Poly(Pro)II Helices in Globular Proteins: Identification and Circular Dichroic Analysis†

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ABSTRACT: A method to identify poly(L-proline)-type (P_{II}) conformation in crystal structures of globular proteins is presented. Short segments of P_{II} structure were identified in globular protein structures, and these form a significant fraction of the residues which are not assigned to α-helix, β-sheet, and β-turns. The fractions of α-helix, β-sheet, β-turns, P_{II}, and unordered, identified in conjunction with the Kabsch and Sander method [(1983) *Biopolymers 22,* 2577], were incorporated in the analysis of circular dichroism (CD) spectra of proteins. The separation of P_{II} fraction from the fraction of residues not assigned to α-helix or β-sheet or -turns resulted in a distinctive P_{II} CD spectrum and an unusual CD spectrum corresponding to the residual unassigned structures. The quality of prediction of P_{II} fraction from CD spectra of proteins was comparable to that of β-sheet and -turns.

The three major types of secondary structures recognized in globular proteins are α-helices, β-sheets, and β-turns. The α-helices and β-sheets are identified by the existence of at least one hydrogen bond between the backbone C=O and N–H groups, while the β-turns may have at most one such hydrogen bond (Pauling *et al.*, 1951; Venkatachalam, 1968; Richardson, 1981). These are also defined, in idealized geometries, by the ϕ and ψ angles making up the structure (Pauling *et al.*, 1951; Venkatachalam, 1968; Cantor & Schimmel, 1980; Richardson, 1981). The secondary structure elements in X-ray-derived structures deviate from the ideal geometry, and algorithms to identify the secondary structure elements in globular proteins have been developed (Levitt & Greer, 1977; Kabsch & Sander, 1983). Up to 25% of the residues in globular protein structures remain unassigned. These residues have been referred to by different terms by different investigators: random coil (Perczel *et al.*, 1991; Böhm *et al.*, 1992); unordered conformation (Chang *et al.*, 1978; Perczel *et al.*, 1991; Sreerama & Woody, 1993); irregular regions (Bolotina *et al.*, 1980); other structures (Hennessey & Johnson, 1981; van Stokkum *et al.*, 1991; Pancoska & Keiderling, 1991); and remainder (Provencher & Glöckner, 1981; Venyaminov *et al.*, 1991). We continue to use the term "unordered" for those residues that are not assigned to a well-defined secondary structure. This term does not imply that these unassigned residues are dynamically unordered or that their conformation varies from one individual protein molecule to another.

There is evidence that some of the unassigned residues show at least short-range order, short segments of poly(Pro)II-helix type (P_{II})[1] structure (Adzhubei *et al.*, 1987a,b; Adzhubei & Sternberg, 1993; Woody, 1992). The P_{II} conformation is a left-handed extended helix with three residues per turn, has the backbone C=O and N–H groups projecting outward, and is favored in proline-rich polypeptides due to the limited conformational flexibility of the proline ring (Cantor & Schimmel, 1980; Woody, 1992).

An analysis of ϕ, ψ angles of 67 globular proteins indicated the presence of a left-handed extended conformation (M-conformation), similar to P_{II} (Adzhubei *et al.*, 1987a,b). Approximately 20% of the residues in the proteins analyzed were in the M-conformation, while those in α-helix and β-sheet were 43% and 20%, respectively. The majority of residues in the M-conformation, however, were isolated residues. The analysis indicated the presence of P_{II} structure in globular proteins, but did not consider the turns and followed the dihedral angle method. The regular segment search (RSS) algorithm developed by Adzhubei and Sternberg (1993), which utilizes the mean distance between the peptide groups in a segment in ϕ, ψ space and the virtual dihedral angle α_i, defined by atoms $C_\alpha(i-1)$–$C_\alpha(i)$–$C_\alpha(i+1)$–$C_\alpha(i+2)$, identified 96 segments of P_{II} structure with more than three peptide units.

Circular dichroism (CD) spectroscopy is a valuable tool for the study of the secondary structure of polypeptides and proteins (Woody, 1977, 1985; Yang *et al.*, 1986; Johnson, 1988, 1990). Its prominence derives from the characteristic CD spectra of α-helix and β-sheet conformations due to the regularity in their structure. The CD spectrum of turns is less characteristic because different sets of ϕ, ψ angles can lead to a β-turn (Woody, 1985). Unordered polypeptides also give a characteristic CD spectrum (Tiffanny & Krimm, 1968, 1969, 1972; Woody, 1992). This has made possible the estimation of secondary structure fractions of proteins from the analysis of CD spectra (Yang *et al.*, 1986; Johnson, 1988, 1990; Sreerama & Woody, 1993). The current methods for analyzing protein CD spectra estimate fractions of α-helix, β-sheet, turns, and unordered.

In this paper, we introduce a new secondary structural class, P_{II} structure, in the CD analysis. We report a method to identify the P_{II} conformation in globular protein structures. The resulting secondary structure assignments were included in the analysis of CD spectra of proteins for estimating secondary structure fractions. Our results indicate that a significant fraction of residues not belonging to α-helix, β-sheet, or turns are in the P_{II} conformation. The quality of prediction of the P_{II} fraction from CD spectral analysis was comparable to that of β-sheet.

METHODS

Identification of the P_{II} Conformation. We identify the P_{II} structure using geometric features defined by the angles τ and ζ, which are as follows: τ_i = virtual bond angle $\{C_\alpha(i-1)$–$C_\alpha(i)$–$C_\alpha(i+1)\}$; ζ_i = virtual dihedral angle $\{O(i-1)$=C-

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[1] Abbreviations: P_{II}, poly(Pro)II-helix type; CD, circular dichroism; *r*, correlation coefficient; δ, root-mean-square deviation.

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$(i-1)$—C(i)=O(i)}. The regular secondary structures with repeating ϕ, ψ angles have typical τ and ζ. Right-handed helical structures have positive ζ, the left-handed structures have negative ζ, and the β-structure has ζ close to 180°. The virtual angle ζ gives the geometric relation between the successive carbonyl groups and indicates the handedness of the propagating chain in a shorter stretch of residues. The τ and ζ angles in regular structures identified from X-ray coordinates, such as α-helices and β-sheets, deviate from their ideal values because of deviations from the ideal geometry. The criteria for identifying P_{II} structure were derived from the values of these virtual angles in idealized structures and their deviations in X-ray structures.

A residue is assigned to the P_{II} structure if the virtual angles τ and ζ do not deviate more than 15° and 25°, respectively, from their ideal values of 120° and −115°, respectively (*i.e.*, $105° < \tau < 135°$; $-140° < \zeta < -90°$). The criteria are relaxed to extend the P_{II} structure as follows: (a) if the previous residue is in P_{II} structure, then the allowed deviations for τ and ζ are relaxed to ±20° and ±35°, respectively; (b) a residue flanked by residues in P_{II} structure is assigned to the P_{II} structure if $100° < \tau < 140°$ (20° deviation from its ideal value) and $\zeta < 0°$ (left-handed propagation).

Proteins. The X-ray structures of the following 16 proteins, which formed our basis set for CD analysis, were taken from the Protein Data Bank (Bernstein *et al.*, 1977). The proteins and the X-ray structures used (PDB code in parenthesis) are as follows: Bence-Jones protein (1rei), prealbumin (2pab), rubredoxin (3rxn), α-chymotrypsin (5cha), elastase (3est), papain (9pap), thermolysin (3tln), lysozyme (7lyz), subtilisin BPN′ (1sbt), glyceraldehyde-3-phosphate dehydrogenase (3gpd), flavodoxin (1fx1), lactate dehydrogenase (5ldh), triosephosphate isomerase (1tim), cytochrome *c* (3cyt), hemoglobin (2mhb), and myoglobin (4mbn). Of these, the first five are $\beta\beta$ proteins, the next three are α/β proteins, the next five are $\alpha+\beta$ proteins, and the last three are $\alpha\alpha$ protiens (Levitt & Chothia, 1976).

Analysis of CD Spectra. The CD spectra associated with various types of secondary structures were deconvoluted from CD spectra of the basis set proteins. Our basis set consisted of 16 proteins and poly(L-Glu), an all-α polypeptide, which was similar to the one used in our previous study (Sreerama & Woody, 1993). The CD spectra of these proteins and poly(L-Glu) were kindly provided by Dr. W. C. Johnson, Jr. The method followed to obtain secondary structure CD spectra was similar to that followed by Compton and Johnson (1986). The matrix containing the basis CD data, **C**, is expressed as a product of three matrices using the singular value decomposition algorithm (Forsythe *et al.*, 1977), $\mathbf{C} = \mathbf{USV}^T$, where **U** and **V** are unitary matrices and **S** is a diagonal matrix. This is incorporated in the matrix equation relating the CD spectra to the secondary structure data matrix, $\mathbf{F} = \mathbf{XC}$. The multiplicative inverse of **X**, which is $\mathbf{FVS}^+\mathbf{U}^T$, gives the spectra corresponding to the secondary structures considered in constructing **F**.

The CD spectrum of the protein analyzed for secondary structure was removed from our basis set, and the secondary structure fractions were predicted using the other members of the basis set, following the self-consistent method (Sreerama & Woody, 1993). In the self-consistent method the spectrum of the protein analyzed is included in the matrix of CD spectral data, and an initial guess, the structure of the protein having the CD spectrum most similar to that of the protein analyzed, is made for the unknown secondary structure. The matrix equation relating the CD spectra to the secondary structure, $\mathbf{F} = \mathbf{XC}$, is solved by the singular value decomposition algorithm (Forsythe *et al.*, 1977). The solution obtained replaces the initial guess, and the process is repeated until self-consistency is reached.

Table 1: Secondary Structure Fractions of Protein Structures Used in the CD Analysis[a]

PDB code	α-helix	β-sheet	turns	P_{II}	unordered
5cha	0.114	0.314	0.222; 0.227	0.163; 0.088	0.186; 0.257
3cyt	0.418	0.000	0.165; 0.189	0.199; 0.150	0.218; 0.243
3est	0.108	0.342	0.225; 0.233	0.154; 0.083	0.171; 0.233
2mhb	0.760	0.000	0.125; 0.129	0.035; 0.007	0.080; 0.105
5ldh	0.390	0.087	0.228; 0.237	0.024; 0.009	0.270; 0.276
7lyz	0.395	0.078	0.310; 0.333	0.085; 0.016	0.132; 0.178
4mbn	0.804	0.000	0.078; 0.078	0.033; 0.013	0.085; 0.105
9pap	0.259	0.170	0.217; 0.226	0.123; 0.071	0.231; 0.274
1sbt	0.302	0.178	0.244; 0.255	0.087; 0.040	0.189; 0.225
1fxl	0.320	0.218	0.279; 0.286	0.061; 0.014	0.122; 0.163
3gpd	0.274	0.208	0.246; 0.251	0.060; 0.028	0.213; 0.238
3pab	0.063	0.449	0.193; 0.197	0.055; 0.031	0.240; 0.260
1tim	0.460	0.168	0.144; 0.154	0.055; 0.012	0.174; 0.206
3tln	0.415	0.165	0.222; 0.234	0.095; 0.051	0.104; 0.136
1rei	0.028	0.491	0.206; 0.215	0.159; 0.126	0.117; 0.140
3rxn	0.173	0.154	0.269; 0.269	0.231; 0.173	0.173; 0.231

[a] The secondary structure fractions were obtained by combining the assignments from the KS method and this work. The fraction of α-helix was obtained by combining the α- and 3_{10}-helix assignments from the KS method, and that of β-sheet was from KS assignments. The two sets of values for turns, P_{II}, and unordered fractions were due to two ways of assigning isolated residues in the P_{II} conformation: the first value was obtained by including isolated P_{II} residues in the P_{II} fraction (SW1); the second value was obtained by including isolated P_{II} residues in turns or unassigned fractions (SW2).

The performance of the analysis is expressed as root-mean-square deviations (δ) and correlation coefficients (r) between the X-ray and CD estimates of secondary structure fractions for different secondary structure assignments. δ and r were calculated using the equations:

$$\delta = \left\{\frac{1}{N}\sum_i (f^x{}_i - f^{cd}{}_i)^2\right\}^{1/2}$$

$$r = \frac{N\sum_i f^x{}_i f^{cd}{}_i - \sum_{ij} f^x{}_i f^{cd}{}_j}{\{[N\sum_i (f^x{}_i)^2 - (\sum_i f^x{}_i)^2][N\sum_i (f^{cd}{}_i)^2 - (\sum_i f^{cd}{}_i)^2]\}^{1/2}}$$

where f^x and f^{cd} are the X-ray and CD estimates of secondary structure fractions of N samples.

RESULTS AND DISCUSSION

Identification of the P_{II} Conformation. The secondary structure fractions obtained from our method in conjunction with Kabsch and Sander (1983) method, for the proteins in our basis set, are given in Table 1. We start with the Kabsch and Sander (1983) assignments of secondary structures (KS), which use hydrogen bond patterns, and assign seven types of secondary structures (α-helix, 3_{10}-helix, π-helix, β-sheet, turns, β-bridge, and bends). The residues assigned to helix and β-sheet were eliminated, and the rest were examined for the P_{II} structure. After assigning P_{II}, the turns, bends, and bridges were assigned among the remaining residues according to the KS method. The bends are regions of high chirality (Kabsch & Sander, 1983), and we include them in the turns fraction; the bridges are isolated β-bridges and were considered in the unordered fraction. In effect, we consider P_{II} to be a higher order structure than turns and reassign some residues assigned to turns, bends, and bridges to P_{II}.

Approximately 10% of the residues were assigned to the P_{II} structure among the proteins in our basis set, and the P_{II} fraction in these proteins varied from 0.024 (5ldh) to 0.231 (3rxn) (Table 1). Generally, proteins with higher α-helix content had less P_{II} structure, and a negative correlation was

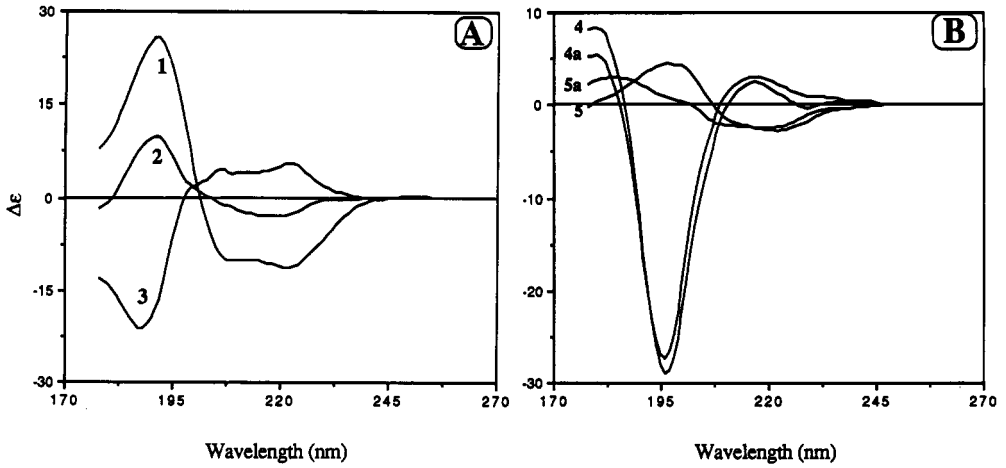

FIGURE 1: CD spectra associated with various types of secondary structures, deconvoluted from protein CD spectra. (A) CD spectra of α-helix (curve 1), β-sheet (curve 2), and turns (curve 3). (B) CD spectra of P_{II}, considering all residues in the P_{II} conformation (curve 4) and only those residues in P_{II} segments of two or more residues (curve 4a), and the corresponding unordered conformation (curves 5 and 5a, respectively).

Table 2: Performance of CD Analyses for Different Secondary Structure Assignments

	α-helix		β-sheet		turns		P_{II}		unordered	
secondary structure assignment[a]	δ	r	δ	r	δ	r	δ	r	δ	r
KS	0.070	0.972	0.074	0.834	0.048	0.812			0.078	0.584
SW1	0.071	0.973	0.073	0.824	0.046	0.801	0.045	0.716	0.078	0.168
SW2	0.068	0.974	0.074	0.823	0.044	0.489	0.044	0.448	0.071	0.207

[a] KS assignments correspond to the assignments from Kabsch and Sander (1983) method with no P_{II} classification, resulting in four secondary structures. SW1 and SW2 assignments have five secondary structure fractions with P_{II} assignments from this work. SW1 includes isolated P_{II} residues in the P_{II} fraction. SW2 does not include isolated P_{II} residues in the P_{II} fraction.

found between the fractions of α-helix and P_{II} (–0.533, Table 1). Among the residues assigned to the P_{II} structure, approximately 50% were isolated residues. Two residues in P_{II} structure would result in three successive C=O groups arranged as in one turn of a P_{II} helix, which has implications for exciton interactions and the resulting CD spectra. However, whether isolated residues assigned to the P_{II} structure need to be considered as P_{II} or left unassigned is not clear. A single residue in the P_{II} conformation cannot be considered as a P_{II} helix, but even in an isolated P_{II} residue two successive amide groups are oriented so that the exciton interaction expected in a P_{II} helix is possible. We evaluated both these possibilities by considering all residues in P_{II} structure (SW1 assignments) and only those in chains of P_{II} structure with two or more residues (SW2 assignments), and using the resulting fractions of secondary structures in the analysis of CD spectra.

We examined the possibility that turns are higher order structures than P_{II} because of the existence of a main-chain hydrogen bond in turns identified from the KS method (results not presented). While more than 85% of the residues in P_{II} belonged to unassigned residues from KS, only 0.5% residues were classified both as turns and as P_{II}, and these were either at the beginning or at the end of a P_{II} helix. These P_{II} assignments differed only a little from those obtained by giving precedence to P_{II}.

Secondary Structure CD Spectra. The CD spectra associated with various types of secondary structures were deconvoluted from the basis CD spectra and are given in Figure 1. These are similar in several cases to the CD spectra of synthetic polypeptides in the corresponding conformations (Figure 1A). The CD spectrum of the α-helix shows characteristic bands at 190, 208, and 220 nm, and that of β-sheet shows bands at 190 and 220 nm. The turns, with different sets of φ and ψ, give different classes of CD spectra, and the spectrum we calculate from the protein CD spectra corresponds to class C′ (Woody, 1985), with an inverted α-helix-like spectrum. Similar β-turn CD spectra were obtained by Chang *et al.* (1978), Bolotina *et al.* (1980), Compton and Johnson (1986), and van Stokkum *et al.* (1990). Neither the type I (III) nor type II β-turns, which are prevalent in proteins, are expected to give such a spectrum (Woody, 1985). For reasons which are unclear, aromatic contributions may segregate with the β-turns in this procedure. The CD spectra corresponding to the P_{II} fraction (Figure 1B), obtained by considering either P_{II} segments of at least two residues (curve 4a) or all residues in P_{II} (curve 4), have a strong negative band at 195 nm and a weak positive band at 215 nm, which are characteristic of the poly(Pro)II helix (Woody, 1992). These bands were observed in the CD spectra of unordered polypeptides but with smaller amplitudes (Woody, 1985, 1992). On the other hand, the CD spectrum we calculate for the unordered fraction, after defining the P_{II} structure, has a weak positive band at 198 nm and a weak negative band at 219 nm and qualitatively resembles the β-sheet CD spectrum. Our definition of the P_{II} structure has resulted in a distinctive P_{II} CD spectrum with features consistent with model polypeptide spectra.

Estimation of Secondary Structure from Protein CD Spectra. The performance indices (r and δ) for SW1 and SW2 assignments of secondary structures for CD analysis are compared with those for KS assignments in Table 2. The quality of prediction of the P_{II} fraction from CD spectra is comparable to that of β-sheet and turns if isolated P_{II} residues are included in the P_{II} fraction (SW1 in Table 2). As always, the α-helix fraction is predicted the best. The RMS differences between the predicted and the X-ray fractions of the members of our basis set are similar for the α-helix, β-sheet, and unordered (~7%), as are those for turns and P_{II} structures (~5%). The average fractions of α-helix, β-sheet, turns, P_{II}, and unordered were 37%, 18%, 20%, 10%, and 15%, respectively, in our basis set for SW1 assignments. The corresponding values for SW2 were 37%, 18%, 21%, 5%, and 19%, respectively. The dynamic range of the secondary structure

fractions, ($f_{max} - f_{min}$), for α-helix, β-sheet, turns, P_{II}, and unordered were 0.972, 0.491, 0.310, 0.231, and 0.240 for SW1 and 0.972, 0.491, 0.333, 0.173, and 0.276 for SW2. Each RMS difference should be divided by the dynamic range of the corresponding fractions (Pancoska *et al.*, 1992) to obtain a better comparison. For the four secondary structures, α-helix, β-sheet, turns, and P_{II}, and the unordered fraction, the RMS differences relative to the dynamic range using the SW1 assignments were 0.07, 0.15, 0.15, 0.19, and 0.33, respectively, indicating that the prediction of P_{II} fraction is on a par with that of β-sheet. Inclusion of isolated P_{II} residues in either the unordered or the turns fraction worsens the predictions of turns and P_{II} (Table 2, SW2). The prediction indices for the unordered fraction are worse than those obtained with KS assignments. The separation of the P_{II} fraction from the unassigned set of KS has left only the residues with no obvious secondary structure in the unordered fraction, which has resulted in the low correlation between the predicted and the X-ray fraction of the unordered fraction.

Should the isolated P_{II} residues be classified as P_{II} or unordered? The P_{II} CD obtained resembles the poly(Pro)II CD, regardless of whether or not the isolated P_{II} residues are included in the P_{II} fraction (Figure 1B, curves 4 and 4a). However, the CD spectrum corresponding to the unordered fraction obtained when isolated P_{II} residues are included in the unordered fraction (Figure 1B, curve 5a) is different from that obtained by considering them as P_{II} (Figure 1B, curve 5). The quality of prediction is improved by including the isolated P_{II} residues in the P_{II} fraction. This suggests that the isolated P_{II} residues should be included in the P_{II} fraction in these analyses.

CONCLUSIONS

We have developed a method to identify poly(Pro)II type structure in globular protein structures and used the resulting secondary structural fractions in the analysis of protein CD spectra. Our attempt to quantitate the P_{II} structure as a significant fraction of the unassigned structure in proteins has been successful because of its characteristic CD spectrum. While the high-α proteins are likely to have less P_{II} structure, no significant correlation was obtained between the high-β proteins and P_{II} structure. However, only a small number of structures were used in this study, and an analysis of available protein structures is in order. Most of the longer P_{II} helices are exposed to solvent, implying stabilization from solvent molecules, and molecular dynamics simulations also point to stabilization by a hydrogen-bonded network (Sreerama & Woody, 1992). The conformational rigidity of proline more than doubles its frequency of occurrence in the P_{II} conformation relative to its average frequency of occurrence in proteins (Adzhubei *et. al.*, 1987a,b). The amino acid propensities toward forming P_{II} structure should be important in sequence-based structure assignment (Chou & Fasman, 1978; Garnier *et al.*, 1978; Holley & Karplus, 1989), and these have been calculated using longer segments of P_{II} structure (Adzhubei & Sternberg, 1993). A survey of all available protein structures for characterizing the amino acid preferences is in order, and studies toward that goal are currently underway.

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