Probability-based protein secondary structure identification using combined NMR chemical-shift data

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Abstract

For a long time, NMR chemical shifts have been used to identify protein secondary structures. Currently, this is accomplished through comparing the observed $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, or $^{13}C'$ chemical shifts with the random coil values. Here, we present a new protocol, which is based on the joint probability of each of the three secondary structural types (β-strand, α-helix, and random coil) derived from chemical-shift data, to identify the secondary structure. In combination with empirical smooth filters/functions, this protocol shows significant improvements in the accuracy and the confidence of identification. Updated chemical-shift statistics are reported, on the basis of which the reliability of using chemical shift to identify protein secondary structure is evaluated for each nucleus. The reliability varies greatly among the 20 amino acids, but, on average, is in the order of: $^{13}C^{\alpha}>^{13}C'>^{1}H^{\alpha}>^{13}C^{\beta}>^{15}N>^{1}H^{N}$ to distinguish an α-helix from a random coil; and $^{1}H^{\alpha}>^{13}C^{\beta}>^{1}H^{N}\sim^{13}C^{\alpha}\sim^{13}C'\sim^{15}N$ for a β-strand from a random coil. Amide ^{15}N and $^{1}H^{N}$ chemical shifts, which are generally excluded from the application, in fact, were found to be helpful in distinguishing a β-strand from a random coil. In addition, the chemical-shift statistical data are compared with those reported previously, and the results are discussed. A JAVA User Interface program has been developed to make the entire procedure fully automated and is available via http://ccsr3150-p3.stanford.edu.

Keywords: Chemical shift; NMR; protein secondary structure; secondary structure identification

Since the late 1960's, NMR chemical shifts have been known to have a strong correlation with secondary structure (Markley et al. 1967; Nakamura and Jardetzky 1968). Although several techniques have been developed to characterize and quantify protein and peptide secondary structure using chemical-shift data (Szilagyi and Jardetzky 1989; Pastore and Saudek 1990; Wishart et al. 1991, 1992; Wishart and Sykes 1994), their protocols remain basically the same — through comparing the observed chemical-shift with the random coil value. Because of the qualitative nature of the data and the simplified procedures used in these methods, the accuracy of the identification is limited. Here we describe a new approach, PSSI (Probability based Secondary Structure Identification), to identify protein secondary structure from NMR chemical-shift data. Unlike the previously reported protocols, PSSI assigns the secondary structure type (β-strand, random coil, or α-helix) to each amino acid on the basis of the joint probability, derived from the observed $^{1}H^{N}$, ^{15}N, $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ chemical-shift data of each secondary structure type. PSSI shows significant improvements in both the accuracy and the confidence of identification. Testing on 36 proteins including >6100 residues, this protocol gave a global accuracy of 88%. For proteins with a well-defined secondary structure and sufficient chemical-shift data, PSSI can readily give >90% accuracy.

In recent years, the improvements in NMR instrumentation, novel multidimensional NMR experiments, higher magnetic field strengths, the wide application of protein isotopic labeling techniques, and the software for automatic assignment have greatly enhanced our ability to assign the chemical shifts for large proteins (Kay and Gardner 1997;

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Goto and Kay 2000). However, completion of chemical-shift assignment is just a first step on the long journey of the tertiary structure determination. Other NMR data, that is, NOE, coupling constant, etc., must be collected to allow an accurate tertiary structure determination. As the size of the protein increases, the difficulty experienced in obtaining these data grows very rapidly due to spin diffusion and resonance overlap. The method presented in this study provides a fast and reliable way to identify secondary structure before the tertiary structure can be actually determined. Not only does the accurate secondary structure identification have potential biological significance (e.g., helping to analyze putative sequences for the existence of function-related motifs and to design site-directed mutants), but it also could help in the model-building phase of experimental 3D-structure determination. In addition, the detailed statistical analysis on the chemical-shift distribution against secondary structure elements shown in this study could also provide a deeper insight into the relationship between chemical-shift and protein secondary structure.

Results and Discussion

Chemical-shift statistics

The averaged $^1H^N$, ^{15}N, $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C^\beta$, and $^{13}C'$ chemical shifts, together with the standard deviations categorized according to three secondary structure types, are listed in Table 1. These statistical data are derived from a carefully prepared database containing >6100 amino acids. Since the late 1980s, several statistical studies on the chemical shifts of the 20 amino acids have been reported with the accumulation of NMR data (Wuthrich 1986; Wishart et al. 1991; Lukin et al. 1997; Schwarzinger et al. 2000). The results presented here expand upon the earlier studies in the following respects.

First, the most recently published chemical-shift assignments and structure data are included in the statistical analysis. All of the proteins selected in this study are large in size (>80 residues), double or triple isotope labeled, and their chemical-shifts are assigned by use of multidimensional NMR techniques. The larger chemical-shift and protein coordinate database allows us to specify and take into account other conditions such as chemical-shift reference, pH, and temperature to avoid their possible effects on chemical shift. Table 2 provides a detailed list of the proteins together with BMRB and PDB entries selected for this study. Although most of the BMRB files contain one or more corresponding PDB access numbers, of the 36 proteins used in this study, only 2 can be found in the list of BMRB entries manually matched to PDB NMR entries. Manually matching chemical-shift data (BMRB entry) with the known protein coordinates (PDB entry) is essential for this study. As shown in this table, sometimes sequence renumbering is necessary to obtain a correct match.

Second, an explicit and exact protocol is applied to define a protein's secondary structure from its 3D coordinates. Accurate assignment of secondary structures in proteins with known 3D structures is crucial for the determination of the relationship between protein secondary structure and the observed chemical shift. However, the correlation always remains subjected to some inaccuracy due to the differences in the concept of secondary structure, as well as errors and inconsistencies in experimental structural data. This was shown in the comparison (Colloch et al. 1993) of three methods for structure determination, DSSP (Kabsch and Sander 1983), DEFINE (Richards and Kundrot 1988), and P-curve (Sklenar et al. 1989) on a set of 154 proteins in which all 3 methods agree only 63% of the time. Another comparison (Cuff and Barton 1999) of DSSP, DEFINE, and STRIDE (Frishman and Argos 1995) on a set of 126 proteins showed an overall agreement of only 71%. Among these various protocols/programs, we tried to choose one by which the secondary structures defined could be in a better agreement with the observed chemical-shift data. For this purpose, three automatic programs, VADAR (Wishart et al. 1995a), DSSP, and STRIDE, were used to define the secondary structure during our statistical study. All three programs agree well with each other in defining both the location and the length of long ($\geq$ 4 residues) and well-formed (in terms of dihedral angle, hydrogen bond, etc.) β-strand/α-helix, but not the short (< 4 residues) β-strand/α-helix and poorly-formed β-strand/α-helix. In most cases, a separated β-bridge (one residue) defined by DSSP/STRIDE is assigned as a three-residue β-strand by VADAR. A separated 3_{10}-helix (only three residues) defined by DSSP/STRIDE is normally assigned to random coil by VADAR. For these two situations, we found that the results from VADAR agree with the observed chemical-shift data. For a long β-strand/α-helix with poorly formed boundary, VADAR defines more β-strand/α-helix. Sometimes, modifications had to be made on one or two residues at the end of β-strand or α-helix defined by VADAR, taking into account the backbone dihedral angle, to which the chemical shift is sensitive (Williamson et al. 1992; Wishart and Nip 1997), as well as the results from DSSP and STRIDE.

Furthermore, the 3D coordinates, from which the secondary structures were extracted and used as reference, were carefully selected and priority was given to high-resolution X-ray structures, which have always been the gold standard for the determination of NMR parameters such as $^3J_{HNH\alpha}$ coupling constants, etc. When NMR structures were used, at least three individual structures were selected to generate a secondary structure reference.

The averaged chemical shifts in this study are compared with the earlier reported values and those showing significant discrepancies are indicated in bold in Table 1. More specifically, $^1H^N$ and $^1H^\alpha$ were compared with Wishart's result (Wishart et al. 1991); ^{15}N, $^{13}C^\alpha$, $^{13}C^\beta$, and $^{13}C'$ with

Table 1. *Averaged chemical shift (in ppm) and standard deviation values (in parentheses) categorized according to secondary structure type*

Amino acid	$^{13}C^{\alpha}$			$^{13}C^{\beta}$			$^{13}C'$		
	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Ala	50.86 (1.28)	52.67 (1.76)	54.86 (0.94)	**21.72** (1.77)	19.03 (1.27)	18.27 (1.08)	175.3 (1.61)	177.39 (1.45)	179.58 (1.39)
Arg	54.63 (1.50)	55.96 (1.94)	59.05 (1.21)	32.36 (1.82)	30.53 (1.77)	30.00 (0.83)	175.04 (1.18)	175.91 (1.27)	**178.11** (1.70)
Asn	52.48 (1.18)	52.94 (1.43)	55.67 (0.99)	40.43 (1.89)	**38.22** (1.47)	38.28 (1.12)	174.55 (1.28)	174.98 (1.38)	176.74 (1.66)
Asp	53.41 (1.15)	54.09 (1.59)	57.04 (1.00)	42.78 (1.75)	40.76 (1.34)	40.50 (1.12)	175.15 (1.54)	176.01 (1.45)	**178.07** (1.80)
Cys	57.64 (1.94)	**58.8** (2.06)	62.86 (1.85)	29.48 (1.97)	29.75 (1.86)[b]	26.99 (0.84)[b]	**173.86** (1.83)[a]	174.77 (1.38)[b]	177.42 (1.35)[b]
	54.19 (1.64)[a]	57.68 (1.43)[a,b]	58.57 (1.59)[a,b]	43.79 (4.04)[a]	38.38 (1.39)[a,b]	40.02 (1.78)[a,b]	172.73 (1.05)[a]	175.85 (1.58)[a,b]	176.84 (0.47)[a,b]
Gln	54.33 (1.39)	55.94 (1.83)	58.61 (1.04)	31.92 (1.74)	**28.67** (1.73)	28.33 (0.79)	174.58 (0.94)	175.88 (1.53)	178.35 (1.15)
Glu	**55.55** (1.45)	56.39 (1.84)	59.30 (1.05)	32.45 (1.96)	30.02 (1.62)	29.20 (0.77)	175.01 (1.24)	176.11 (1.47)	178.46 (1.34)
Gly	45.08 (1.20)	45.34 (1.17)	47.02 (0.90)				**173.01** (2.59)	174.30 (1.80)	**176.31** (1.50)
His	54.8 (1.75)	55.78 (2.02)	59.62 (1.57)	32.2 (2.52)	29.62 (1.99)	29.91 (1.67)	**173.80** (2.24)	174.88 (1.68)	176.83 (1.16)
Ile	60.00 (1.51)	60.64 (2.08)	64.68 (1.66)	40.09 (1.85)	38.26 (2.06)	37.59 (1.08)	174.79 (1.41)	175.46 (1.65)	**177.49** (1.62)
Leu	53.94 (1.19)	54.85 (1.79)	57.54 (0.98)	44.02 (1.99)	**41.87** (1.70)	41.40 (1.11)	**175.16** (1.31)	**176.61** (1.77)	178.42 (1.70)
Lys	55.01 (1.00)	56.40 (1.80)	59.11 (1.19)	34.86 (1.79)	32.57 (1.30)	32.31 (1.08)	174.93 (1.25)	176.15 (1.40)	177.79 (2.22)
Met	**54.10** (1.46)	**55.12** (1.79)	58.45 (1.66)	**34.34** (2.44)	32.93 (3.05)	**31.70** (1.72)	**174.64** (1.47)	175.93 (1.54)	177.76 (1.77)
Phe	56.33 (1.31)	**56.94** (1.98)	60.74 (1.63)	41.64 (1.65)	39.43 (1.93)	38.91 (1.49)	174.15 (1.93)	175.28 (1.88)	**176.42** (1.74)
Pro	62.79 (1.22)	63.53 (1.26)	65.52 (1.01)	32.45 (0.93)	31.87 (0.96)	31.08 (0.84)	**176.41** (1.50)	176.91 (1.72)	178.34 (1.53)
Ser	57.14 (1.11)	58.35 (1.78)	60.86 (1.27)	65.39 (1.48)	63.88 (1.24)	62.81 (0.58)	**173.52** (1.55)	174.33 (1.22)	**176.51** (1.40)
Thr	61.10 (1.71)	61.59 (2.04)	65.89 (1.55)	70.82 (2.11)	69.75 (1.29)	68.64 (0.98)	173.47 (1.39)	174.62 (1.45)	176.62 (1.24)
Trp	56.28 (1.52)	57.62 (1.25)	60.03 (1.94)	**31.78** (1.76)	29.27 (1.10)	28.74 (1.15)	175.10 (1.80)	175.91 (1.32)	**177.81** (1.62)
Tyr	56.56 (1.59)	57.72 (2.14)	61.07 (1.72)	40.79 (1.77)	38.71 (2.00)	38.38 (0.89)	174.65 (1.64)	175.32 (1.54)	177.05 (1.51)
Val	60.72 (1.59)	61.80 (2.25)	65.96 (1.39)	33.81 (1.79)	32.68 (1.76)	31.41 (0.74)	174.66 (1.36)	175.76 (1.63)	177.75 (1.49)

Amino acid	$^{1}H^{N}$			$^{1}C^{\alpha}$			^{15}N		
	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Ala	**8.59** (0.76)	8.11 (0.68)	7.99 (0.57)	**4.87** (0.46)	4.25 (0.35)	4.03 (0.31)	125.57 (4.80)	**132.52** (3.51)	121.65 (2.52)
Arg	8.57 (0.69)	8.17 (0.77)	8.03 (0.56)	**4.85** (0.47)	4.33 (0.37)	4.00 (0.33)	**122.60** (4.74)	**120.59** (4.42)	118.99 (2.56)
Asn	8.70 (0.55)	8.33 (0.72)	8.20 (0.66)	**5.26** (0.41)	4.60 (0.38)	4.45 (0.20)	**122.70** (4.18)	118.48 (4.58)	**117.60** (2.37)
Asp	8.56 (0.62)	8.39 (0.66)	8.05 (0.55)	5.01 (0.36)	4.64 (0.29)	4.44 (0.22)	123.82 (4.70)	120.69 (4.45)	119.90 (2.03)
Cys	9.00 (0.45)	7.81 (0.62)	8.22 (0.53)	5.18 (0.57)	4.63 (0.37)[b]	4.16 (0.25)[b]	123.27 (5.69)	117.01 (2.50)[b]	117.47 (3.04)[b]
	8.68 (0.98)[a]	8.53 (0.59)[a,b]	8.58 (0.48)[a,b]	5.21 (0.49)[a]	4.44 (0.29)[a,b]	4.53 (0.18)[a,b]	121.81 (4.34)[a]	118.62 (4.25)[a,b]	119.51 (2.44)[a,b]
Gln	8.51 (0.83)	8.25 (0.75)	8.11 (0.52)	4.97 (0.43)	4.26 (0.39)	4.03 (0.23)	123.14 (4.89)	119.73 (3.85)	118.59 (2.59)
Glu	8.66 (0.60)	8.29 (0.53)	8.32 (0.63)	4.76 (0.44)	4.28 (0.30)	3.99 (0.21)	123.52 (4.29)	120.87 (3.94)	119.89 (2.85)
Gly	8.27 (1.06)	8.34 (0.83)	8.23 (0.78)	4.09[c] (0.46)	3.95[c] (0.40)	3.84 (0.43)[c]	110.19 (4.20)	109.94 (4.09)	**107.34** (2.82)
His	**8.76** (0.79)	8.09 (0.83)	8.03 (0.68)	5.07 (0.50)	4.50 (0.51)	4.06 (0.54)	**121.65** (5.16)	**118.87** (4.98)	118.09 (3.17)
Ile	8.74 (0.66)	7.94 (0.66)	8.06 (0.56)	4.72 (0.42)	4.13 (0.36)	3.66 (0.30)	124.12 (4.93)	121.07 (5.17)	120.22 (2.75)
Leu	8.63 (0.67)	8.12 (0.72)	8.02 (0.56)	4.85 (0.43)	4.35 (0.36)	4.00 (0.27)	125.69 (4.14)	121.53 (4.30)	120.18 (2.46)
Lys	8.54 (0.63)	8.13 (0.66)	8.04 (0.61)	4.96 (0.46)	4.28 (0.31)	3.98 (0.26)	123.29 (4.76)	121.44 (4.19)	119.90 (2.93)
Met	8.43 (0.65)	8.37 (0.51)	8.05 (0.48)	4.94 (0.48)	4.55 (0.38)	4.03 (0.35)	121.67 (4.12)	120.19 (3.46)	118.69 (2.36)
Phe	8.80 (0.70)	7.95 (0.90)	8.21 (0.66)	5.17 (0.47)	4.62 (0.42)	4.11 (0.40)	121.95 (4.38)	119.41 (4.75)	119.12 (4.05)
Pro				4.72 (0.45)	4.41 (0.30)	4.13 (0.39)			
Ser	8.57 (0.65)	8.26 (0.74)	8.11 (0.50)	5.08 (0.48)	4.48 (0.35)	4.20 (0.19)	117.44 (4.19)	115.94 (4.13)	**114.78** (2.39)
Thr	8.50 (0.58)	8.22 (0.74)	8.10 (0.55)	4.81 (0.46)	4.33 (0.38)	4.02 (0.27)	**118.09** (4.86)	114.41 (5.70)	115.30 (3.72)
Trp	8.83 (0.73)	**7.59** (0.84)	8.24 (0.82)	**5.24** (0.41)	4.54 (0.24)	4.35 (0.40)	124.04 (5.43)	**120.57** (3.58)	120.48 (2.89)
Tyr	8.69 (0.73)	**7.90** (0.79)	8.10 (0.70)	5.00 (0.51)	4.55 (0.45)	4.14 (0.36)	122.55 (4.70)	120.05 (4.23)	119.67 (3.19)
Val	8.73 (0.61)	7.88 (0.75)	7.99 (0.63)	4.66 (0.42)	4.14 (0.40)	3.57 (0.34)	123.27 (5.05)	**119.66** (5.62)	119.53 (3.19)

[a] Cys in the oxidized form.

[b] Number of the chemical shifts used in the statistical analysis is less than 10.

[c] Averaged value fro Gly.

Those that show significant discrepancies ($^{15}N > 1.0$ppm, $^{1}H^{\alpha} > 0.2$, $H^{N} > 0.3$, $^{13}C' > 0.5$) when compared with earlier reported value (see Results and Discussion) are indicated in bold.

that of Lukin's (Lukin et al. 1997). The statistics of the chemical-shift difference, Δδ (averaged chemical-shift value in this study minus previously reported value), are also listed in Table 3. As shown in Tables 1 and 3, despite significant differences for certain amino acids, on average, the $^{1}H^{N}$, $^{1}H^{\alpha}$, and $^{13}C^{\alpha}$ chemical shifts reported in this study are in good agreement with the earlier results, whereas for ^{15}N, $^{13}C^{\beta}$, and $^{13}C'$ chemical shifts, the discrepancies are

Table 2. *Listing of proteins used in chemical shift statistical analysis*

Protein (no. of residues)	BMRB entries (conditions)[a]	PDB entries (Exp Method)	PDB entries Manually Matched in BMRB
β2-GP1 domain V (86)	4981 (pH6.0, 298K)	1G4F (NMR)	N/A
Vam3p N-terminal (123)	**4945 (pH6.0, 302K)**[b]	**1HS7 (NMR)**[b]	N/A
Mouse doppel (132)	**4938 (pH5.2, 299K)**[b]	**1I17 (NMR)**[b]	N/A
DLC8 (89)	4931 (pH7.0, 303K)	1F95 (NMR)	N/A
ERp29 C-domain (120)	**4920 (pH4.9, 308K)**[b]	**1G7D (NMR)**[b]	N/A
ERp29 N-domain (137)	**4919 (pH4.9, 308K)**[b]	**1G7E (NMR)**[b]	N/A
Dynein light chain 8 (89)	4911 (pH7.0, 303K)	1F96 (NMR)	N/A
Human galectiin-3, C-terminal (143)	4909 (pH7.4, 303K)	1A3K (X-ray)	N/A
CDC4P (141)	4851 (pH6.5, 303K)	1GGW (NMR)	N/A
Hepatitis A 3C protease (217)	4836 (pH5.4, 298K)	1QA7 (X-ray)	N/A
EPPIb (164)	4765 (pH6.2, 308K)	2NUL (X-ray)	N/A
Xylanase (185)	4705 (pH7.4, 298K)	1C5I (X-ray)	N/A
Apo CRBP II (134)	4681 (pH7.4, 298K)	1OPB (X-ray)	N/A
Pathogenesis-related protein (159)	4671 (pH7.0, 298K)	1E09 (NMR)	N/A
HTLV-I capsid protein (134)	4649 (pH6.0, 302K)	1G03 (NMR)	N/A
Phosphoglycerate mutase (211)	4648 (pH6.4, 310K)	1FZT (NMR)	N/A
Human prion protein (146)	4641 (pH4.6, 299K)	1F07 (NMR)	N/A
Anti-dansyl Fv fragment (237)	**4580 (pH6.0, 310K)**[b]	**2DLF (X-ray)**[b]	N/A
Bet v 1-L (159)	4417 (pH7.0, 298K)	1B6F (NMR)	1B6F
HPrP (210)	4402 (pH4.5, 293K)	1QLX (NMR)	N/A
Cyclic AMP receptor protein (209)	4388 (pH6.0, 313K)	1RUN (X-ray)	N/A
CA RSV (262)	4384 (pH6.0, 303K)	1D1D (NMR)	N/A
Rabphilin_3_C2B (140)	4360 (pH6.1, 304K)	3RPB (NMR)	N/A
FvNPN43C9 (230)	**4349 (pH6.8, 298K)**[b]	**43C9 (X-ray)**[b]	N/A
α-β T cell receptor (249)	4330 (pH5.0, 298K)	1D9K (X-ray)	N/A
p55 (166)	4321 (pH6.5, 300K)	5GCN (NMR)	N/A
HTLV-I capsid (214)	4311 (pH6.0, 303K)	1QRJ (NMR)	N/A
CaM:SEF2-1 (148)	4310 (pH6.0, 306K)	1CDL (X-ray)	N/A
P14a (135)	4301 (pH5.5, 303K)	1CFE (NMR)	N/A
XRCC1 N-terminal (183)	4282 (pH6.8, 298K)	1XNA (NMR)[c]	N/A
Superoxide Dismutase (153)	4202 (pH5.0, 298K)	1MFM (X-ray)	1BA9 (NMR)
Intimin cell adhesion domain (280)	**4111 (pH5.0, 310K)**[b]	**1FOO (X-ray)**[b]	N/A
metallo-β-lactamase (232)	4102 (pH7.0, 295K)	2BMI (X-ray)	N/A
Core binding factor (143)	4092 (pH6.6, 293K)	2JHB (NMR)	N/A
Protoporphyrin IX (147)	4038 (pH5.0, 297K)	1VRF (NMR)	N/A
Human carbonic anhydrase I (260)	4022 (pH6.2, 303K)	1HUG (X-ray)	N/A

[a] DSS was used directly or indirectly as zero chemical shift reference of ^{1}H, ^{15}N, and ^{13}C for all of the proteins.
[b] Manually renumbering of the sequence is needed to match the BMRB entry to PDB entry.
[c] Coordinate is not available for the random coil region (resides 152–183) in the PDB file.

relatively larger for both the overall averaged chemical shift and certain individual amino acids. Because the random coil chemical shift is usually used as a reference in the identification of secondary structure, a number of random coil data sets have been published. A comparison of the random coil chemical shift obtained in this study with those most recently obtained by Schwarzinger et al. (2000) on Ac-GGXGG-NH2 hexapeptides in pH 2.3, 8 M urea is listed in Table 4. As shown in Table 4, despite the difference in the methods applied, on average, the random coil chemical shifts of ^{1}H^{N}, ^{1}H$^{\alpha}$, ^{13}C$^{\beta}$, and ^{13}C$^{\alpha}$ reported in this study are in good agreement with those of Schwarzinger et al. (2000). However, as is apparent from this table, systematic deviation of nearly 1.0 ppm is observed between the two sets of data for ^{13}C′ for most of the amino acids except Asp and Cys. As the data of Schwarzinger et al. (2000) were obtained at very low pH (2.3), the CO may be partly protonated and, hence, affect the observed the chemical shifts. It is of great interest to find that significant deviations also exist for ^{13}C$^{\beta}$ chemical shift on Asp and Cys. One possible reason for this could be the formation of an intra-residue hydrogen bond, OH-CO for Asp and SH-CO for Cys. The other few significant deviations could be attributed to the different method used.

Most important is that the standard deviation of the chemical-shift distribution, which is ignored in earlier reports, is reported for each individual amino acid in this study. As we show later, the standard deviation plays an important role in evaluating the reliability of the identification. As shown in Table 1, the value of standard deviation

Table 3. *Statistic result of Δδ (averaged chemical shift in this study—previously reported value[a])*

	$^{1}H^{N}$			$^{1}H^{\alpha}$			^{15}N		
Amino acid	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Average	0.07	−0.07	0.03	0.11	0.02	0.00	0.14	−0.57	−0.58
Std	0.16	0.16	0.11	0.10	0.11	0.07	0.88	0.74	0.66
Max	0.32	0.22	0.26	0.3	0.32	0.15	1.56	1.21	0.58
Min	−0.27	−0.44	−0.11	−0.04	−0.16	−0.16	−1.18	−1.68	−2.0
		$^{13}C^{\alpha}$			$^{13}C^{\beta}$			$^{13}C'$	
Amino acid	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix	β-strand	Random coil	α-helix
Average	−0.10	−0.09	−0.07	0.16	−0.32	−0.25	−0.09	−0.11	−0.31
Std	0.25	0.46	0.34	0.46	0.38	0.33	0.50	0.32	0.52
Max	0.5	0.89	0.69	0.95	0.19	0.13	1.23	0.37	1.16
Min	−0.5	−1.21	−0.98	−0.9	−1.16	−1.45	−0.9	−0.73	−1.03

[a] $^{1}H^{N}$ and $^{1}H^{\alpha}$ are from Wishart et al. (1991); ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ are from Lukin et al. (1997).

varies greatly with the amino acid as well as the secondary structure type. In earlier studies, the standard deviation value was either not considered (Wishart et al. 1992; Wishart and Sykes 1994) or used as an averaged value (Lukin et al. 1997). As shown in Figure 1, the chemical-shift distribution varies from one amino acid to another. Using the standard deviation data specified for each of the individual amino acid and secondary structure type certainly increases the accuracy of the secondary structure identification. The statistics of the chemical-shift data in Table 1, we believe, will have other potential applications, such as automatic chemical-shift assignment.

Table 4. *The difference of the random coil chemical shifts (previously reported value[a] minus that from this study)*

Amino acid	$^{13}C'$	$^{13}C^{\beta}$	$^{13}C^{\alpha}$	$^{1}H^{N}$	$^{1}H^{\alpha}$	^{15}N
Ala	1.11	0.23	0.15	0.24	0.1	1.48
Arg	1.19	0.40	0.52	0.22	0.05	0.61
Asn	1.12	0.87	0.39	0.18	0.19	0.52
Asp	−0.11[b]	−2.43[b]	−1.1	0.17	0.18	−1.59
Cys	0.17[b]	−4.10[b]	0.56	0.6	−0.04	1.22
Gln	0.92	0.86	0.28	0.19	0.12	0.77
Glu	0.69	−1.14	−0.3	0.11	0.14	−0.67
Gly	0.60		0.05	0.07	0.02	−2.44
His	0.22	−0.5	−0.39	0.47	0.29	−0.77
Ile	1.64	0.65	0.98	0.23	0.08	−0.67
Leu	1.59	0.59	0.62	0.16	0.03	0.87
Lys	1.25	0.64	0.31	0.23	0.08	0.16
Met	1.17	0.01	0.65	0.05	−0.03	0.11
Phe	1.32	0.32	1.15	0.36	0.03	1.29
Pro	0.89	0.35	0.17		0.04	
Ser	1.07	0.18	0.32	0.17	0.03	−0.44
Thr	0.98	0.26	0.42	0.03	0.1	−2.41
Trp	1.19	0.48	−0.02	0.63	0.16	1.53
Tyr	1.38	0.23	0.56	0.36	0.03	0.85
Val	1.24	0.14	0.81	0.28	0.02	−0.36
Average	0.98	0.27	0.31	0.24	0.08	0.00
SD	0.46	0.49	0.51	0.17	0.08	1.18

[a] Schwarzinger et al. (2000).

[b] Data is not included in the calculation of average and S.D.

Reliability of identification

When using observed chemical shifts to identify secondary structure, we would like to know the reliability of the result. In this study, the reliability of using an observed chemical shift to identify secondary structure is investigated. This is accomplished by evaluating *R*, the resolution of the chemical-shift distribution between the three secondary structure types. The calculated $R_{strand\ vs.\ coil}$ and $R_{helix\ vs.\ coil}$ values (see Materials and Methods for definitions) for the 20 amino acids are listed in Table 5. The higher the $R_{strand\ vs.\ coil}$ or $R_{helix\ vs.\ coil}$ value, the higher the reliability of distinguishing a β-strand or an α-helix from a random-coil. A High-Low-Average chart of $R_{strand\ vs.\ coil}$ and $R_{helix\ vs.\ coil}$ is shown in Figure 2. Both $R_{strand\ vs.\ coil}$ and $R_{helix\ vs.\ coil}$ vary greatly with the nucleus and the amino acid. On the basis of the calculated values of $R_{strand\ vs.\ coil}$ and $R_{helix\ vs.\ coil}$, on average, the reliability is in the order of: $^{13}C^{\alpha}>^{13}C'>^{1}H^{\alpha}>^{13}C^{\beta}>^{15}N>^{1}H^{N}$ to distinguish an α-helix from a random coil; and $^{1}H^{\alpha}>^{13}C^{\beta}>^{1}H^{N}\sim^{13}C^{\alpha}\sim^{13}C'\sim^{15}N$ to distinguish a β-strand from a random coil. In this study, we also found that the amide ^{15}N and $^{1}H^{N}$ chemical shifts, which were generally excluded in prior work, have almost the same ability/resolution as those of $^{13}C^{\alpha}$ and $^{13}C'$ to distinguish a β-strand from random coil. It is of interest that the $R_{strand\ vs.\ coil}$ values of amide $^{1}H^{N}$ are relatively higher for amino acids Cys, Phe, Ile, Val, Trp, Tyr, and His. Most of these amino acids are of high β-strand propensity (Kim and Berg 1993).

Probability-based secondary structure identification

In this study, the secondary structure is identified by comparing the joint probabilities derived from the observed $^{13}C^{\alpha}$, $^{13}C'$, $^{1}H^{\alpha}$, $^{13}C^{\beta}$, ^{15}N, and $^{1}H^{N}$ chemical shifts. After the secondary structure type of each residue is assigned,

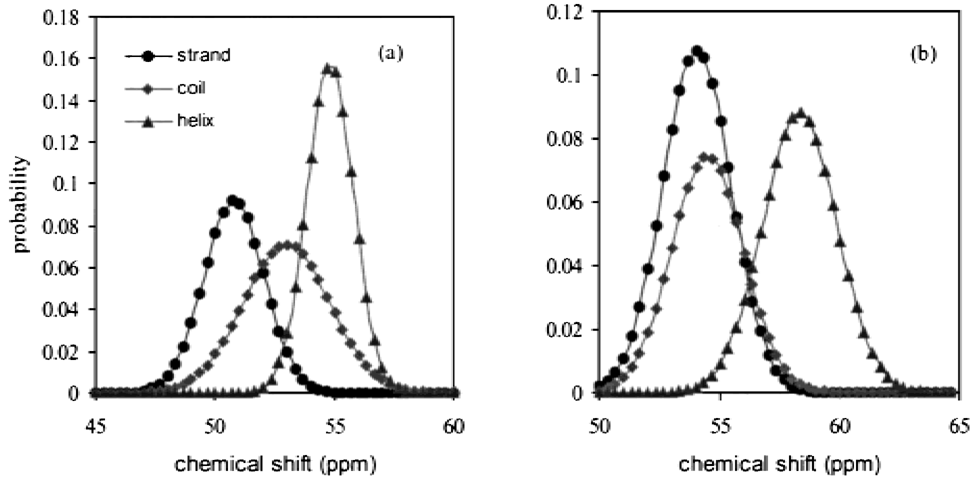


Fig. 1. Simulated $^{13}C^{\alpha}$ chemical-shift distribution of (*a*) Ala and (*b*) Met. (●) Strand; (♦) coil; (▲) helix.

empirical pattern filters/functions are used to smooth poorly defined secondary structure fragments. Testing this probability-based method on a set of 30 proteins gave an accuracy (on a residue per residue basis) of 89% for β-strand, 84% for random-coil, and 89% for α-helix. For all of the residues (>6100), the global accuracy Q3 (see Materials and Methods for definition) is 88%. Testing on the same set of proteins, the current CSI methods (Wishart et al. 1992; Wishart and Sykes 1994) gave a global accuracy of 81%. This improvement in accuracy, we believe, is due to the over-simplified protocol used by the CSI method, which assigns the secondary structure type to each residue by simply comparing the observed chemical-shift with the random coil value. More specifically, if the measured chemical-shift value is within the range of the random coil (± 0.1 ppm for ^{1}H, ± 0.5 ~0.7 ppm for ^{13}C), then the residue is assigned as random coil, otherwise, it is assigned as either β-strand or α-helical. The over-simplified procedure used in CSI methods certainly causes a loss of accuracy. In addition, formerly ignored amide ^{15}N and $^{1}H^{N}$ chemical shifts are included in this study, and they improve the accuracy and the confidence of identification.

Table 5. *Calculated $R_{strand\ vs.\ coil}$ and $R_{helix\ vs.\ coil}$ for the 20 amino acids*

	$R_{strand\ vs\ coil}$						$R_{helix\ vs\ coil}$					
Amino acid	$^{1}H^{\alpha}$	$^{13}C^{\beta}$	$^{1}H^{N}$	$^{13}C^{\alpha}$	$^{13}C'$	^{15}N	$^{13}C^{\alpha}$	$^{13}C'$	$^{1}H^{\alpha}$	$^{13}C^{\beta}$	^{15}N	$^{1}H^{N}$
Ala	0.85	0.92	0.3	0.79	0.66	0.2	0.66	0.61	0.28	0.3	0.29	0.12
Cys	0.46	0.22	0.6	0.53	0.38	0.6	0.34	0.8	0.45	0.12	0.08	0.19
Asp	0.65	0.7	0.07	0.25	0.28	0.36	1.02	0.88	0.36	0.11	0.13	0.27
Glu	0.74	0.81	0.27	0.32	0.35	0.37	1.03	0.83	0.52	0.29	0.12	0.05
Phe	0.55	0.65	0.56	0.15	0.24	0.29	1.08	0.33	0.65	0.09	0.08	0.16
Gly	0.18		0.09	0.11	0.24	0.03	0.83	0.65	0.13		0.37	0.01
His	0.57	0.61	0.5	0.23	0.27	0.35	1.08	0.6	0.4	0.04	0.02	0.03
Ile	0.77	0.43	0.58	0.18	0.23	0.33	1.02	0.39	0.62	0.35	0.03	0.02
Lys	0.88	0.83	0.34	0.47	0.43	0.17	0.95	0.39	0.57	0.23	0.18	0.11
Leu	0.58	0.57	0.37	0.33	0.44	0.46	0.81	0.49	0.5	0.15	0.27	0.08
Met	0.38	0.32	0.09	0.16	0.36	0.17	1.24	0.56	0.9	0.25	0.25	0.25
Asn	0.86	0.68	0.21	0.22	0.18	0.5	1.1	0.41	0.19	0.08	0.2	0.18
Pro	0.51	0.37		0.52	0.15		0.79	0.7	0.37	0.42		
Gln	0.77	0.84	0.18	0.42	0.41	0.43	0.91	1.15	0.38	0.19	0.24	0.1
Arg	0.65	0.6	0.26	0.32	0.3	0.17	0.96	0.69	0.35	0.18	0.23	0.08
Ser	0.69	0.62	0.22	0.43	0.28	0.18	0.79	0.76	0.48	0.48	0.13	0.07
Thr	0.6	0.37	0.19	0.16	0.41	0.37	1.21	0.61	0.43	0.54	0.13	0.09
Val	0.65	0.31	0.58	0.28	0.36	0.37	1.06	0.53	0.74	0.52	0.02	0.02
Trp	1.12	0.9	0.69	0.57	0.01	0.25	0.53	0.79	0.14	0.2	0.17	0.31
Tyr	0.44	0.6	0.54	0.32	0.2	0.26	0.83	0.48	0.53	0.1	0.03	0.19

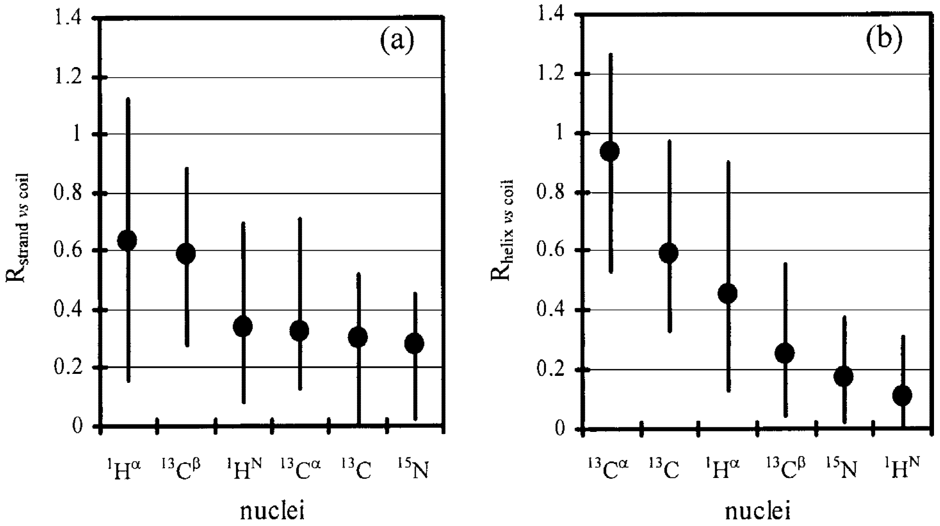


Fig. 2. High-Low-Average charts displaying the distribution of (*a*) $R_{stand\ vs.\ coil}$ and (*b*) $R_{helix\ vs.\ coil}$ among the 20 amino acids.

For proteins with well-defined secondary structure and sufficient ^{1}H, ^{13}C, and ^{15}N chemical-shift assignments, PSSI can give an accuracy of identification as high as 95% (i.e., HTLV-I Capsid Protein). We notice that almost all of the misidentifications occur between β-strand/random coil or α-helix/random coil. Among the misidentifications, those between β-strand/α-helix are <1%. Amide ^{15}N and ^{1}H^{N} chemical shifts, which were easily obtained but excluded from application, were found actually useful, specifically for distinguishing β-strand from random coil. Including these two nuclei into the calculation allows a 1.6% increase of Q3 value for the proteins we tested.

If a residue is in a well-defined secondary fragment, in most cases it has a probability of that secondary structure type above 0.9 (the sum of the probabilities of all the secondary structure types are normalized to 1) and is readily distinguished from the other two types. Plotted in Figure 3, are the normalized joint probabilities calculated from ^{1}H, ^{15}N, and ^{13}C chemical-shifts for a fragment of pathogenesis-related protein P14a as well as the secondary structures extracted from its 3D coordinates. The probability distributions shown in this figure provide high-confidence identification of the β-strand, random coil, and α-helix. Those with a poorly defined secondary structure type, for example, the isolated short β-bridge and 3_{10}-helix, are clearly indicated by the ambiguity of the probability between the three secondary structure types.

A JAVA user interface graphic program, PSSI, has been developed to make the process automatic. The chemical-shift data can be read from a file in the NMR-str format (essential for the deposition of the chemical-shift data in the BioMagResBank) or input by the user directly. Its output includes: (1) The normalized joint probability of each secondary structure type, the basis of which the secondary structure is automatically assigned. As we discussed earlier, in this study, empirical filters/functions are used to smooth the poorly defined secondary structure fragments. However, users can also make judgements by themselves on the basis of the probabilities provided by PSSI. (2) The secondary structure, which is editable, derived from chemical-shift data. (3) A graphic output displaying the identified secondary structure. A graphic output from PSSI showing the secondary structure derived from NMR chemical-shift data is shown in Figure 4. Because chemical-shift references, other than DSS, could be used, a function to calibrate the chemi-

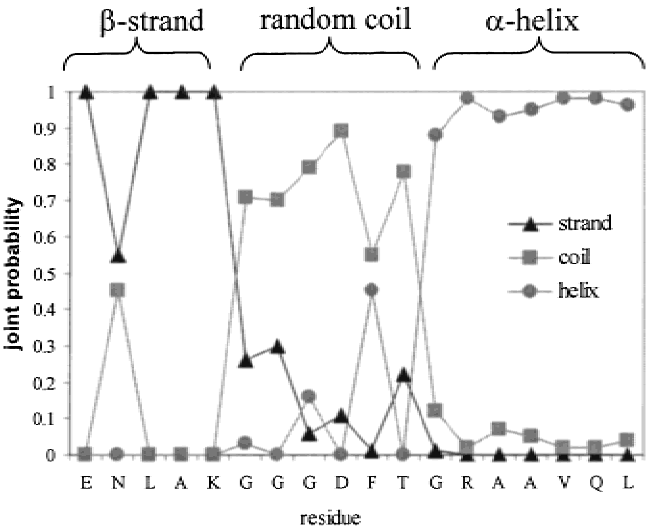
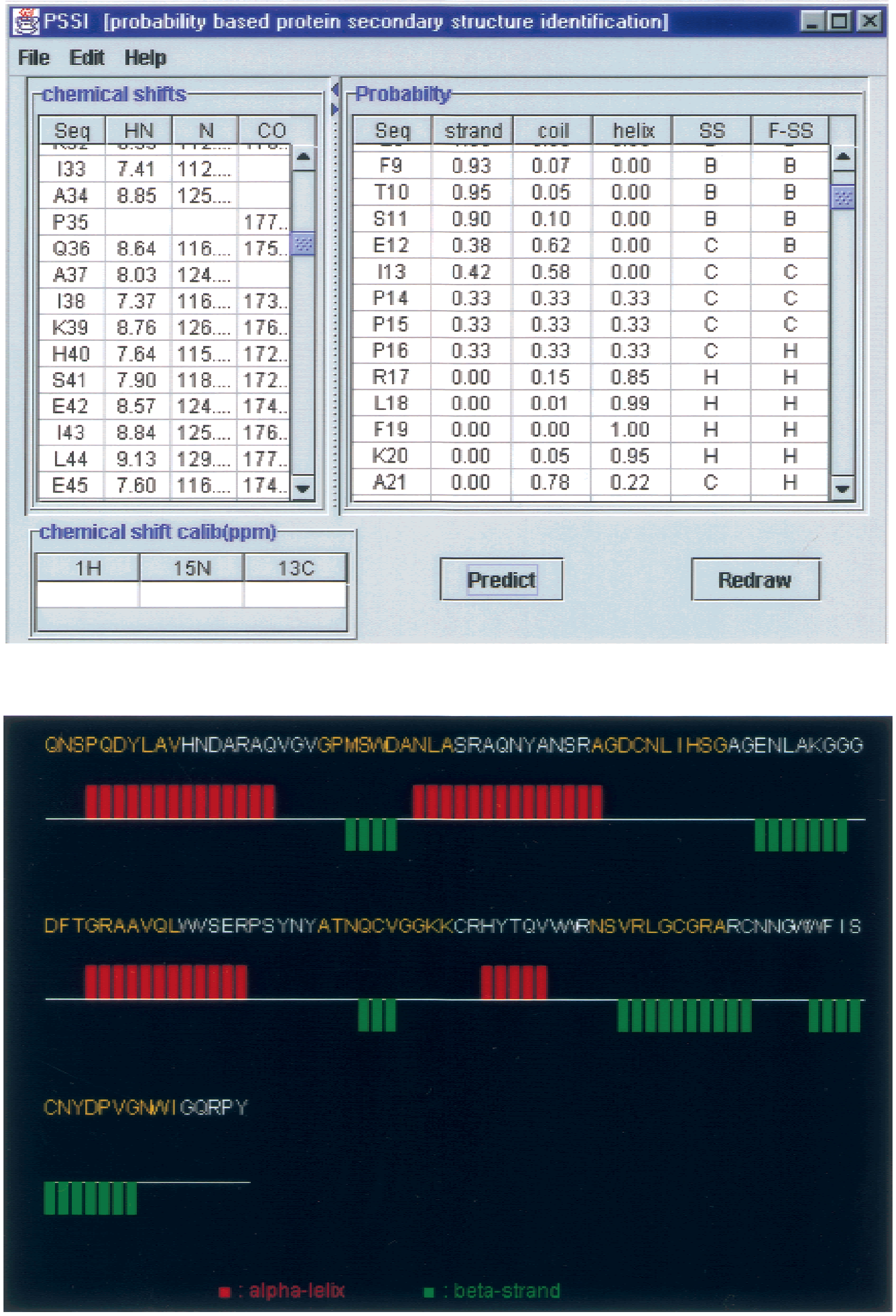


Fig. 3. Normalized joint probabilities of the three secondary structure types for a fragment from Pathogenesis-related Protein P14a. The secondary structures derived from its 3D coordinates are marked at *top*. (▲) Strand; (■) coil; (●) helix.


PSSI [probability based protein secondary structure identification]
File
Edit
Help
chemical shifts
Probabilty
chemical shift calib(ppm)
Predict
Redraw

Seq	HN	N	CO
I33	7.41	112....	
A34	8.85	125....	
P35			177..
Q36	8.64	116....	175..
A37	8.03	124....	
I38	7.37	116....	173..
K39	8.76	126....	176..
H40	7.64	115....	172..
S41	7.90	118....	172..
E42	8.57	124....	174..
I43	8.84	125....	176..
L44	9.13	129....	177..
E45	7.60	116....	174..

Seq	strand	coil	helix	SS	F-SS
F9	0.93	0.07	0.00	B	B
T10	0.95	0.05	0.00	B	B
S11	0.90	0.10	0.00	B	B
E12	0.38	0.62	0.00	C	B
I13	0.42	0.58	0.00	C	C
P14	0.33	0.33	0.33	C	C
P15	0.33	0.33	0.33	C	C
P16	0.33	0.33	0.33	C	H
R17	0.00	0.15	0.85	H	H
L18	0.00	0.01	0.99	H	H
F19	0.00	0.00	1.00	H	H
K20	0.00	0.05	0.95	H	H
A21	0.00	0.78	0.22	C	H

1H	15N	13C

QNSPQDYLAVHNDARAQVGVGPMSWDANLASRAQNYANSRAGDCNL IHSGAGENLAKGGG
DFTGRAAVQLWWSERPSYNYATNQCVGGKKCRHYTQVWWRNSVRLGCGRARCNNGWWF IS
CNYDPVGNWI GQRPY
🟥 : alpha-lelix
🟩 : beta-strand

Fig. 4. PSSI (*top*), and its graphic output (*bottom*) showing the secondary structure derived from NMR chemical-shift data.

cal-shift reference is also provided. The whole package is available at http://ccsr3150-p3.stanford.edu.

Materials and methods

A data-set containing chemical shifts and secondary structures was established as follows: the whole BioMagResBank database was downloaded and entries meeting the following criteria were selected first. (1) ^{1}H, ^{15}N, and ^{13}C chemical shifts were referred to DSS (Wishart et al. 1995b); (2) the temperature at which the NMR data were collected was 293–313 K; (3) pH of the sample is 4.5–8.5; and (4) the sequence length is >80. For each initial chemical-shift entry obtained, the corresponding coordinate file was carefully identified either on the basis of the information provided in chemical-shift file and/or by a key words search from Protein Data Bank (PDB). The second selection was based on availability of the 3D coordinates, number of assignments, and quality of the structures. In cases in which several PDB files were available for one protein, the priority of selection was given to the high-resolution X-ray structure. If only NMR structures were available, the consensus result from at least three structures was used. For some proteins, modification of the sequence numbering had to be made to precisely match the chemical-shift data with the coordinate data. Finally, a data-set of 36 distinct, nonparamagnetic proteins including >6100 amino acid residues, was set up for the statistical analysis.

Three programs, VADAR, DSSP, and STRIDE were compared and used to define the reference secondary structure from the 3D coordinate file. Consensus results from the three programs, taking into account the backbone ϕ and ψ dihedral angles of each residue, were used as the secondary structure reference.

During the statistical analysis of the chemical-shift distribution against secondary structure (β-strand, random coil, and α-helix), extremely large or small values (>4.5 standard deviation) were automatically removed. The chemical-shift data of the very first N-terminal and last C-terminal residues of each protein were also excluded to avoid possible terminal effects. The averaged chemical shift, standard deviation, and the distribution resolutions of each nucleus were calculated by use of either the JAVA program or MS Excel for all of the 20 amino acids. The resolution is defined as:

$$R_{helix\ vs.\ coil} = \frac{|\bar{\delta}_\alpha - \bar{\delta}_c|}{|\sigma_\alpha - \sigma_c|}$$

$$R_{strand\ vs.\ coil} = \frac{|\bar{\delta}_\beta - \bar{\delta}_c|}{|\sigma_\beta - \sigma_c|}$$

in which the averaged chemical shift and standard deviations are categorized according to the three secondary structure types (β-strand, random-coil, and α-helix).

Secondary structure identification

Two steps were used to identify the secondary structure elements from the observed NMR chemical-shift data.

Step l: Joint probability

Given a $\{\delta_n\}$ $(n = \delta_N, \delta_{CA}, \delta_{CO}, \delta_{CB}, \delta_{HA}, \delta_{HN})$ are the observed chemical shifts for amino acid *i;* the secondary structure type of this amino acid is adjudged by comparing the joint probability of the three secondary structure types, *Ps,i*.

$$P_{s,i}(\{\delta_n\}) = F_{s,i} \prod_n G_{s,i}(\delta_n)$$

$F_{s,i}$ represents the probability for amino acid *i* at the secondary structure type *s* (*s* = β-strand, random coil, or α-helix); $G_{s,i}$ is the Gaussian distribution.

$$G_{s,i}(\delta_n) = \frac{1}{\sqrt{2\pi\sigma_{n,s,i}}} \exp\left[-\frac{(\delta_n - \bar{\delta}_{n,s,i})^2}{2\sigma^2_{n,s,i}}\right]$$

During the calculation, $G_{s,i}(\delta_n)$ is set to 1 if δ_n is not available. G of three secondary structure types is normalized so that its sum is equal to 1.0, as is the joint probability, Ps, for each residue. The secondary structure type is initially assigned to B, C, or H (representing β-strand, random coil, and α-helix, respectively) based on joint probability of each type, that is, B if $P_{\beta,i} > P_{c,i}$ and $P_{\beta,i} > P_{\alpha,i}$.

Step 2: Smoothing/filtering

This step is optional. Users can make their own adjustments on the basis of the calculated probabilities. The result from step 1 can be further smoothed/filtered by use of empirical patterns and smoothing functions. For example, (1) if a local density of either B or C exceeds one-half for a five-residue window, its secondary structure type is adjusted on the basis of Ps values, that is, a BBCBC segment will be adjusted to BBBBB (if P_β of the last residue is >0.35) or BBBBC (if P_β of the last residue = <0.35); (2) if a C-type residue is not covered by the smoothing/filtering in (1) and is located either at the end or in the middle of B or H segment, its Ps is recalculated and its secondary structure type is readjudged accordingly. For example, for a CHHH segment, the $P_{s,i}$ of the first residue is recalculated as $P_{s,i} = 0.5{*}P_{s,1} + 0.3{*}P_{s,2} + 0.2{*}P_{s,3}$ for the second N-terminal residue; (3) separated short (less than two residues) B or H segments are smoothed to C.

The global accuracy of identification Q3 (Schulz and Schimer 1979) is defined as:

$$Q_3 = \frac{\Sigma_s P_s}{T}$$

in which P_s is the number of residues identified in the *s* (β-strand, random coil, and α-helix) state, effectively observed in the *s* state; T is the total number of residues.

The JAVA user interface program (PSSI), which makes the entire procedure fully automated, was developed by one of the authors (Y.J. Wang)

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