides to ice. Definite experimental verification of this hypothesis has not been achieved.

G. Morphine-like Activity in a Neuropeptide: Enkephalin

Enkephalin was initially isolated from pig brain as a mixture of two pentapeptides: H-Tyr-Gly-Gly-Phe-Met-OH ([Met^5]-enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH ([Leu^5]-enkephalin).[333] Enkephalins have been demonstrated to bind to brain, gut, and spinal cord opiate receptors,[333] as well as to compete with naloxone, a strong opiate antagonist, for receptor binding.[333] Because of the likelihood that a detailed understanding of the molecular structure of enkephalin might lead to the rational design of effective and non-addictive[334] analgesics and to the elucidation of the intricacies of the opiate receptor, the structural features of this molecule have been intensively examined by ^{1}H-[335-339] and ^{13}C-NMR,[337,340,341] fluorescence energy transfer,[342] X-ray diffraction,[44] model building,[343] and theoretical energy calculations[224-227] (see Table 16).

Bradbury et al.[343] originally proposed on theoretical grounds that the conformation of enkephalin was likely to be a β-turn with an intramolecular hydrogen bond between the NH of Phe and the C=O of Tyr. Hence the Gly^2-Gly^3 sequence of enkephalin would occupy the i + 1th and i + 2th positions, respectively, of the "G-G" β-turn.[225] Such a structure also resulted in significant spatial overlap with the functional groups in morphine-like opiates,[227] known to be necessary for opiate activity.[344] Since this conformation was shown by conformational energy calculations to be a high energy structure, it should not be the favored conformation for the molecule.[225,227] In spite of this apparent discrepancy, the crystal structure of [Leu^5]-enkephalin has been demonstrated to be a "G-G" β-turn with dihedral angles consistent with a type I′ conformation[44] (see Section II). Thus, if the resemblance of enkephalin to rigid opiates is a requirement for binding and if large perturbations of the conformation of enkephalin at the opiate receptor do not occur, then a "G-G" β-turn conformation is highly likely to be involved in favorable interactions with the receptor.[227] In contrast, overwhelming evidence accumulated from ^{1}H-NMR indicated that the conformation of enkephalin, although a folded conformation, was probably a "G-P" β-turn with the Gly^3-Phe^4 sequence occupying the central positions of the turn and with an intramolecular hydrogen bond between the amide proton of the methionyl (or leucyl) residue and the carbonyl of Gly^2. Results gleaned from theoretical energy calculations also suggested that such a "G-P" β-turn was a low energy structure and hence a favored conformation.

These differences between the conformation of enkephalin deduced from ^{1}H-NMR and theoretical energy calculations, as compared to the conformation determined from

Table 16
EXPERIMENTAL AND THEORETICAL EVIDENCE FOR THE EXISTENCE OF A β-TURN IN ENKEPHALIN AND ITS ANALOGS

Enkephalin analog	Method of analysis	Proposed conformation[a]	Hydrogen bond location[b]	Ref.
[Leu^5]	X-ray diffraction	β-turn (I′)	4 → 1	44
[Met^5]	Empirical energy calculations	β-turn (II′)	5 → 2 Tyr^2 → 3	224, 225
[Met^5] and [D-Ala^2,Met^5]	Empirical energy calculations; quantum-mechanical calculations (requirement for overlap with rigid opiate)	β-turn	4 → 1	227
[Met^5]	Empirical energy calculations	β-turn (II′)	4 → 1	226
		β-turn (II)	5 → 2	
		β-turn (I)	5 → 2	
[Met^5] and [Leu^5]	Model building	β-turn	4 → 1	343
[Met^5] and [D-Ala^2,Met^5]	^{1}H-NMR	β-turn (I)	5 → 2	335, 336, 338
[Met^5] and [Leu^5]	^{1}H-NMR	β-turn (II)	5 → 2	339
		Inverse γ-turn	3 → 1 Tyr^1 → 3	
[Met^5] and [Gly^3-d$_2$, Met^5]	^{13}C-T$_1$	Tyr restricted at C$^\beta$	—	337
[2-^{13}C Gly^2, Met^5] and [2-^{13}C Gly^3, Met^5]	^{13}C-T$_1$	Gly^3 more restricted than Gly^2	—	341
[Met^5]	^{13}C-T$_1$	Consistent with Ref. 336		340
[Met^5]	Fluorescence energy transfer	β-turn (I)	5 → 2	342

[a] The numeral within the parenthesis refers to the type of β-turn as defined by Reference 18.
[b] Residue number refers to the position in the enkephalin. As written (m → n) the m residue N–H is hydrogen bonded to the n residue C=O.

model building and X-ray diffraction analysis, highlight the limitations of each technique in elucidating the structure of a biologically active (i.e., receptor-bound) peptide. However, the fact that all the experimental evidence accumulated with this peptide indicated that a β-turn conformation of one type or another occurs as the predominant structural feature of this linear peptide argues strongly for the importance of the contribution of this conformation to the biological activity of this neuropeptide and the potential of existing techniques to elucidate the structural detail of β-turn-containing peptides.

H. Peptide Hormones Containing Reverse Turn Conformations

It must be emphasized that the detection of a reverse turn conformation among the solution conformations available to a peptide hormone may or may not be related to the conformation required for receptor binding. This same caution applies to theoretical energy calculation predictions of low energy structures, since such calculations are carried out assuming a low dielectric constant (approaching *in vacuo* conditions) and without consideration of solvation or charged amino or carboxyl termini (see Section III.E). As discussed in Section III.A , the validity of a direct extrapolation from an X-ray diffraction-determined conformation to the preferred receptor conformation is also uncertain, since both the statistical distribution and the frequency of conversion among the various solution conformations are completely ignored. Despite these pro-

visos, the occurrence of a reverse turn in solution, in a crystal or *in vacuo*, does argue that such a conformation is a stable structural feature and that its recognition may be involved in its interaction with the receptor. At present there are no experimental approaches available that allow the detailed assessment of the conformation of a peptide hormone when it is interacting with its receptor within the complex macromolecular array of a membrane. Until such techniques have been developed which prove unequivocally whether flexible or inflexible (i.e., conformationally restricted) molecules are involved in peptide hormone-receptor interactions, the generalization that such interactions always rely on precise structural complementary should not be made.

As shown in Table 17, data have been accumulated which imply that a reverse-turn conformation in various peptides may be important in eliciting the biological activity associated with these molecules in vivo. However, as pointed out by Williams,[362] this may be an oversimplification, and the intrinsic flexibility of a peptide hormone, rather than some unique conformation, may be the signal feature governing its interaction with a receptor.

1. Melanotropin Release Inhibiting Factor (MSH-R-IF)

MSH-R-IF, *H*-L-Pro-L-Leu-Gly-NH_2, has been isolated from bovine pituitary extracts[363] and was shown to be an oxytocin-derived enzymatic cleavage product, which inhibited the release of pituitary melanotropin.[364] This peptide has been examined by high resolution (i.e., 300 Mhz) ^{1}H-NMR,[345] X-ray diffraction,[46] and energy calculations,[221,347] and the preferred conformation of this peptide was suggestive of a type II β-turn with a i + 3 → i hydrogen bond between the glycinamidyl carboxamide and the prolyl carbonyl (see Table 17). The ^{13}C-T_1 measurements completed on this molecule, although these measurements gave no direct evidence favoring a β-turn, indicated that the peptide was a flexible and seemingly compact structure in aqueous solution.[111] Conspicuously neglected in the above ^{1}H-NMR study[345] was an examination of the temperature coefficient of the carboxamide chemical shift which would have allowed an assessment of the proposed intramolecular hydrogen bonding (see Section III.B.1.). When this approach was finally used, Frič et al.[346] failed to demonstrate an intramolecular hydrogen bond. However, their choice of a markedly elevated temperature range (30 to 75°C) for that study may have contributed to their negative results. However, their CD data, which indicated a spectral minimum at 230 nm and a maximum at 210 nm for the peptide in methanol, were compatible with CD parameters for other type II β-turn-containing model peptides[346] (see Table 9).

2. Thyrotropin Release Factor (TRF)

TRF, *p*-Glu-L-His-L-Pro-NH_2, was originally isolated from bovine hypothalami and has been demonstrated to control the secretion of pituitary thyroid stimulating hormone (TSH).[365,366] Although the initial semiempirical energy calculations done on this peptide suggested that its preferred conformation was a β-turn with an intramolecular hydrogen bond between the prolinamide NH_2 moiety and the carbonyl of the pyroglutamyl residue,[222] additional empirical[223] and quantum mechanical[231] energy calculations, as well as ^{1}H-[348,349] and ^{13}C-NMR[350] data, indicated that an extended conformation was the preferred conformation (see Table 17).

3. Contraceptive Peptide

Contraceptive peptide, H-L-Thr-L-Pro-L-Arg-L-Lys-OH, has been isolated from the lumen of oviducts of progravid hamsters.[367] The possible structural relationship of this tetrapeptide to a sequence variant, H-L-Thr-L-Lys-L-Pro-L-Arg-OH (tuftsin) has been pointed out[160,228] (see Section V.A.3). Energy calculations on contraceptive peptide have demonstrated that the lowest energy calculation is an open reverse turn with two

Table 17
EXPERIMENTAL AND THEORETICAL EVIDENCE FAVORING THE OCCURRENCE OF REVERSE TURNS IN VARIOUS PEPTIDE HORMONES

Peptide hormone	Sequence[a]	Method of analysis[b]	Proposed conformation[b,c,d]	Ref.
Melanotropin release inhibiting factor (MSH-RIF)	H-L-Pro-L-Leu-Gly-NH_2	X-ray diffraction	β-turn	46
		^{1}H-NMR	β-turn	345
		^{1}H-NMR	NT	346
		^{13}C-T_1	E	111
		CD	β-turn (II) or inverse γ-turn	346
		Energy calculations	β-turn (II)	221,347
Thyrotropin release factor (TRF)	*p*-Glu-L-His-L-Pro-NH_2	Energy calculations	β-turn	222
		Energy calculations	NT	223,231
		^{1}H-NMR	NT	348,349
		^{13}C-NMR	E	350
Contraceptive peptide	H-L-Thr-L-Pro-L-Arg-L-Lys-OH	Energy calculations	Open reverse turn	228
Angiotensin II (A II)	H-L-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe-OH	CD	β-turn	150
		Energy calculations	Inverse γ-turn (or β-turn)	21
		^{1}H-NMR	NT	351
		^{13}C-NMR	E	112
Bradykinin	H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH	CD	Inverse γ-turn	161
		Fluorescence energy transfer	Inverse γ-turn	352
		ESR	Inverse γ-turn	352
Oxytocin	H-L-Cys-L-Tyr-L-Ile L-Cys-L-Asn-L-Gln L-Pro-L-Leu-Gly-NH_2	^{1}H-NMR	β-turn	131-134,353
		^{13}C-T_1	E	354
		X-ray diffraction	β-turn (I)	35
Luteinizing hormone releasing factor (LRF or LH-RH)	*p*-Glu-L-His-L-Trp-L-Ser-L-Tyr-Gly-L-Leu-L-Arg-L-Pro-Gly-NH_2	Charge-transfer	β-turn (II)	190,335
		^{1}H-NMR	NT	356

Somatostatin[a]	H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp ⋮ HO-Cys-Ser-Thr-Phe-Thr-Lys	Energy calculations Model building	NT β-turn (II′)	357 358,359

[a] --- Indicates disulfide linkage.
[b] Abbreviations used: CD, circular dichroism; E, equivocal (i.e., technique cannot rule out reverse turn); ESR, electron spin resonance; NMR, nuclear magnetic resonance; NT, nonturn conformation; T_1, spin lattice-relaxation time.
[c] Numeral within parentheses corresponds to β-turn type as defined by Venkatachalam.[18] (See Section II of this review.)
[d] Inverse γ-turn conformation defined in Section II of this review.
[e] The nonglycyl residues of this peptide are in the L-configuration.

side chain-backbone hydrogen bonds.[228] Experimental confirmation of this predicted conformation has not been achieved.

4. Angiotensin II (A II)

Although a number of conformations have been proposed for A II, one likely structure is the inverse γ-turn proposed by Printz et al.[21] (see Table 17). Although they suggested that the inverse γ-turn conformation was the most likely structure, they also accepted the possibility that a β-turn conformation was a possible alternative. CD data have also been interpreted as indicating the presence of two β-turns within the molecule,[150] although the contribution of the tyrosyl and phenylalanyl chromophores to the observed CD leads to certain ambiguity in this assignment (see Section III.C.1). In contrast, the ^{1}H-NMR study by Glickson et al.[351] revealed that A II either exists in a conformation unlike the previously proposed β- or inverse γ-turn models, or occurs as a statistical average of various conformations including β- and inverse γ-turns. The exact solution conformation of this hormone remains unknown.

5. Bradykinin

Bradykinin is a pharmacologically active substance, produced by the kinin-generating system, which causes vasodilatation, increased capillary permeability, and either contraction or relaxation of extravascular smooth muscle. The substance was originally discovered by Rocha e Silva,[368] although the structure was established 11 years later by Elliott et al.[369] Two CD studies have indicated that the molecule contains an inverse γ-turn conformation[161,352] (see Table 17). Such an inverse γ-turn conformation is experimentally consistent with various intramolecular distances estimated from fluorescence energy transfer[352] and electron spin resonance.[352]

6. Oxytocin

Oxytocin is a powerful stimulant of uterine contraction, which is secreted by the posterior pituitary.[370] Overwhelming ^{1}H-NMR evidence has been accumulated which indicates that the loop, formed by a disulfide linkage between residues 1 and 6 of this peptide, contains a β-turn structure,[131-134,353] (see Table 17). In addition, X-ray diffraction obtained from a model peptide for the oxytocin tail, H-L-Cys (Bzl) L-Pro-L-Leu-Gly-NH_2,[35] indicated that this portion of the molecule occurs as a type I β-turn, at least in the crystal state (see Table 3).

7. Luteinizing Hormone-Releasing Hormone (LH-RH)

LH-RH (or LRF) is a hypothalamic factor responsible for stimulating the secretion of the hormones from the anterior pituitary, which regulate ovulation.[371] Experimental evidence from charge-transfer experiments indicates that the molecule contains a type II β-turn[190,355] (see Section III.D.3.). However, high resolution ^{1}H-NMR experiments have yielded no evidence to substantiate the existence of a β-turn (i.e., an intramolecular hydrogen bond).[356] Empirical energy calculations have been completed for this molecule, and there was also no indication of a favored β-turn conformation.[357]

8. Somatostatin

Somatostatin is a 14-amino acid peptide isolated initially from bovine hypothalami.[372] It has been established to have diverse biological activities including the inhibition of the release of insulin, growth hormone, glucagon, gastric acid, and pancreatic exocrine secretions.[373,374] From direct and difference CD studies it was concluded that somatostatin contains a β-turn conformation.[360,361] This observation is also consistent with the results from model building based on the biological activity of

various synthetic bicyclic analogs of somatostatin, which led to the suggestion that this peptide hormone contains a β-turn involving residues 7 through 10.[358,359] Definitive proof for this β-turn conformation remains to be established by ^{1}H-NMR.

I. Protein Folding: β-Turns as Possible Nucleation Sites

Protein folding is the molecular mechanism which converts an open polypeptide chain into the characteristic three-dimensional structure of the native protein molecule.[7] Although numerous reviews dealing with general aspects of protein conformation and of protein folding have been written, the role of the β-turns in the folding of proteins has been discussed adequately in only two reviews.[7,242] The interested reader should consult both of these for further details. With the exception of presenting certain of the most accepted concepts which implicate β-turns in the mechanism of protein folding, the prodigious literature dealing with this subject will of necessity be neglected herein.

During the process of protein folding, the folding chain frequently changes direction. The regions in which these changes of direction occur are called β-turns. In addition, the number of β-turns in a protein is a linear function of the number of amino acid residues.[258] In such β-turn-containing regions, short range interactions would be expected to dominate and to lead to an ordering of a short segment of the polypeptide chain. Such ordered segments may then function as nucleation sites where folding begins and then continues until the folding process is complete.[6-8] Although this general concept has been espoused qualitatively over the years by several workers, a quantitative evaluation first resulted from the application of Monte Carlo, predictive methods by Tanaka and Scheraga.[375] In their subsequent paper,[376] they proved that β-turn conformations in rubredoxin, lysozyme, and ferricytochrome C played a significant role in the initial stages of protein folding.

Hiltner and Walton[213] have favored an alternative viewpoint that chain folding at reverse turns occurred not because specific residues in the turn favor the folding, but because these regions are such that they can have their intrinsic destabilization overcome by the driving force of long-range interactions.

Regardless of which model is demonstrated experimentally to be correct (and the evidence is certainly not conclusive at present), it can be stated that certain β-turn regions in globular proteins are probably nucleation sites either because of their intrinsic short-range interactions or because of additional interactions which lead to apparent nucleation involving a β-turn, although the interactions themselves might not be a consequence of initial β-turn formation. This may account for the remarkably high degree of conservation of β-turns in proteins, demonstrated for 10 mammalian proinsulins,[9] for 7 proteinase inhibitors,[9] and for 12 pancreatic ribonucleases, as well as for immunoglobulin light and heavy chain.[246]

In summary, β-turns and protein folding appear on theoretical grounds to be inextricable. However, the experimental proof from spectroscopic experiments investigating the tendency of short, linear peptides to form stable β-turn conformations (as assessed by CD or NMR) has been equivocal.

J. Reverse Turns in Antibiotics, Toxins, Antitoxins, Ionophores, and Metabolic Products

1. Gramicidin S and the Tyrocidines

Gramicidin S, a cyclic decapeptide with a repeating pentapeptide sequence L-Val-L-Orn-L-Leu-D-Phe-L-Pro, which was first isolated from *Bacillus brevis* by Gause and Brazhnikova,[377] shows antimicrobial activity. This antibiotic has played an interesting role in the history of peptide conformational analysis. It was the subject of studies

using various methods including X-ray diffraction,[10, 378] NMR,[15,379,380] and conformational energy calculations,[206-208] but a consistent interpretation of the conformation was elusive until Ovchinnikov and co-workers[63] postulated, primarily from ^{1}H NMR, the currently accepted C_2-symmetric β-turn-containing structure, which has the D-Phe-L-Pro residues occurring in the i + 1th and i + 2th positions of type II′ β-turns. This conformation has now been established as the favored one in crystals by X-ray diffraction.[381]

It has been found that the activity of gramicidin S depends critically on the proximity of the two charged ornithine side chains.[382,383] The average distance between these groups was estimated using electron spin resonance of a spin-labeled derivative to be 8 to 10 Å with alcohol as a solvent. This finding, together with the observation that gramicidin S solubilizes lecithin, led to a model for its antibiotic activity which requires a step involving association of the positively-charged gramicidin S side chains and the negative phosphates of the phospholipid head groups.[382,383] Essential for this step would be a rigid conformation of the cyclic peptide, such that the ornithine side chains remain in proximity. The presence of β-turns and intramolecular hydrogen bonding are principal factors in the maintenance of the peptide conformation.

Tyrocidines A-E are closely related to gramicidin S, since they are cyclic decapeptides which share a common pentapeptide fragment with gramicidin S. Although a conformation similar to that of gramicidin S is suggested for the tyrocidines,[144,384] no structure-activity studies have been reported.

2. *Valinomycin*

Valinomycin is a cyclic dodecadepsipeptide antibiotic, isolated from *Streptomyces fulvissimus*,[385] which has been extensively studied with regard to both its structure and function.[386] It acts on membranes, causing a large and specific increase in their K^+ permeability.[387,388] This ionophoric role is attributed to the formation by valinomycin of a strong, selective, hydrophobic complex with K^+.[386] The K^+ in the complex can then move readily through the membrane, "carried" by valinomycin. The valinomycin-K^+ complex is made of six β-turns (alternating types II and II′), arranged so that the six non-hydrogen bonded carbonyls are oriented toward a central cavity. Uncomplexed valinomycin adopts different conformations depending upon solvent; many contain β-turns. These conformations and the cation complexes of valinomycin have been studied in detail by NMR[66,84,93,125,126,389] and by X-ray diffraction.[32,34,390,391] In addition, the relationship between structure and function in this ionophore has been extensively discussed.

3. *Toxic and Antitoxic Peptides from Amanita phalloides*

For over a century, extracts of the deadly poisonous *Amanita* mushrooms have been studied in efforts to determine the active components and to elucidate their modes of action. Three classes of peptides have been isolated from these toxic mushrooms and characterized as to structure and function. A comprehensive review of the chemistry and toxicology of these peptides has been written by Wieland and Faulstich.[392]

Two types of toxic peptides, the octapeptide amatoxins and the heptapeptide phallotoxins, are bicyclic, with unusual cross-linkages between Trp and Cys side chains. Nonetheless, their mechanisms of toxicity are reported to be distinct.[382,392] The former group is considered to be the cause of death in humans after consumption of *Amanita* mushrooms.[392]

The conformation of one phallotoxin, phalloidin, has been investigated by NMR.[393] No unique structure was proposed, although it was concluded that the molecule is rigid, and has two N-Hs which are either buried or intramolecularly hydrogen-bonded. X-ray diffraction analyses of the phallotoxins have not been reported.

The structure of β-amanitin, an amatoxin, has been solved by X-ray crystallography.[394] The results agree fairly well with an NMR study,[395] and reveal that a type II Ile-Gly β-turn is present.

The most extensively studied peptide from *Amanita phalloides* is, interestingly, a phalloidin antagonist, antamanide, which is a cyclic decapeptide. Studies based on NMR,[128,129,382] energy calculations,[229] and X-ray diffraction[41,396,397] agree in general on a conformation which contains two β-turns for the alkali metal complex of antamanides (and for a C_2-symmetric, biologically active analog, [Phe⁴,Val⁶] antamamide). Antamanide is thought to be membrane active,[382] and, as was the case for gramicidin S and valinomycin, an important feature of the conformation is likely to be the arrangement in space of hydrophobic side chains. The adoption of a rigid, β-turn-containing conformation is consistent with this requirement. Uncomplexed [Phe⁴,Val⁶] antamanide in crystals does not contain β- or γ-turns.[398,399] The N-Hs either hydrogen bond to water molecules or are involved in i + 4 → i intramolecular interactions. In solution, a conformational equilibrium has been proposed.[382]

4. Ferrichromes

Siderochromes constitute a group of hydroxamic acid-containing iron chelators.[382] One class of these compounds, the ferrichromes, isolated from the smut fungus *Ustilago sphaerogena*,[400] has as a major structural feature a cyclic hexapeptide backbone. X-ray diffraction analysis[45] and nuclear magnetic resonance[127] have established that the ferrichromes adopt a conformation containing an i + 3 → 1 intramolecular hydrogen bond in a type II β-turn. The extreme rigidity of ferrichrome and various analogs, and the apparent close resemblance between the conformation of the chelate in crystals and in solution has led to extensive use of these peptides as models in conformational analysis.[72,81,83,110,127]

Ferrichromes show potent growth factor activity, and are thought to act as cellular transport factors for iron,[401] functioning via a cellular membrane transport system which is specific for the metal-bound hydroxamic acid.

5. Other Antibiotics and Metabolic Products

The crystal structures of two reverse turn-containing peptides with tuberculostatic activity, isolated from bacterial cultures of *Streptomyces griseoverticullatus* var. *tuberacticus*, have been reported.[402,403] Tuberactinomycin[402] and viomycin[403] have similar sequences, and both antibiotics take up crystal conformations containing pseudo-type II β-turns with dehydro amino acids in the i + 2th position, which is usually occupied by a D-residue in this type of β-turn.

Bouvardin is a metabolic product of the plant *Bouvardia ternifolia*, which has been found to have tumor-inhibiting activity.[404] It is composed of a cyclic hexapeptide ring with an ether linkage between two tyrosine side chains. The resulting rigid conformation, as revealed by an X-ray analysis,[404] is stabilized by one intramolecular hydrogen bond of the i + 3 → i, β-turn type, although the ϕ,ψ angles differ appreciably from the predicted values,[18] presumably due to constraints from the bicyclic system.

Two natural peptides with i + 2 → i intramolecular hydrogen bonding have been examined: dihydrochlamydocin, a cyclic tetrapeptide containing two γ-turns;[28] and cyclosporin A, a cyclic undecapeptide with both a β-turn (type I) and an inverse γ-turn.[56] The ϕ,ψ angles of the corner residue in the γ- or inverse γ-turn are given in Table 3.

Ilamycin B_1, an antibiotic whose crystal structure has been reported, and evolidine, a metabolic product from the tree *Evodia xanthoxyloides*, are both cyclic heptapeptides which contain all L-residues.[382] Two β-turns were described in the crystal conformation of ilamycin B_1, but the i + 1th and i + 2th residues are linked by *cis* peptide

bonds.[405] In addition, one of the hydrogen bonds is bifurcated, with the i^{th} C=O interacting with both the i + 3^{th} and i + 2^{th} N-Hs. NMR studies on both ilamycin B_1[406] and evolidine[407] suggested the existence of intramolecular hydrogen bonding in solution, although structural details were not conclusively elucidated.

Several other natural products have been proposed to contain reverse turns (e.g., stendomycin,[408] telomycin,[409,410] patricin A,[3] vernamycin B,[3] and polymixin B[3]), but their conformations are not definitively established.

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