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^{13}C NMR chemical shifts can predict disulfide bond formation

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Abstract

The presence of disulfide bonds can be detected unambiguously only by X-ray crystallography, and otherwise must be inferred by chemical methods. In this study we demonstrate that ^{13}C NMR chemical shifts are diagnostic of disulfide bond formation, and can discriminate between cysteine in the reduced (free) and oxidized (disulfide bonded) state. A database of cysteine ^{13}C C^{α} and C^{β} chemical shifts was constructed from the BMRB and Sheffield databases, and published journals. Statistical analysis indicated that the C^{β} shift is extremely sensitive to the redox state, and can predict the disulfide-bonded state. Further, chemical shifts in both states occupy distinct clusters as a function of secondary structure in the C^{α}/C^{β} chemical shift map. On the basis of these results, we provide simple ground rules for predicting the redox state of cysteines; these rules could be used effectively in NMR structure determination, predicting new folds, and in protein folding studies.

Introduction

Disulfide bonds play a pivotal role in protein structure, function, folding, and stability. The importance of disulfide bonds has been extensively studied, but invariably involves either breaking or forming a disulfide bond. Further, it is not the disulfide bond but the effect of the disulfide bond on the rest of the structure that has been studied. The disulfide bond has many degrees of freedom, and recent studies have shown that they are dynamic and undergo significant conformational exchange (Srinivasan et al., 1990; Otting et al., 1993). Therefore there is a need to develop methodologies that can directly measure disulfide bond properties. NMR spectroscopy is ideal for this purpose; furthermore, it is well established that the chemical shifts are exquisitely sensitive to the microenvironment and secondary structure. Statistical treatment of protein chemical shift databases has provided useful empirical relationships between chemical shifts and various structural parameters (Wishart et al., 1991, 1994; Iwadate et al., 1999). We report here the chemical shift properties of cysteines using a database approach, and show that the ^{13}C chemical shifts are excellent probes for detecting disulfide bonds. This study provides the groundwork for unraveling the relationship between the chemical shifts and the structural characteristics of the disulfide bond.

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Methods

Data collection and analysis: A database of cysteine ^{13}C C^{α} and C^{β} chemical shifts was constructed from the BioMagResBank (BMRB) (http://www.bmrb.wisc.edu) and Sheffield (http://www.shef.ac.uk/uni/academic/1-M/mmb/nmr/chemshifts.html) databases and published literature. A Visual Basic program was specifically written to download, sort and extract the cysteine ^{13}C chemical shifts, and related information such as experimental conditions and referencing. In case of multiple BMRB entries of a given protein, the entry with the most complete assignments was used. The great majority were referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) and sodium 3-(trimethyl-silyl) propionate (TSP). Chemi-

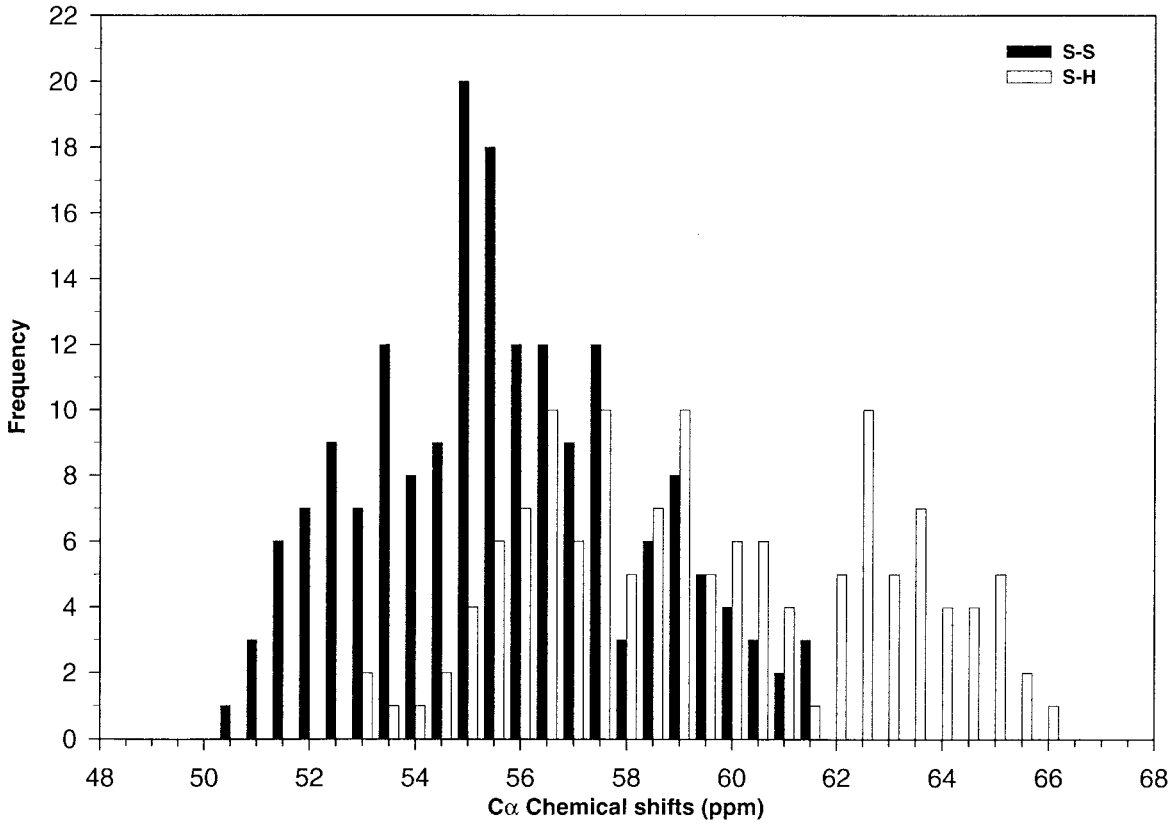

Figure 1. Distribution of cysteine C^{α} chemical shifts as a function of redox state. The frequency of occurrence is plotted in 0.5 ppm intervals in Figures 1, 2, and 3.

cal shifts not referenced to DSS and TSP were corrected according to established protocols (Wishart et al., 1995a). Processing and statistical analysis of the chemical shifts were carried out using Sigmaplot and Sigmastat, respectively (Jandel Scientific). Chemical shifts were grouped in 0.5 ppm intervals for calculating the statistics of occurrence (Figures 1–3).

Redox and secondary structure assignment: The oxidation state was deduced from the BMRB entry and/or published articles. In all cases, the redox state was confirmed from the visualization of NMR and/or crystal structures. Further, sample conditions, such as use of DTT were also taken into consideration. For a few entries, the thiol state was confirmed from personal communications. All shifts were classified into one of three categories: α-helix (H), β-strand (B), and others (O). The secondary structures were assigned as indicated in the original source, and if possible were also confirmed from the crystal structures. For the 316 cysteines analyzed, 26% was assigned to α-helix and 32% to β-strand.

Table 1. Summary of cysteine ^{13}C chemical shifts

Proteins	No. of proteins	^{13}C chemical shifts	
		C^{α}	C^{β}
Oxidized	38	179	159
Reduced	74	137	115
Redox/M^{n+}/paramagnetic[a]	17	55	53
Incomplete data[b]	3	4	10
Total	132	375	337

[a]Cysteine shifts from redox, metallo and paramagnetic proteins.
[b]Cysteine shifts from proteins with incomplete assignments and in one case data was collected in non-aqueous buffer.

Results

A total of 375 C^{α} and 337 C^{β} resonances were collected from 132 proteins. The distribution of the shifts is summarized in Table 1. Shifts from redox, metallo, and paramagnetic proteins were excluded from

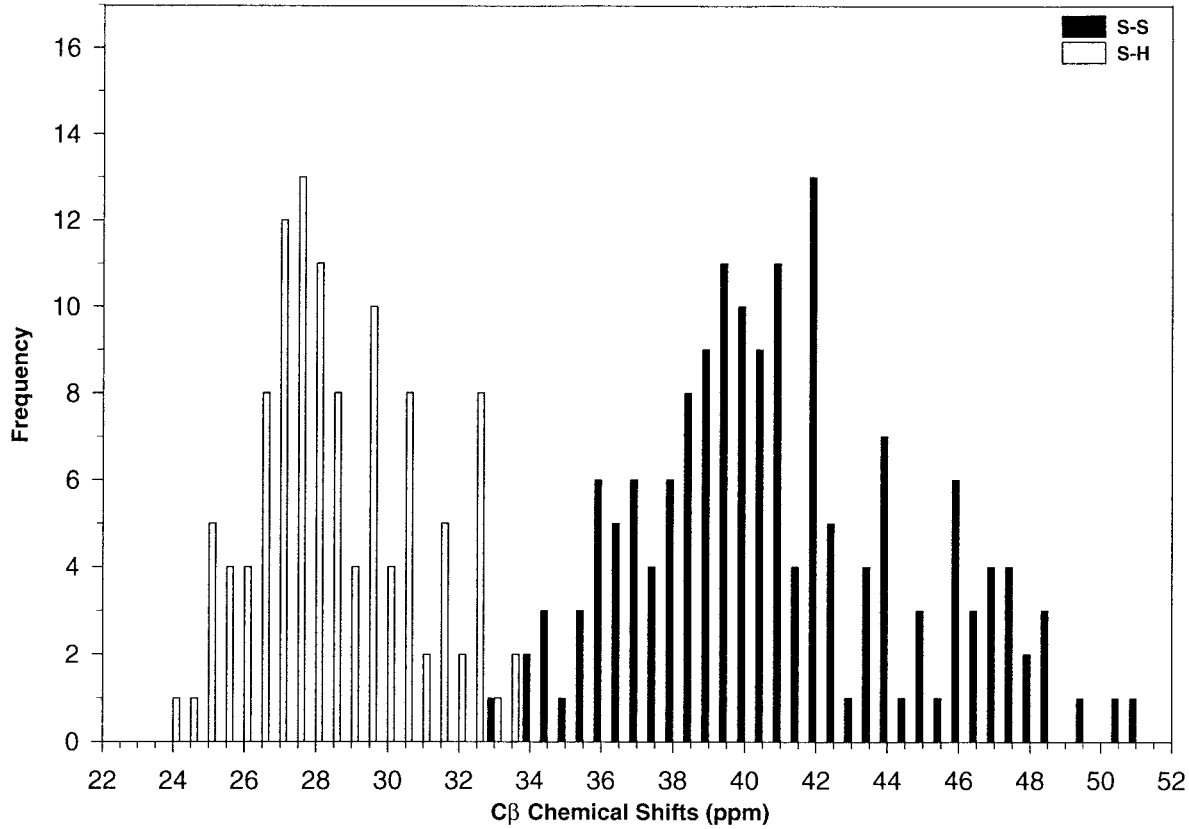

Figure 2. Distribution of cysteine C^{β} chemical shifts as a function of redox state.

the statistical analysis. C^{α} and C^{β} chemical shifts fall in two distinct domains (Figures 1 and 2). The reduced cysteine C^{α} and C^{β} shifts are the most downfield- and upfield-shifted, and therefore well separated. The disulfide C^{α} and C^{β} shifts are clustered, but there is marginal overlap around 51 ppm (Figures 1 and 2). The C^{α} chemical shifts of reduced and oxidized cysteine range from 50.5 to 65.6 ppm (Figure 1). Although there is considerable overlap between these shifts ($\sim$ 70%), the shifts for oxidized cysteine lie in the lower end (55.5 $\pm$ 2.5 ppm) and for reduced cysteine lie in the upper end of the spectrum (59.3 $\pm$ 3.2 ppm) (Table 2). The C^{β} chemical shifts of reduced and oxidized cysteine span a wider range (23.8 to 50.9 ppm), but clearly fall in two distinct domains (Figure 2). The observed C^{β} shift for oxidized cysteine is 40.7 $\pm$ 3.8 ppm, and for reduced cysteine is 28.4 $\pm$ 2.4 ppm (Table 3). A few of the C^{β} shifts were found to overlap in a narrow range of 33.0–34.0 ppm.

C^{α} and C^{β} chemical shifts were analyzed as a function of secondary structure (Figure 3); statistics are summarized in Tables 2 and 3. The data indicate that, on average, the C^{α} shifts are downfield shifted for α-helix and upfield shifted for β-strand in both the oxidation states (Figure 3), similar to what has been observed for all other amino acids (Spera and Bax, 1992; Wishart et al., 1994). The C^{α} shifts for reduced cysteine fall in two distinct regions with minimal overlap (α-helix: 62.6 $\pm$ 1.7 and β-strand: 56.6 $\pm$ 1.8 ppm),

Table 2. Statistics for the C^{α} chemical shifts in oxidized and reduced cysteine

	C^{α} (S-S)			C^{α} (S-H)		
	Total	Helix	Beta	Total	Helix	Beta
Size	179	39	56	136	44	45
Minimum	50.5	51.3	50.8	52.6	58.9	52.6
Maximum	61.2	61.2	59.1	65.6	65.6	61.1
Range	10.7	9.9	8.3	13.0	6.7	8.5
Median	55.4	57.4	54.9	59.0	62.7	56.4
Mean	55.5	57.6	54.8	59.3	62.6	56.6
Std. dev.	2.5	2.3	2.1	3.2	1.7	1.8

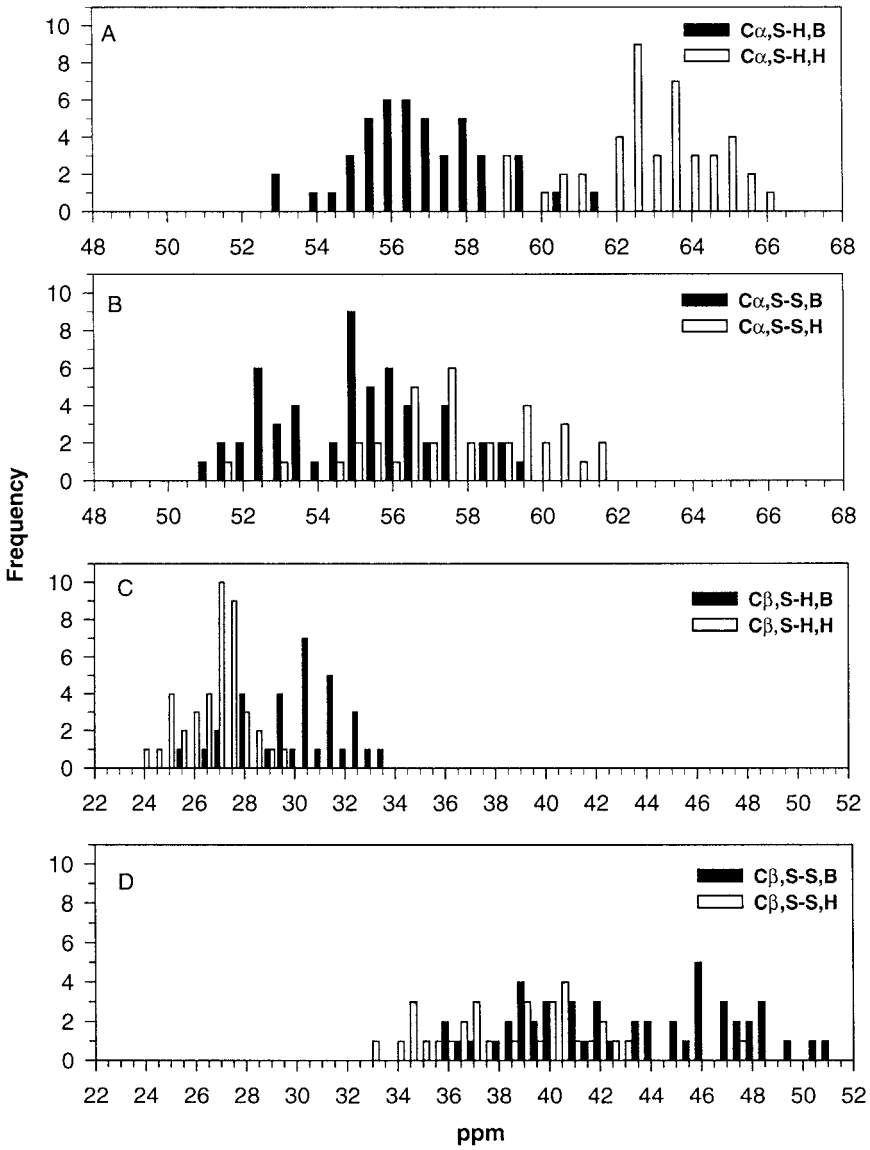

Figure 3. Distribution of C^{α} chemical shifts as a function of secondary structure in reduced (A) and oxidized (B) states, and distribution of C^{β} chemical shifts as a function of secondary structure in (C) reduced and (D) oxidized states.

whereas for oxidized cysteine there is significant overlap (α-helix: 57.6 ± 2.3 and β-strand: 54.8 ± 2.1 ppm; Table 2). The C^{β} shifts are upfield shifted for α-helix and downfield shifted for β-strand in both oxidation states (Figure 3). In reduced cysteine, the observed C^{β} shifts for α-helix and β-strand are 26.5 ± 1.1 ppm and 29.7 ± 2.0 ppm, respectively, whereas for oxidized cysteine they are 38.4 ± 3.2 ppm and 43.0 ± 4.2 ppm, respectively. A plot of C^{β} versus C^{α} shifts reveals that the shifts are clustered both as a function of redox state and secondary structure (Figure 4). Clearly the information content in Figure 4 is substantially higher, as the redox state and secondary structure are correlated to both C^{α} and C^{β} shifts. Therefore, the ability to assign the redox state of cysteines becomes unambiguous, especially for the C^{β} shifts that lie in the overlap region.

Assigning the redox state of cysteines: Our results demonstrate that C^{β} shifts can determine the redox state for the majority of the proteins and are diagnostic of disulfide bond formation (Figure 2). Shifts in the overlap region are usually from oxidized cysteines in

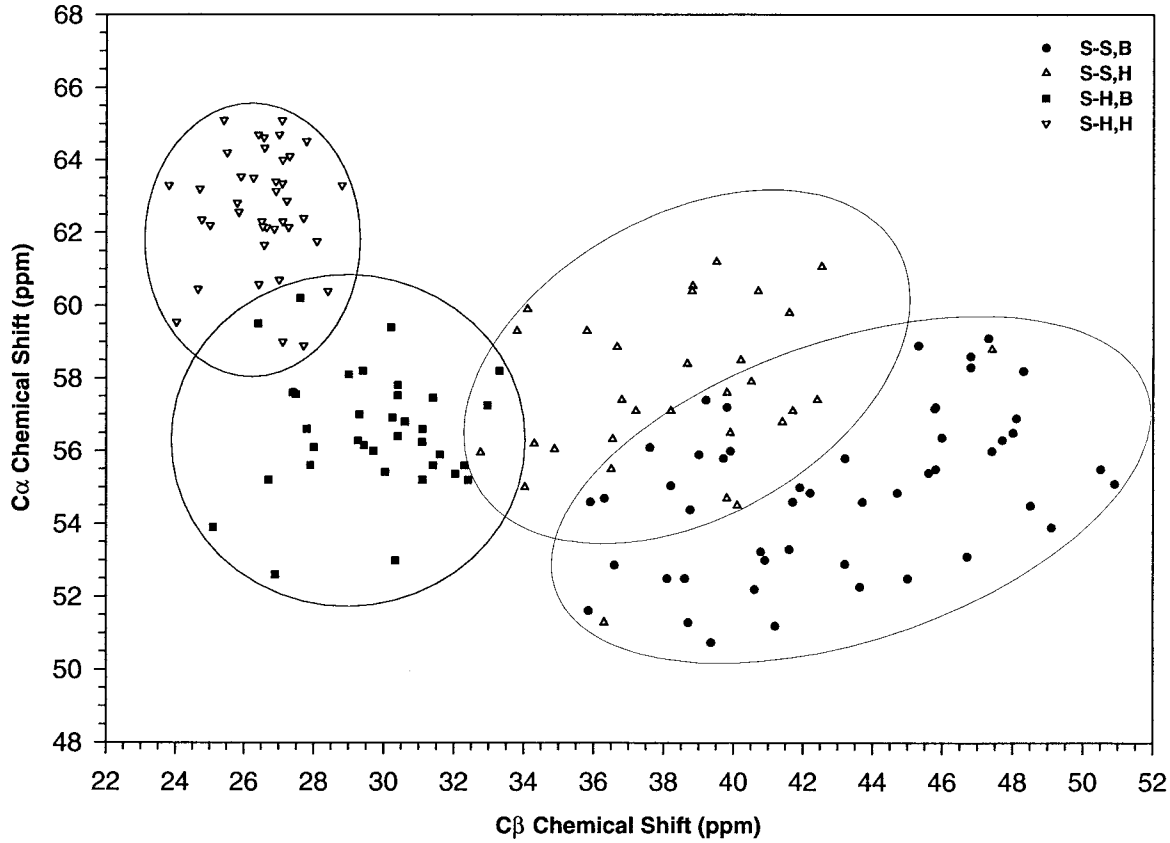

Figure 4. A plot of C^{α}/C^{β} chemical shifts as a function of both redox state and secondary structure.

Table 3. Statistics for the C^{β} chemical shifts in oxidized and reduced cysteine

	C^{β} (S-S)			C^{β} (S-H)		
	Total	Helix	Beta	Total	Helix	Beta
Size	159	33	49	113	39	35
Minimum	32.8	32.8	35.9	23.8	23.8	25.1
Maximum	50.9	47.4	50.9	33.3	28.8	33.3
Range	18.1	14.6	15.0	9.5	5.0	8.2
Median	40.2	38.8	43.2	27.9	26.6	30.2
Mean	40.7	38.4	43.0	28.3	26.5	29.7
Std. dev.	3.8	3.2	4.2	2.2	1.1	2.0

α-helices and reduced cysteines in the β-strands. Further, we observe that C^{α}/C^{β} shifts are clustered as a function of redox state and secondary structure (Figure 4). It was our experience that the redox state of all cysteines in this database could be assigned using our ground rules. We demonstrate here with a few examples that the redox state of any cysteine, especially those in the overlap region, can be determined using Figures 2 and 4.

The C2B-domain of Rabphilin 3 has three cysteines (BMRB accession # 4360), and one of the C^{β} shifts (33.0 ppm) lies in the overlap region. The C^{β} chemical shifts of the other two cysteines (30.4 and 26.6 ppm) lie in the reduced region (Figure 2), implying that this cysteine is also reduced. In MPIF-1 (unpublished results), a C^{β} shift of 34.3 ppm lies in the overlap region. The C^{β} chemical shifts of the other five cysteines (39.5, 41.8, 38.4, 48.3 and 37.2 ppm) lie in the oxidized region (Figure 2), implying that this cysteine is also oxidized. Native hen egg white lysozyme has eight cysteines (BMRB accession # 4562). The C^{β} shifts of five cysteines indicate that they are oxidized, and the other three lie in the overlap region (34.0, 34.9 and 35.1 ppm). If we take secondary structure into consideration (Figure 4), the shifts at 34.0 and 34.9 ppm can be assigned to the oxidized region, as

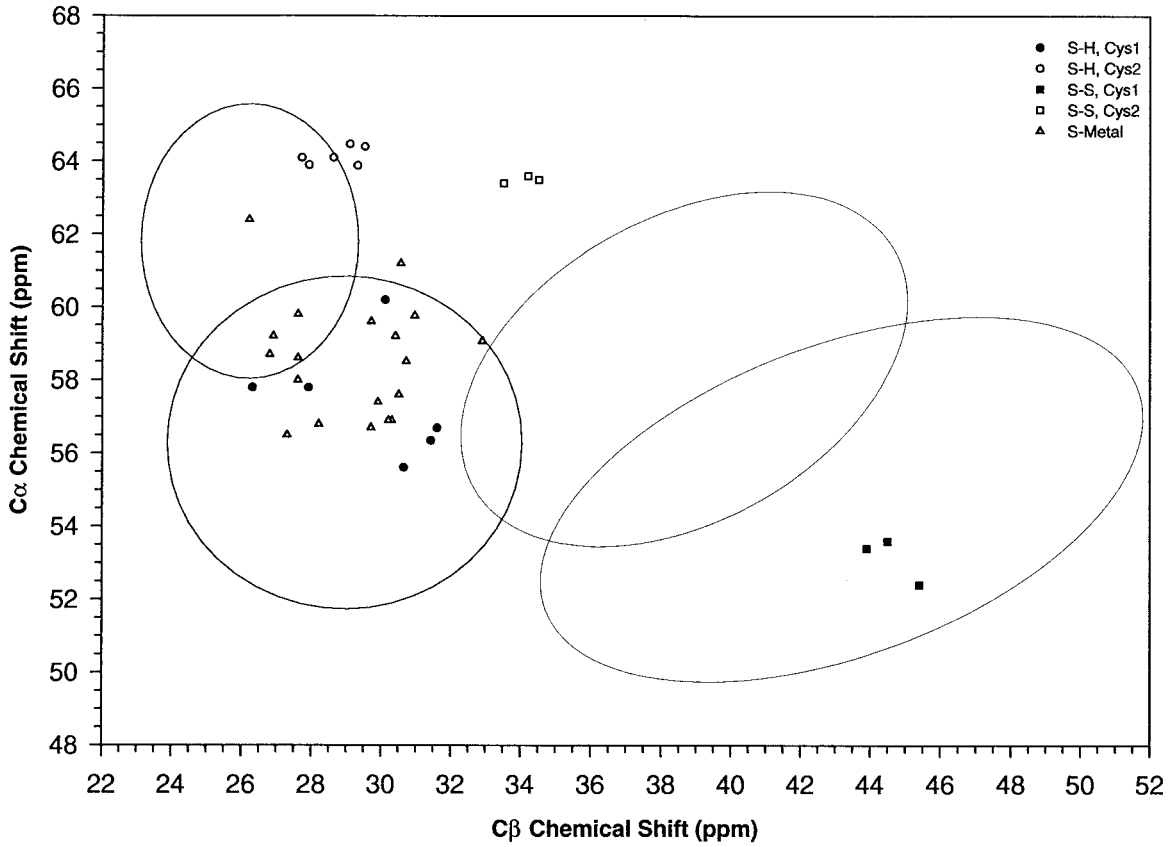

Figure 5. A plot of C^{α}/C^{β} chemical shifts of redox and metalloproteins. The redox protein active site is characterized by the 'Cys^1-X-X-Cys^2' motif, where X is any amino acid.

these cysteines are helical, and therefore the remaining cysteine at 35.1 ppm should be oxidized.

Discussion

We show here that the ^{13}C NMR chemical shifts can distinguish between free cysteine and disulfide bond. We also provide statistics for the cysteine chemical shifts in both redox states, and as a function of secondary structure (Tables 2 and 3). In principle, the secondary structure of the cysteines in both states can be assigned using the chemical shift index approach (CSI) (Wishart et al., 1991, 1994). However, assigning a single random coil shift value has been difficult, as the cysteine chemical shifts are sensitive to pH and solution conditions (Wishart et al., 1995b). Therefore, chemical shift statistics from this study, in conjunction with the CSI approach, could provide secondary structure in a more reliable manner. The statistics should also prevent incorrect assignments, especially for proteins kept in the reduced state by external addition of a reducing agent. Some of the chemical shift assignments in the BMRB database were inconsistent with statistics from this study, and we could determine that these assignments were wrong or ambiguous by personally communicating with the authors.

We also analyzed the chemical shifts of cysteines in redox proteins and in metalloproteins. All redox proteins have the characteristic Cys^1-X-X-Cys^2 motif, and the structural basis of their function remains an area of intensive study. ^{13}C chemical shifts were available for both redox states for three proteins (*E. coli* thioredoxin, human thioredoxin, and *E. coli* DsbA) and shifts for the reduced state alone for three more proteins (*E. coli* glutaredoxin, Vaccinia glutaredoxin, protein disulfide isomerase). C^{α}/C^{β} chemical shifts of the second cysteine alone, that is helical in all structures, are characteristic in both redox states, and are especially unusual and unique in the oxidized state (Figure 5). The ability to correlate the observed cysteine chemical shifts, redox potential, pK_a, and the

molecular structure could provide valuable insights toward understanding the chemistry of this important family of proteins. In the case of metalloproteins, only diamagnetic proteins were considered, as chemical shifts in paramagnetic proteins are complicated due to paramagnetic contribution. It is observed that the chemical shifts of cysteines coordinated to metal are similar to those of a reduced cysteine in the general database. The mean C^{α} and C^{β} chemical shifts of 20 cysteines from 5 metalloproteins are 58.6 ± 1.6 and 29.1 ± 1.8 ppm.

Ground rules for deducing the redox state of cysteines in an unknown protein

The redox state of cysteines in all proteins except those that are paramagnetic can be determined using the following ground rules. To ensure that the redox states are correctly assigned, we suggest that all of the steps are used.

Step 1: Ensure that the C^{α} and C^{β} assignments of cysteine are correct.

Step 2: If the C^{β} shift is less than 32.0 ppm or greater than 35.0 ppm, the redox state is assigned to reduced or oxidized, respectively. If the shift lies in the overlap region (32.0–35.0 ppm), the redox state can be assigned using step 3.

Step 3: For proteins with a single cysteine showing a C^{β} shift in the overlap region, the cysteine must be reduced. For proteins with multiple cysteines, of which one of the C^{β} shifts lies in the overlap region, the oxidation state of this cysteine is established from the C^{β} shifts of the other cysteines in the protein using Figure 2 and further confirmed from Figure 4. For proteins with multiple cysteines of which more than one lie in the overlap region, the redox state is assigned using Figure 4. In this case, Figure 2 alone cannot unambiguously assign the redox state.

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