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The ^{13}C Chemical-Shift Index: A simple method for the identification of protein secondary structure using ^{13}C chemical-shift data*

David S. Wishart and Brian D. Sykes**

Protein Engineering Network of Centres of Excellence, Department of Biochemistry, University of Alberta, Edmonton, AB, Canada T6G 2S2

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SUMMARY

A simple technique for identifying protein secondary structures through the analysis of backbone ^{13}C chemical shifts is described. It is based on the Chemical-Shift Index [Wishart et al. (1992) *Biochemistry*, **31**, 1647–1651] which was originally developed for the analysis of $^{1}H^{\alpha}$ chemical shifts. By extending the Chemical-Shift Index to include $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and carbonyl ^{13}C chemical shifts, it is now possible to use four independent chemical-shift measurements to identify and locate protein secondary structures. It is shown that by combining both ^{1}H and ^{13}C chemical-shift indices to produce a 'consensus' estimate of secondary structure, it is possible to achieve a predictive accuracy in excess of 92%. This suggests that the secondary structure of peptides and proteins can be accurately obtained from ^{1}H and ^{13}C chemical shifts, without recourse to NOE measurements.

INTRODUCTION

The introduction of 3D and 4D heteronuclear NMR to the field of biomolecular NMR spectroscopy has greatly enhanced our ability to study proteins with molecular weights in excess of 15 kDa (Fairbrother et al., 1992; Ikura et al., 1992; Wittekind et al., 1992). These powerful techniques now make it possible to sequentially assign ^{13}C- and ^{15}N-labeled proteins without recourse to NOE analysis (Ikura et al., 1990; Powers et al., 1992). Even though these new methods allow us to completely discard NOE measurements during the sequential assignment of proteins, we

*Supplementary material is available in the form of a 10-page table (Table S1) describing the exact location of secondary structures in all 20 proteins as determined using the methods described in this paper. Requests for Table S1 should be directed to the authors.

**To whom correspondence should be addressed.

still must rely on ^1H–^1H NOEs when it comes to actual structural determination. The problem with NOE measurements lies in the fact that, as the number of residues (and thus the number of NOEs) increases, the difficulty experienced in identifying, assigning and measuring these ^1H NOEs grows very rapidly. Furthermore, as the size of the protein increases, the cross-relaxation times progressively shorten and the effects of spin-diffusion make ^1H NOE measurements much more difficult to analyze in terms of internuclear distances. As a result, we are often unable to determine the secondary and tertiary structures of larger proteins with the same ease and accuracy possible with smaller proteins, using more conventional homonuclear 2D ^1H NMR spectroscopy.

Clearly, it would be of considerable help if techniques could be developed which would allow the determination of protein secondary (and tertiary) structure, independent of NOE information. In the case of heteronuclear 3D and 4D spectroscopy, it would be most useful to apply J-coupling data (Mierke et al., 1992; Vuister et al., 1993) or chemical-shift information (i.e. ^1H, ^{13}C or ^{15}N chemical shifts) to this problem. With respect to the latter approach, it has already been demonstrated that ^1H chemical shifts can be used to determine secondary structures both qualitatively and quantitatively in peptides and proteins (Dalgarno et al., 1983; Pastore and Saudek, 1990; Williamson, 1990; Wishart et al., 1991). One technique in particular, the Chemical-Shift Index (Wishart et al., 1992), has been widely used for the quantitative identification and location of secondary structures in many peptides and proteins (Jorgensen et al., 1992; Mott et al., 1992; Romier et al., 1993). In this report we describe how the concept of the Chemical-Shift Index (CSI) can be adapted for use in identifying secondary structures based on backbone ^{13}C (and ^1H) chemical-shift information. We believe that this application of the CSI could be of considerable use to those wishing to rapidly and quantitatively analyze peptide and protein spectra obtained through heteronuclear NMR techniques.

MATERIALS AND METHODS

It is well known that both C^α and carbonyl carbons experience a downfield shift when they are located in helices and an upfield shift when they are located in β-strands (Saito, 1986; Wishart et al., 1991). It is also well known that C^β resonances experience an upfield shift when located in helices and a downfield shift when located in β-strands (Spera and Bax, 1991). These observations have essentially been confirmed for all 20 amino acids (Spera and Bax, 1991; Wishart et al., 1991). The strong correlation between chemical shift and secondary structure suggested to us that backbone ^{13}C chemical shifts could be used in a similar fashion to ^1H chemical shifts (via the CSI) for quantitatively determining secondary structure. To test this hypothesis we have derived CSI reference values and thresholds specific to $^{13}C^\alpha$, $^{13}C^\beta$ and carbonyl ^{13}C nuclei and applied the technique to a test set of 20 different proteins.

To develop our initial set of reference values and thresholds, we collected extensive experimental data on a subset of 12 selected proteins (representing 1525 residues). These are marked by an asterisk in Table 3. All data were corrected so that carbon chemical shifts were referenced to TSP at 0.0 ppm. This required adjustment of any data referenced to dioxane at 67.8 ppm (Richarz and Wüthrich, 1978) by adding 1.6 ppm to the values originally quoted (see Table 1). Each chemical-shift value was tabulated according to residue type and one of three kinds of secondary structure (helix, β-strand or coil). Secondary structures were assigned by combining the authors' original

TABLE 1
^{13}C CHEMICAL-SHIFT REFERENCES AND THEIR RESPECTIVE CHEMICAL SHIFTS RELATIVE TO TSP

Compound	Conditions[a]	Chemical shift (ppm)
TSP	Aqueous, pH 4.2, 25 °C	0.00
TSP	Aqueous, pH 8.5, 25 °C	0.03
DSS	Aqueous, pH 2–11, 25 °C	0.15
TMS	In chloroform, 25 °C (ext.)	2.81
TMS	Neat, 25 °C (ext.)	2.84
Acetone	Aqueous, 25 °C	33.10
Dioxane	Aqueous (1%), 25 °C	69.43
Dioxane	Aqueous (10%), 25 °C	69.46
Dioxane	In chloroform, 25 °C (ext.)	69.90
Dioxane	Neat, 25 °C (ext.)	70.00

[a] (ext.) indicates that an external capillary was used in these measurements.

assignments with those obtained by more objective measurements using X-ray coordinate data where available (Kabsch and Sander, 1983; Richards and Kundrot, 1988 — as integrated into the program VADAR — Wishart, D., Willard, L. and Sykes, B., unpublished). Average 'coil' chemical-shift values for C^{α} and carbonyl carbons for each of the 20 amino acids were extracted from this initial database. The statistically derived chemical-shift data were further supplemented with experimental information taken from our own measurements of 'random coil' $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ chemical shifts (Wishart, D., Bigam, C. and Sykes, B., manuscript in preparation) along with those reported by Spera and Bax (1991). This preliminary set of backbone 'coil' chemical shifts was iteratively refined against the full dataset (20 proteins, representing 2520 residues) to produce a reference set of chemical-shift indices for the backbone carbon nuclei ($^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$). The final, optimized CSI values are listed in Table 2.

The concept behind the CSI has been described in detail in an earlier publication (Wishart et al., 1992) but it may be summarized as follows: It is a two-stage digital filtration technique which can be used to objectively identify secondary structures. In the first stage, a chemical-shift index (a ternary index having values of −1, 0 or 1) is assigned to all identifiable residues on the basis of their observed chemical shifts. In the second stage, secondary structures are delineated and identified on the basis of the values and local 'densities' of these chemical-shift indices. The exact CSI protocol, as it applies to $^{13}C^{\alpha}$ and carbonyl ^{13}C chemical shifts, proceeds as follows:

(1) Be sure to adjust your chemical-shift reference to DSS or TSP. If you used TMS referenced to 10% dioxane at 67.8 ppm as a standard, you must add 1.6 ppm to all of your measured chemical shifts (Wishart, D. and Sykes, B., in press; see also Table 1).

(2) Using the ^{13}C chemical-shift reference values in Table 2 as a guide, carry out the following procedure for each residue in the protein: (a) If the measured C^{α} (or carbonyl carbon) chemical shift is greater than the range given in Table 2 for that residue, mark a '1' beside it; (b) If the measured C^{α} (or carbonyl carbon) chemical shift is less than the range given in Table 2 for that residue, mark a '−1' beside it; (c) If the measured C^{α} (or carbonyl carbon) chemical shift is within the range given in Table 2 for that residue, mark a '0' beside it.

The above procedure defines the chemical-shift index for each residue in the protein. Using

these chemical-shift indices for C^{α} and carbonyl carbons, one may identify the secondary structures as follows:

(3) Any 'dense' grouping of four or more '1's' not interrupted by a '−1' is a helix. Any dense grouping of three or more '−1's' not interrupted by a '1' is a β-strand. All other regions are designated as coil.

(4) A local 'density' of nonzero chemical-shift indices which exceeds 70% is required when defining helical or β-strand structures. The local density may be measured over a window of four or five residues. A minimum of three consecutive '−1's' is needed to define a β-strand, and a minimum of four '1's' is needed to define a helix. All remaining regions not identified as either helix or β-strand, or those regions where the local density of '1's' or '−1's' falls below 70%, are defined as 'coil'.

(5) Termination points (at either end) of helices or β-strands can often be recognized by the first appearance of chemical-shift indices that are opposite in magnitude to those of the corresponding secondary structure. In cases where this does not occur, the first appearance of two consecutive zero-valued chemical-shift indices marks the termination point.

The CSI protocol, as it applies to C^{β} atoms, follows exactly the same procedure as given in steps (1) and (2) above. However, because C^{β} resonances actually shift oppositely from C^{α} and carbonyl carbons, and because C^{β} chemical shifts from both helices and β-strands overlap quite considerably (Spera and Bax, 1991), we have found it necessary to modify steps (3)–(5) above. In fact, because of this overlap problem, it appears that C^{β} resonances can only be used to identify stretches of β-strands and that the consistent identification of helices seems to be impossible. Consequently, one can use the C^{β} chemical-shift index to identify β-sheet structure (and *only* β-sheet structure) as follows:

(6) Any 'dense' grouping of four or more '1's' not interrupted by a '−1' is a β-strand. All other regions are designated as coil or 'non-β'.

(7) A local 'density' of '1's' which exceeds 70% is required when defining a β-strand. The local density may be measured over a window of four or five residues. A minimum of three consecutive '1's' is needed to define a β-strand. All remaining regions not identified as a β-strand, or those regions where the local density of '1's' falls below 70%, are defined as coil (or 'non-β').

(8) Termination points (at either end) of β-strands can be recognized by the first appearance of chemical-shift indices that are opposite in magnitude to those of the corresponding secondary structure. In cases where this does not occur, the first appearance of two consecutive zero-valued chemical-shift indices marks the termination point.

In order to make the CSI protocol as objective and as error-free as possible, we have computerized the entire procedure. The program CSI (authors: L. Willard, T. Jellard and D. Wishart) is a menu-driven software package designed to read chemical-shift assignment files from peptides and proteins (containing any combination of ^{1}H and/or ^{13}C chemical shifts) and to automatically output a file containing the corresponding chemical-shift indices and secondary structure assignments. The CSI program also produces a consensus secondary structure assignment whenever the chemical shifts for three or more kinds of nuclei are present in the assignment file. In addition, this package is designed to produce high-quality CSI histograms (see Fig. 1) that can be written either to the terminal and/or any postscript-compatible printer. CSI also includes a subroutine which allows the user to automatically adjust or rereference chemical shifts from a variety of zero-point standards (dioxane, TMS, DSS, acetone — see Table 1) to the appropriate TSP standard.

TABLE 2
^{13}C CHEMICAL-SHIFT VALUES (ppm) USED IN THE DETERMINATION OF SECONDARY STRUCTURE (RELATIVE TO TSP)[a]

Residue	$^{13}C^{\alpha}$ range	$^{13}C'$ range	$^{13}C^{\beta}$ range
Alanine	52.5 ± 0.7	177.1 ± 0.5	19.0 ± 0.7
Cysteine (red)	58.3 ± 0.7	174.8 ± 0.5	28.6 ± 0.7
Cysteine (ox)	58.0 ± 0.7	175.1 ± 0.5	41.8 ± 0.7
Aspartic acid	54.1 ± 0.7	177.2 ± 0.5	40.8 ± 0.7
Glutamic acid	56.7 ± 0.7	176.1 ± 0.5	29.7 ± 0.7
Phenylalanine	57.9 ± 0.7	175.8 ± 0.5	39.3 ± 0.7
Glycine	45.0 ± 0.7	173.6 ± 0.5	(CSI of $^{1}H^{\alpha}$)[b]
Histidine	55.8 ± 0.7	175.1 ± 0.5	32.0 ± 0.7
Isoleucine	62.6 ± 0.7	176.9 ± 0.5	37.5 ± 0.7
Lysine	56.7 ± 0.7	176.5 ± 0.5	32.3 ± 0.7
Leucine	55.7 ± 0.7	177.1 ± 0.5	41.9 ± 0.7
Methionine	56.6 ± 0.7	175.8 ± 0.5	32.8 ± 0.7
Asparagine	53.6 ± 0.7	175.1 ± 0.5	39.0 ± 0.7
Proline	62.9 ± 4.0	176.0 ± 4.0	31.7 ± 4.0
Glutamine	56.2 ± 0.7	176.3 ± 0.5	30.1 ± 0.7
Arginine	56.3 ± 0.7	176.5 ± 0.5	30.3 ± 0.7
Serine	58.3 ± 0.7	173.7 ± 0.5	62.7 ± 0.7
Threonine	63.1 ± 0.7	175.2 ± 0.5	68.1 ± 0.7
Valine	63.0 ± 0.7	177.1 ± 0.5	31.7 ± 0.7
Tryptophan	57.8 ± 0.7	175.8 ± 0.5	28.3 ± 0.7
Tyrosine	58.6 ± 0.7	175.7 ± 0.5	38.7 ± 0.7

[a] If spectra are referenced indirectly to dioxane at 67.8 ppm, they must be adjusted by 1.6 ppm.
[b] Glycine has no C^{β}. It is, therefore, necessary to use its $^{1}H^{\alpha}$ chemical-shift index as a proxy for the missing C^{β} chemical shift.

The CSI program is written in C and is compatible with most UNIX-based architectures including SUNs, SGIs and NeXT workstations. The graphical output is, however, limited to SGI machines only. The entire software package, including the source code, the installation routines, the manual and the databases (which include chemical-shift assignment files for 17 proteins) is freely available and may be obtained via anonymous FTP at the following address: canopus.biochem.ualberta.ca (129.128.6.158). The CSI program can be found in the /pub directory and must be untarred and uncompressed upon receipt.

RESULTS AND DISCUSSION

To illustrate how successful the CSI can be in identifying and locating secondary structures, we present the results obtained after running the CSI program on chemical-shift assignments taken from a total of 20 different proteins, see Table 3. In this table we present the results from a pairwise comparison between the 'predicted' secondary structure (derived from various chemical-shift indices, including the consensus index) and the 'observed' secondary structure for each of the 20 proteins. The 'observed' structure represents the secondary structure determined by careful evaluation of the original assignments, in conjunction with the results from computer analyses of X-ray coordinates and published NOE and/or J-coupling data of identical or homologous pro-

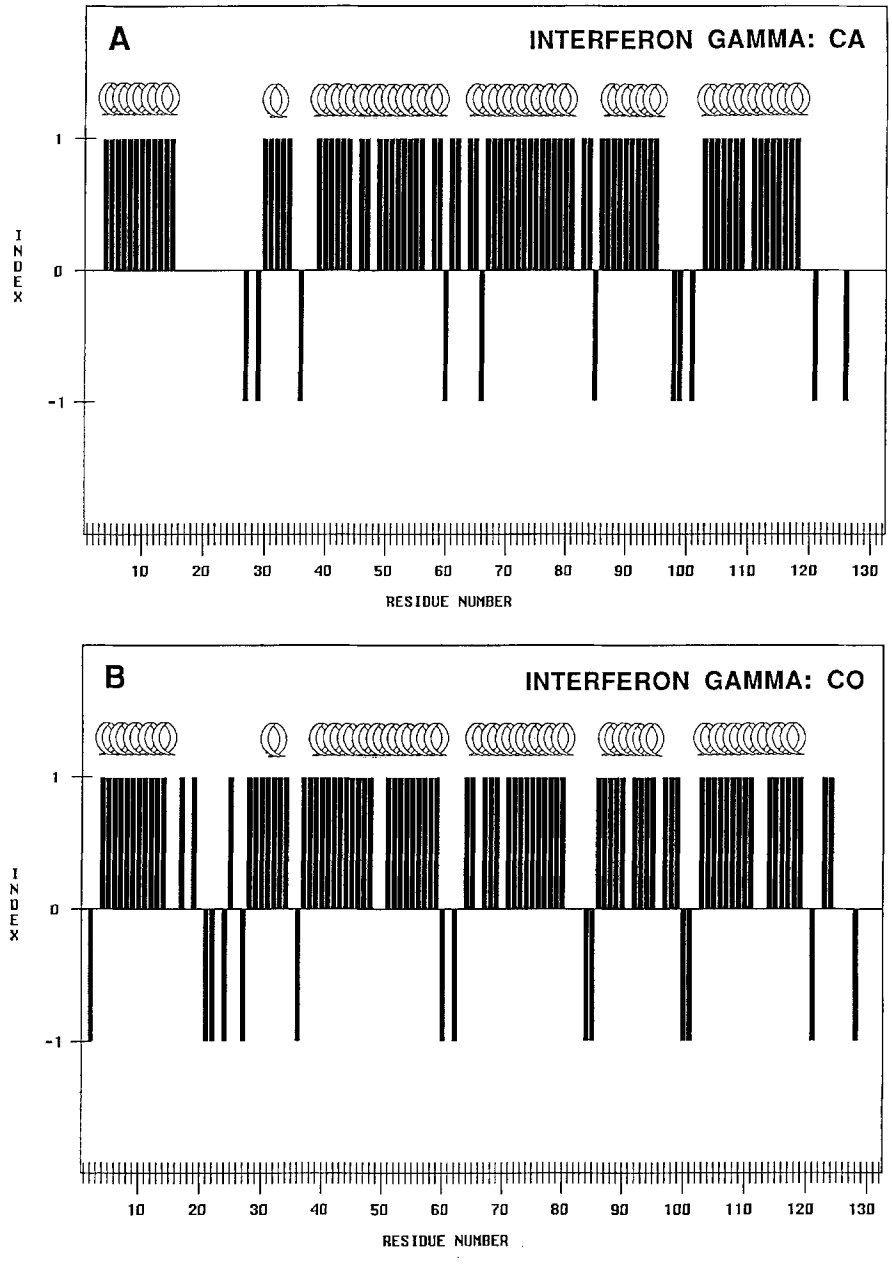

Fig. 1. Backbone ^{13}C, ^{1}H and consensus CSI plots of interferon γ, prepared using the program CSI. (A) Chemical-Shift Index derived from $^{13}C^{\alpha}$ chemical shifts; (B) Chemical-Shift Index derived from $^{13}C'$ chemical shifts.

teins. A more detailed breakdown of these results is available in the supplementary material (Table S1).

A quick inspection of Table 3 reveals that the two most accurate indices correspond to the $^{13}C^{\alpha}$ and $^{1}H^{\alpha}$ nuclei with average scores of 86 and 84%, respectively. The $^{13}C'$ index seems to be slightly

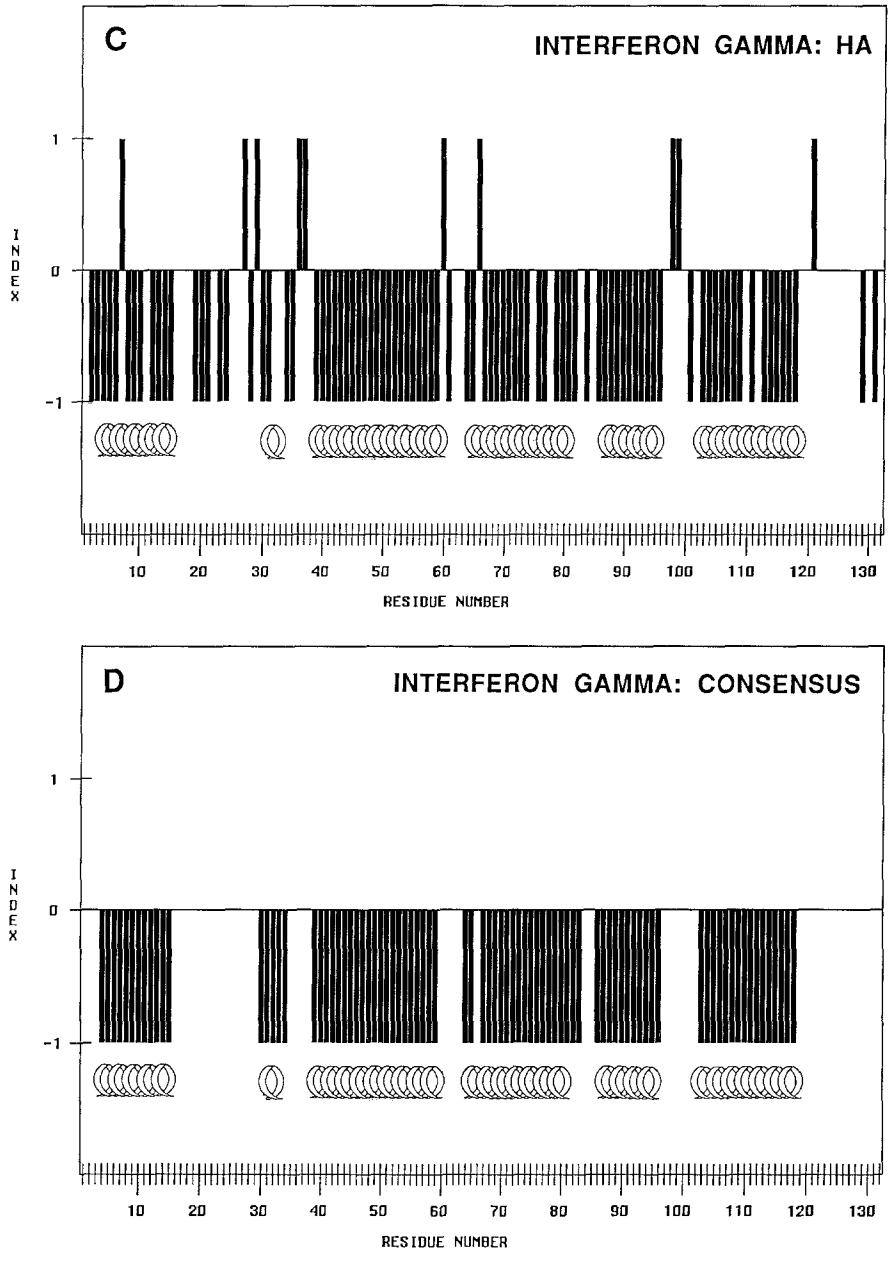

Fig. 1. (continued). (C) Chemical-Shift Index derived from $^{1}H^{\alpha}$ chemical shifts; (D) Consensus Chemical-Shift Index. The observed secondary structure is marked in each histogram using a 'helix' icon.

less accurate (81%), especially for proteins with a high level of β-sheet. This lower level of reliability may stem from the carbonyl carbon's extra sensitivity to hydrogen bonding and solvent exposure, which could lead to a higher proportion of spurious chemical shifts. The $^{13}C^{\beta}$ index scores very high (88%), but this is primarily because it is only a two-state (β or non-β) predictor.

What is particularly notable is the high overall performance of the consensus predictions (92%). These consensus predictions represent the secondary structure as determined by a simple 'majority rules' algorithm (two out of three, or three out of four), applied whenever at least three different chemical-shift indices are available. This high level of concordance between the CSI consensus secondary structure and what is actually observed is comparable to the level of agreement found when one compares highly resolved X-ray structures with highly resolved NMR structures of the same protein (LeMaster and Richards, 1988; Driscoll et al., 1990; Ikura et al., 1991; Grzesiek et al., 1992). Furthermore, a close inspection of precisely where the disagreements occur reveals that many of these differences seem to lie in the N- or C-terminus of helices or β-strands. Given that the structure of most terminal residues is often difficult to define, even in ideal circumstances, it is not hard to understand why this small amount of disagreement seems to persist in many of our comparisons (Richardson and Richardson, 1988). In other words, the precision (as opposed to the accuracy) with which secondary structures can be identified probably does not exceed 95% and so a concordance of 92% clearly represents a significant achievement in secondary structure identification. Based on these results, we believe that the consensus CSI approach may be as accurate as any NMR method now available for determining secondary structure in proteins.

While the accuracy of all four CSI methods — taken individually — is generally quite good, it is important to remember that the CSI protocol is fundamentally a statistical technique. Conse-

TABLE 3
ACCURACY OF CHEMICAL-SHIFT INDICES (%) FOR 20 FULLY ASSIGNED PROTEINS

Protein (reference)	$^{13}C^{\alpha}$ CSI	$^{13}C'$ CSI	$^{13}C^{\beta}$ CSI	$^{1}H^{\alpha}$ CSI	Consensus (%)
Gal 4 (Shirakawa et al., 1993)	98	–[a]	85	90	100
Troponin C* (C. Slupsky)[b]	93	91	–	91	97
Interferon gamma* (Grzesiek et al., 1992)	92	88	–	89	94
Calmodulin* (Ikura et al., 1990, 1991)	91	76	98	82	94
Ribonuclease H* (Yamazaki et al., 1991, 1993)	86	75	94	85	94
Interleukin 4* (Powers et al., 1992)	90	93	99	90	93
hn RNP c* (Wittekind et al., 1992)	88	89	–	84	93
SH2 Domain-plγ (J. Forman-Kay)[b]	88	–	83	83	93
FKB 506 (Xu et al., 1993)	88	74	88	89	93
Digoxin antibody (Constantine et al., 1993)	88	–	87	87	92
Tendamistat* (Kessler et al., 1990)	85	–	78	85	91
Glucose Perm. IIA* (Fairbrother et al., 1992)	82	–	83	81	90
Cellulose BP (L. Kay)[b]	83	77	85	76	90
Profilin (Archer et al., 1993)	84	79	81	81	90
Staph. Nuclease (T. Yamazaki)[b]	81	70	84	87	87
Phosphocarrier III* (Pelton et al., 1991)	82	76	82	75	86
Ras P-21 (Campbell-Burk et al., 1992)	83	–	–	86	–
Interleukin 1β* (Clore et al., 1990)	85	–	–	84	–
IRAP* (Stockman et al., 1992)	82	–	–	81	–
BPTI* (Wagner and Bruhwiler, 1986)	78	–	–	78	–

* Indicates that the chemical shifts from these proteins were used in compiling the preliminary reference set of CSI values.
[a] Chemical-shift data unavailable or not published.
[b] Personal communication.

quently, any single method (say the $^{13}C^{\alpha}$ index, alone) has a finite probability of failure over some region in a protein sequence. This failure may be due to a spurious chemical shift arising from some unique backbone geometry, or it could be due to a stretch of chemical shifts which just happen to fall at the wrong edge of the chosen threshold (± 0.7 ppm or ± 0.5 ppm). A case in point is Staphylococcal Nuclease (see Table 3) where it is clear that the CSI for one nucleus (^{1}H) can do quite well while the CSI for another nucleus ($^{13}C'$) can do quite poorly. This suggests that one should try to rely on more than a single chemical-shift index (just as one relies on more than a single type of NOE) when determining secondary structures in proteins.

CONCLUSIONS

By expanding the CSI protocol from an exclusively $^{1}H^{\alpha}$ technique to a heteronuclear (^{1}H and ^{13}C) technique, we have essentially quadrupled its resolving power. The inclusion of $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ along with $^{1}H^{\alpha}$ chemical shifts allows four independent, 'orthogonal' approaches to be used in identifying and locating secondary structures. This is essentially the same as having four interresidue NOEs for each assigned residue in the protein — a statistic which compares favorably to the level of interresidue NOE redundancies found in the most accurately determined NMR structures (LeMaster and Richards, 1988).

We believe that the CSI protocols described here and elsewhere (Wishart et al., 1992) are both simple and effective. Within our own laboratory we have already used these heteronuclear CSI techniques to determine the secondary structure of several $^{13}C/^{15}N$-labeled calcium-binding proteins. As more and more proteins of greater and greater size are subjected to higher dimensional heteronuclear NMR analysis, it is likely that the problems of assignment and, in particular, structure determination will steadily increase. We believe that the ^{13}C CSI — when used in conjunction with the ^{1}H CSI — offers a simple and convenient solution to at least some of these difficulties.

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