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# Complete assignment of lysine resonances in $^1$H NMR spectra of proteins as probes of surface structure and dynamics

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A strategy is presented for complete identification of $^1$H spin systems of lysine residues using sophisticated 2D NMR experiments. Relayed and remote connectivities within each spin system are determined for spin subsystems based at the backbone amide and $C^\epsilon$ proton resonances. When complete spin system identification is combined with sequence-specific assignment, protein surface structure and dynamics can be probed in a site-specific manner. The interaction between the five lysine residues of French bean plastocyanin and a model redox partner $Cr(CN)_6^{3-}$ has been examined using this approach.

Protein surface; Lysine; 2D $^1$H NMR; Plastocyanin

## 1. INTRODUCTION

Lysine residues play an important role in the structure and function of many proteins, but it is usually difficult to obtain information about their conformations, microenvironments and dynamic properties by direct physical measurements. Surface lysines are often disordered in the electron density maps obtained by X-ray crystallography as a result of their high degree of mobility. In $^1$H NMR spectra of proteins, the lysine $C^\epsilon H_2$ resonances are frequently observed as a prominent, unresolved peak near 3.0 ppm but the resonances of the other lysine side chain protons occur in crowded spectral regions and cannot be assigned by conventional NMR methods. Thus, despite its potential for probing the conformational preferences and dynamics of the surface residues of proteins in solution, NMR has been of rather limited use in studying lysine side chains.

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Recent advances in two-dimensional NMR spectroscopy dramatically improve the resolution of the spectrum and provide methods for unequivocal assignment of resonances, even in crowded spectral regions. We report here on the use of phase-sensitive $^1$H total correlation spectroscopy (TOCSY) [1], relayed and double-relayed COSY (R-COSY and DR-COSY) [2], and 2-quantum spectroscopy (2Q) [3] to resolve and assign completely the $^1$H resonances of lysine residues in plastocyanin, a small ($M_r$ 10500) copper-containing protein active in the chloroplast electron-transport chain. When these spin system assignments can be combined with the complete sequence-specific assignment of the backbone resonances [4] this opens up the possibility of probing important interactions at the protein surface in a site-specific manner. We demonstrate this application by examining the interaction between plastocyanin and the inorganic probe, $Cr(CN)_6^{3-}$, a paramagnetic analogue of the electron-transfer reagent $Fe(CN)_6^{3-}$. Such paramagnetic reagents are frequently used as probes of surface structure in proteins [5].

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## 2. EXPERIMENTAL

Plastocyanin was isolated from leaves of French bean (*Phaseolus vulgaris*) as described [6]. Sample and experimental conditions are described in detail in [7], and summarized in the figure legends.

## 3. RESULTS AND DISCUSSION

The strategy proposed here is a specific application of an integrated approach for obtaining complete spin system identification in proteins [7,8] designed to address the problem of obtaining assignments for the particularly long side chain of lysine residues. Such strategies have become possible as a result of the development of experiments which provide relayed and remote connectivities within a spin system, i.e. between spins that are not directly coupled. There are three relevant aspects to the strategy for obtaining complete assignments for lysine spin systems:

(i) Two points of departure for assignments are utilized, the backbone amide proton and the $C^{\epsilon}H_2$ resonances, both of which are normally found in characteristic, isolated regions of the spectrum.

(ii) Sets of spin subsystems based at the resonance frequency of these two termini are established via observation of a maximum number of relayed and remote connectivities sequentially along the side chain.

(iii) Corresponding amide-based and $C^{\epsilon}$-based subsystems are matched by noting the coincidence of $C^{\beta}$ and $C^{\gamma}$ proton resonances.

This strategy is effective despite the extremely crowded nature of the spectral region containing the intervening $C^{\alpha}$, $C^{\beta}$, $C^{\gamma}$ and $C^{\delta}$ proton resonances because two networks of overlapping connectivities are observed for each spin system. The experimental protocol utilizes TOCSY to delineate the sets of backbone amide- and $C^{\epsilon}$-based spin subsystems and R-COSY, DR-COSY and 2Q for assignment of resonances to specific proton positions along the side chain. The experiments are complementary because the latter specifically provide connectivities only two (and three in DR-COSY) coupling steps along the spin system, whereas TOCSY is unrestricted and can in principle provide connectivities to all spins in the spin system. (Remote connectivities in R-COSY and 2Q occur through successive stages of coherence transfer of single spin modes, whereas in TOCSY they occur directly from collective spin modes during the spin-locking period [1].) In addition, 2Q is of critical importance for unambiguous identification of magnetic equivalence, which is often observed towards the end of the lysine side chain.

Connectivities from backbone amide and $C^{\alpha}$ resonances to the $C^{\epsilon}H_2$ resonance and from $C^{\epsilon}H_2$ and $C^{\delta}H_2$ resonances to the backbone NH resonance were observed for some but not all of the spin systems in the TOCSY spectra. The two-stage method of working out from the amide and in from the $C^{\epsilon}H_2$ is clearly a more general strategy for identification of the entire spin system. The inability to observe specific connectivities could result from small spin-spin coupling constants; for the lysine residues in plastocyanin, this effect can be directly correlated with the presence of a small $J_{HN\alpha}$ or $J_{\alpha\beta}$. As expected for these long side chain residues, protons further out along the side chain did not exhibit small coupling constants, due to rapid rotational averaging.

The strategy is demonstrated for the K54 spin system of plastocyanin in figs 1 and 2, which show small sections from the backbone amide ($\omega_2$ dimension) regions of DR-COSY (fig.1A) and TOCSY (fig.1B) experiments, and the corresponding parts of the $C^{\epsilon}H_2$ ($\omega_2$ dimension) regions of the same TOCSY (fig.1C) and a 2Q experiment (fig.2). The mutual overlap of four relayed connectivities observed at the amide at 7.24 ppm in fig.1B and at the $C^{\epsilon}H_2$ resonance at 3.04 ppm in fig.1C clearly establishes the association of the two spin subsystems based at these frequencies. Although relayed connectivities to the backbone amide proton resonances are not shown, examination of fig.1C and table 1 indicates that complete spin system identification by the same method is possible for the other four lysines. Fig.1A identifies the strong resonance at 1.98 ppm as one of the $C^{\beta}$ protons and strongly suggests that the resonance at 1.75 ppm observed in the TOCSY experiment (fig.1B) is that of $C^{\delta}H_2$. The resonances at 1.42 and 1.57 ppm could be tentatively assigned to $C^{\beta}$ or $C^{\gamma}$ protons, however specific assignments could not be made until a detailed analysis of the 2Q spectrum was carried out.

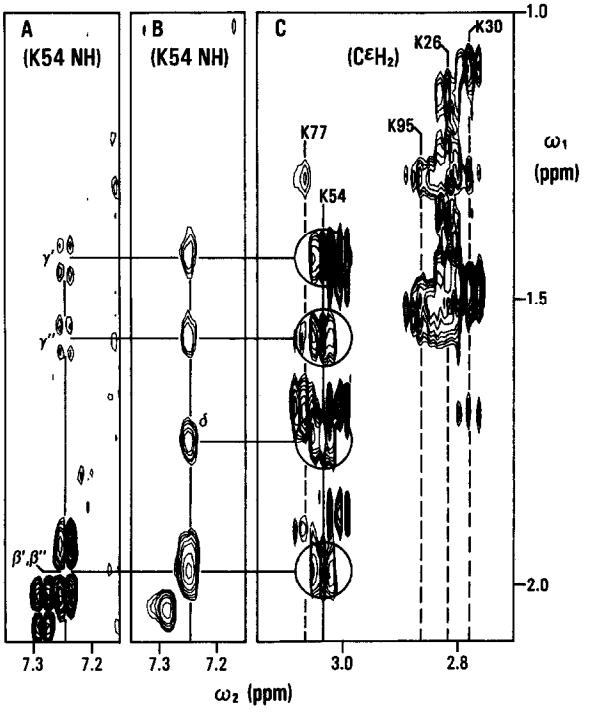


Fig.1. Complete assignment of $^1H$ lysine spin systems of plastocyanin via coincidence of remote connectivities observed at the backbone NH and $C^{\epsilon}H_2$ resonances. Connectivities observed at the backbone NH resonance ($\omega_2$ dimension) of K54 are shown from DR-COSY (A) and TOCSY (B) experiments. $C^{\beta}H_2$, $C^{\gamma}H_2$ and $C^{\delta}H_2$ connectivities observed at $C^{\epsilon}H_2$ resonances ($\omega_2$ dimension) of all five lysines are shown from the same TOCSY experiment (C). Spectra were obtained at 500.13 MHz on a Bruker AM-500 spectrometer at 303 K, from a 4 mM (95% $H_2O$/5% $^2H_2O$) solution of reduced protein (100 mM phosphate, pH 6.2). The TOCSY experiment was acquired with the carrier on the $^1H_2O$ signal, a radio-frequency field strength of 6.25 kHz, and a 100 ms MLEV-17 mixing period [12]. The optimized [9] relay periods in the DR-COSY were 20 and 31 ms. Solid vertical lines correlate the series of connectivities observed for the K54 spin system, solid horizontal lines indicate the coincidence of connectivities, circles highlight cross-peaks in the crowded $C^{\epsilon}H_2$ region of the TOCSY spectrum, and dashed vertical lines correlate connectivities observed at $C^{\epsilon}H_2$ resonances of the other four lysine spin systems.

In the 2Q spectrum obtained with a 30 ms excitation period (fig.2), a large number of direct and remote connectivities are observed, that are easily distinguished in a phase-sensitive display on the basis of the opposite signs (− + or + −) of the two principal lobes of the peaks [10]. Pairs of direct connectivities are seen between the strong lysine $C^{\epsilon}H_2$ resonances near $\omega_2$ = 2.80 and 3.05 ppm and $C^{\delta}H_2$ resonances near $\omega_2$ = 1.50 and 1.75 ppm, associated by their common $\omega_1$ frequencies ($\omega_{\epsilon}+\omega_{\delta}$) near 4.3 and 4.8 ppm. At each of the $C^{\epsilon}H_2$ frequencies there is also a remote peak at the sum of the $C^{\delta}H$ resonance frequencies. As most clearly shown in the region of these remote peaks, there are only five $C^{\epsilon}H$ resonances for the five lysine residues, identifying the expected magnetic equivalence of the $C^{\epsilon}$ protons. In addition, since the remote peaks occur at a chemical shift in $\omega_1$ which is exactly twice the $\omega_2$ chemical shift of the $C^{\delta}H$ resonances, they indicate magnetic equivalence of the $C^{\delta}H$ protons. Magnetic equivalence of these protons is expected due to rapid rotation about the corresponding $C^{\epsilon}$–$C^{\delta}$ and $C^{\delta}$–$C^{\gamma}$ bonds at the end of the side chain.

The next step in the analysis of the spin systems is to interpret the additional peaks observed at the $\omega_2$ frequencies of the $C^{\delta}$ protons to assign the $C^{\gamma}$ protons. The $C^{\delta}H/C^{\gamma}H$ direct and remote peaks are located in an extremely crowded spectral region and require careful analysis. Remote peaks at $\omega_1 = \omega_{\epsilon} + \omega_{\gamma}$ for one or both $C^{\gamma}$ protons are particularly useful, since they are located in a less crowded

Table 1

$^1H$ Chemical shifts of lysines in French bean plastocyanin

| | K77 | K54 | K95 | K26 | K30 |
|---|---|---|---|---|---|
| $C^{\epsilon}H_2$ | 3.08 | 3.04 | 2.84 | 2.82 | 2.78 |
| $C^{\delta}H_2$ | 1.69 | 1.75 | 1.53 | 1.47 | 1.49 |
| $C^{\gamma'}H$ | 1.29 | 1.42 | | 1.15 | |
| | | | 1.29 | | 1.10 |
| $C^{\gamma''}H$ | 1.57 | 1.57 | | 1.26 | |
| $C^{\beta'}H$ | 1.74 | 1.98 | | 1.37 | 1.29 |
| | | | 1.57[a] | | |
| $C^{\beta''}H$ | 1.90 | 2.02 | | 1.55 | 1.70 |
| $C^{\alpha}H$ | 4.02 | 4.22 | 5.11 | 4.25 | 4.78 |
| NH | 8.86 | 7.24 | 8.34 | 8.19 | 9.29 |

[a] Magnetic equivalence unambiguously identified by observation of remote peak in the 2Q spectrum

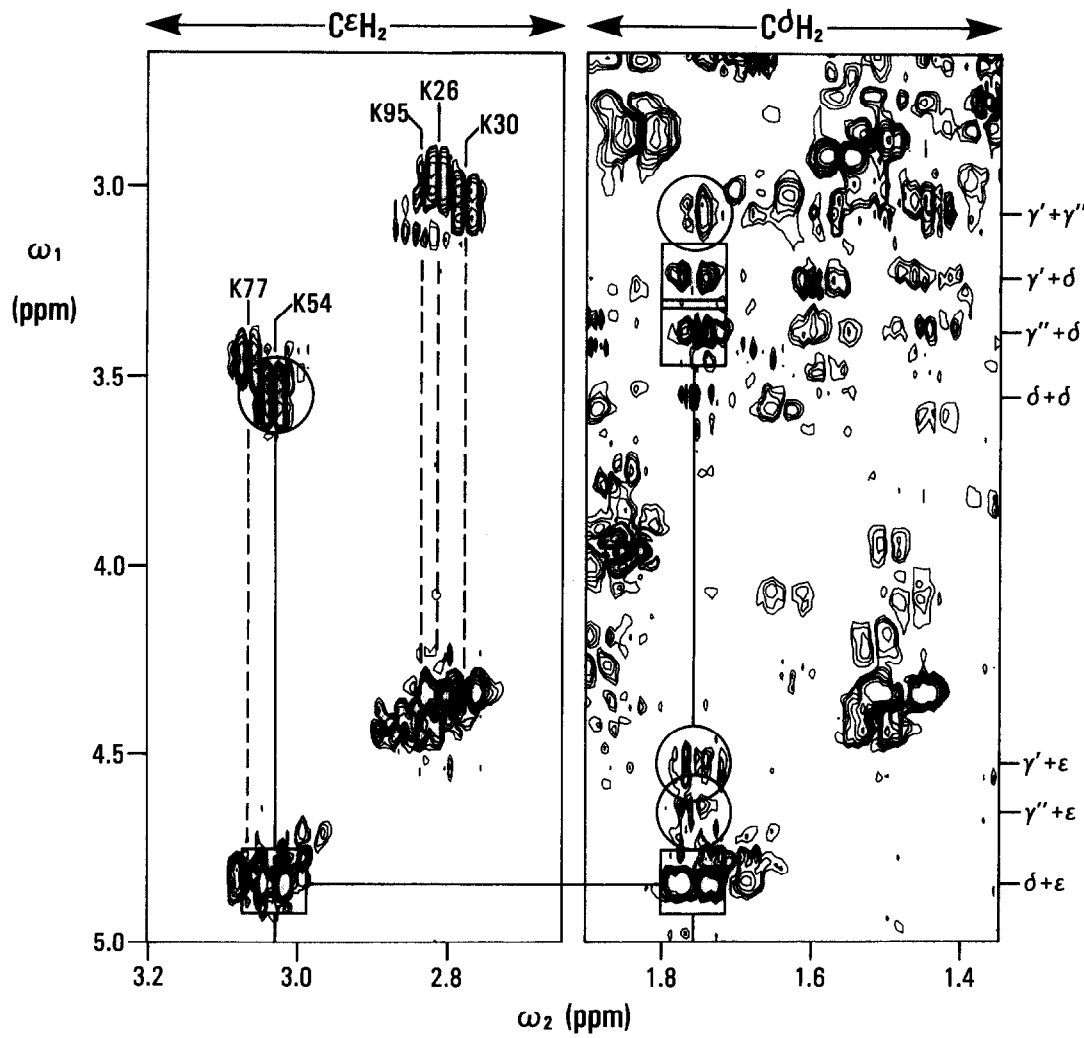


Fig.2. Method for assignment of $C^{\gamma}$, $C^{\delta}$ and $C^{\epsilon}$ protons of plastocyanin from the $C^{\epsilon}H$ (left) and $C^{\delta}H$ (right) regions of the 2Q spectrum. Resonances are assigned to specific positions along the side chain on the basis of direct (− + pattern) and remote (+ − pattern) peaks which are highlighted with squares and circles, respectively, for K54. For other spin systems, corresponding direct and remote peaks in the $C^{\epsilon}$ section are connected with vertical dashed lines. The spectrum was acquired on a Bruker AM-500 with a 30 ms excitation period from a 10 mM $^2H_2O$ solution of reduced protein (100 mM phosphate, pH 7.0) at 303 K.

region of the spectrum. The existence of only a single remote peak for some of these $C^{\gamma}H_2$ resonances reflects differences in scalar coupling patterns, except when the $C^{\gamma}$ protons are magnetically equivalent. Note that even though the $C^{\gamma}H$ resonances cannot be directly observed, their chemical shifts can be calculated from the $\omega_1$ shift of the remote peaks.

For K54, two direct peaks at $\omega_1 = \omega_{\delta} + \omega_{\gamma'}$ and $\omega_1 = \omega_{\delta} + \omega_{\gamma}$ and a remote peak at $\omega_1 = \omega_{\gamma'} + \omega_{\gamma''}$ can be identified, unambiguously assigning the two $C^{\gamma}$ proton resonances (table 1). The assignment is confirmed by the observation of two additional remote connectivities at $\omega_1 = \omega_{\epsilon} + \omega_{\gamma'}$ and $\omega_1 = \omega_{\epsilon} + \omega_{\gamma''}$. This unambiguously demonstrates that the $C^{\gamma}H$ resonances are not magnetically equivalent, and suggests that the $C^{\beta'}H$ and $C^{\beta''}H$ resonances are nearly degenerate as subsequently

confirmed by observation of a remote peak at $\omega_1 = \omega_{\beta'} + \omega_{\beta''}$, $\omega_2 = \omega_\alpha$ (not shown). The assignment of the K54 spin system can thus be completed. Similar methods were used to assign the resonances of the other lysines (table 1).

In order to obtain information about specific sites on the protein surface, sequence-specific assignments are required. These have been obtained for the five lysine residues of plastocyanin using the sequential assignment procedure [5] and details will be reported with the complete sequence-specific assignments for plastocyanin [11].

Assignment of the lysine spin systems provides some insight into the dynamics of these side chains. For all five lysines in plastocyanin the $C^\epsilon H_2$ and $C^\delta H_2$ protons are magnetically equivalent indicating rapid internal rotations about the $C^\epsilon$–$C^\delta$ and $C^\delta$–$C^\gamma$ bonds. For K95 and K30 the $C^\gamma$ protons, and for K95 even the $C^\beta$ protons, are also magnetically equivalent, indicating rapid motions along C–C bonds closer to the backbone. Thus, as might be expected, these lysine side chains enjoy a high degree of motional freedom on the surface of the protein. Motions of K77, K26 and in particular K54 appear to be more restricted, as indicated by the considerable magnetic inequivalence of the $C^\gamma$ protons. Future work will be directed towards correlating this variation in side chain dynamics with surface conformational features. The observation of two groups of lysine $C^\epsilon H_2$ and $C^\delta H_2$ resonances for plastocyanin indicates that the chemical shifts of these resonances are rather more sensitive to the lysine environment than was previously supposed.

The ability to assign proton resonances of lysine side chains provides a powerful new probe of the surface structure and dynamics of proteins in solution. It should now be possible to investigate directly, using NMR methods, the changes in surface conformation and dynamics induced by the binding of small molecules to protein surfaces. The methods may even be applicable to studies of interactions between small proteins. We demonstrate this application by examining the highly specific interaction between plastocyanin and $Cr(CN)_6^{3-}$, an inactive analogue of the electron transfer reagent $Fe(CN)_6^{3-}$. We have followed the progressive titration of plastocyanin with increasing amounts of $Cr(CN)_6^{3-}$; the $C^\epsilon H$ region of a titration difference spectrum is shown in fig.3. The sequence-specific assignments of all five lysine residues are indicated in the reference spectrum (fig.3A). The selective broadening of K26 is clearly observed in the difference spectrum (fig.3B), obtained by subtraction of the reference spectrum from that obtained after adding a molar equivalent of $Cr(CN)_6^{3-}$. The specificity of the interaction with the K26 side chain is indicated by the significant broadening of only these $C^\epsilon H_2$ resonances. Though not shown, we note that effects are also observed at a number of other residues and these will be described later in a detailed account of the interactions between plastocyanin and a variety of charged inorganic complexes.

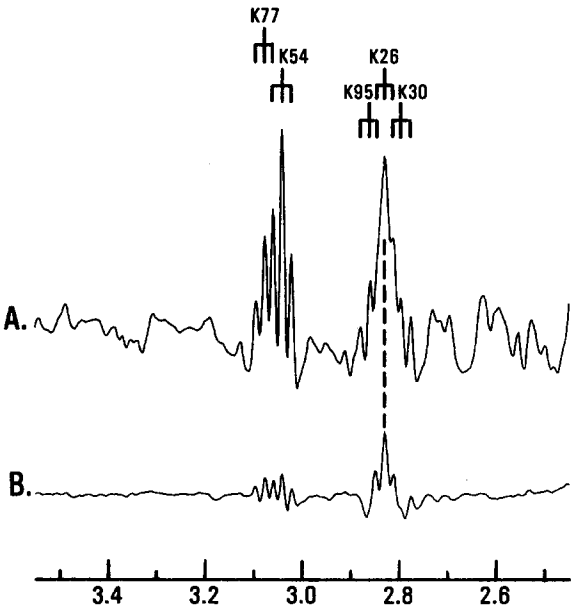


Fig.3. Highly selective effect on K26 of plastocyanin (from French bean) upon titration with $Cr(CN)_6^{3-}$ detected by $^1H$ NMR. The $C^\epsilon H_2$ resonances are shown from a reference spectrum (A) and from a titration difference spectrum (B) obtained by subtracting spectrum A from a spectrum acquired after adding a molar equivalent of $Cr(CN)_6^{3-}$. Spectra were acquired at 400.13 MHz on a Bruker WH-400 spectrometer from a 1 mM solution (100 mM phosphate, pH 6.0) at 298 K. The 16K of data points were treated to enhance resolution of the overlapped $C^\epsilon H$ resonances.

## 4. CONCLUSION

We have demonstrated a general strategy for the assignment of lysine spin systems in $^1H$ NMR spec-

tra of proteins that can be studied by two-dimensional methods. The strategy relies on the application of sophisticated experiments that provide relayed and remote spin connectivity. The observation of a network (rather than single) of correlations with mutual overlap implies that this process is highly reliable. We have applied this strategy to the assignment of the five lysine spin systems of plastocyanin (from French bean), and demonstrated the power of the method for determining the specificity of the interaction of the inorganic reagent, $Cr(CN)_6^{3-}$, with the protein. The new method is of particular importance in that it allows detailed investigations of the structure, dynamics and interactions at specific charged sites on the surfaces of small proteins.

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