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A TWO-DIMENSIONAL NMR METHOD FOR ASSIGNMENT OF IMIDAZOLE RING PROTON RESONANCES OF HISTIDINE RESIDUES IN PROTEINS

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The use of two-dimensional scalar correlated NMR spectroscopy for the assignment of histidine imidazole ring proton resonances is described. The method allows unequivocal assignment of both the C-2 and C-4 proton resonances belonging to individual histidine residues. Applications to the small copper protein, plastocyanin, and to myoglobin, a protein containing many histidines, are described. This method should be generally applicable to histidine-containing proteins of moderate size.

INTRODUCTION

^{1}H NMR has been widely used to study the histidine residues of proteins [1]. Resonances from the C-2 proton can often be resolved to low field of the aromatic proton envelope even for quite large proteins. Assignment of C-2 proton resonances is aided by their pH-dependence, for titratable histidines, and by the ease of deuteration at the C-2 position [1]. In addition, both the C-2 and C-4 proton resonances usually appear as sharp singlets which may be readily resolved using a spin-echo pulse sequence for multiplet selection [2]. It has previously been difficult to assign both the C-2 and C-4 proton resonances of individual imidazole rings in proteins containing more than one histidine residue. Such assignments are most commonly made on the basis of the correspondence of pK_a values determined separately for C-2 and C-4 proton resonances [3]. This method can be ambiguous, especially when the protein contains several histidines, e.g., ribonuclease [4, 5]. Other methods which have been used to assign C-2 and C-4 proton resonances to individual histidines include measurement of very small nuclear Overhauser effects between the C-2 and C-4 protons,

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measurement of nuclear Overhauser effects from imidazole ring NH protons under favourable circumstances [6] and observation of specific perturbations, e.g., paramagnetic broadening [7], of resonances from individual histidines.

The C-2/C-4 proton coupling constant is much smaller than the resonance linewidth in protein NMR spectra. This long-range coupling is thus unobservable in conventional one-dimensional spectra. However, we have recently observed that the long-range coupling produces relatively strong cross-peaks in the two-dimensional scalar correlated (COSY) [8 - 10] spectrum. This provides a direct method for unequivocal assignment of the C-2 and C-4 proton resonances belonging to individual histidines.

MATERIALS AND METHODS

French bean plastocyanin was isolated by a modification of the method of Ramshaw et al. [11]. Sperm whale metmyoglobin (Sigma type II) was used without further purification. NMR spectra were recorded on a Bruker WM-400 spectrometer. COSY spectra were acquired with dual quadrature detection [12] and were Fourier transformed with the standard Bruker 2D NMR program. COSY spectra of 1024 x 1024 data points were obtained from 512 individual experiments of 2048 data points by zero filling in one time domain before Fourier transformation. Accumulation of 64 free induction decays per individual experiment resulted in a total acquisition time of 12h for 7mM protein samples in D_2O solution. Quoted pH values are uncorrected meter readings.

RESULTS AND DISCUSSION

Figure 1 shows a contour plot of the aromatic region of the COSY spectrum of reduced plastocyanin. The spectrum was acquired with a conventional $(T_w\text{-}90^o\text{-}t_1\text{-}90^o\text{-}t_2\text{-})_n$ pulse sequence [13]. Relatively strong cross-peaks occur between the C-2 and C-4 proton resonances of both histidines in the protein. Cross-peaks are observed between the C-2 and C-4 proton resonances of His 87 at 7.66 and 6.97 ppm respectively, while the C-2 and C-4 proton resonances of His 37 correlate between 7.57 and 7.16 ppm. The C-2/C-4 coupling constant responsible for these cross-peaks is 1.8 Hz as measured by two-dimensional J-resolved spectroscopy (G. King and P.E. Wright, unpublished results). The observed correlations confirm previous assignments made using extrinsic paramagnetic broadening probes [7] and by measurement of a nuclear Overhauser effect from the His 37 imidazole ring NH proton (G. King and P.E. Wright, unpublished results).

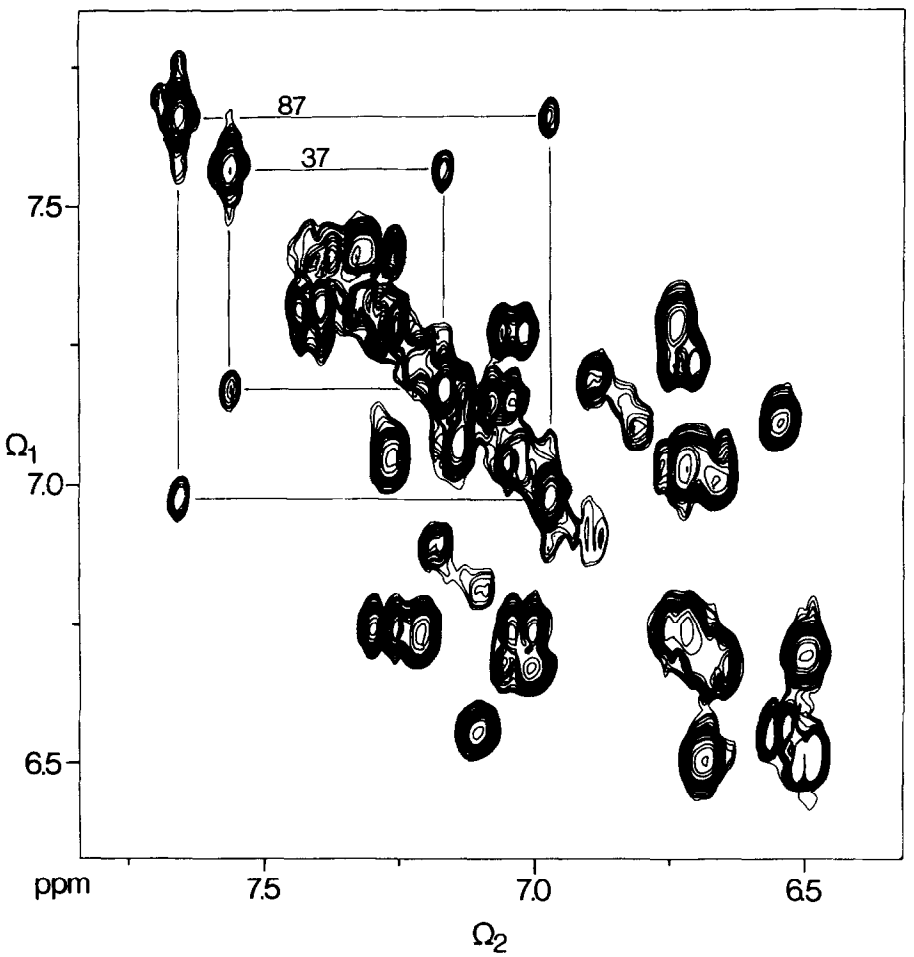

Figure 1. Contour plot of the aromatic region of the COSY spectrum of reduced French bean plastocyanin. Protein concentration was 7mM in 100mM deuterated phosphate buffer, pH 7.6, 313 K. Pulse sequence: $(T_w\text{-}90^o\text{-}t_1\text{-}90^o\text{-}t_2\text{-})_n$. Resolution was enhanced by sine-squared bell multiplication in both domains. Histidine correlations are indicated.

The COSY spectrum in Figure 1 features intense cross-peaks between coupled ring protons of tyrosine and phenylalanine residues. In larger proteins at high pH, these aromatic proton correlations may obscure the histidine imidazole proton correlations. We have found that an acquisition scheme suggested by Bax and Freeman to emphasize long-range couplings in small molecules [14] also selects for histidine C-2/C-4 proton correlations in proteins. Figure 2 shows the COSY spectrum resulting from a $(T_w\text{-}90^o\text{-}\tau\text{-}\ t_1\text{-}90^o\text{-}\tau\text{-}t_2\text{-})_n$ pulse sequence, where an extra delay τ = 100ms has been introduced. The histidine proton correlations have been enhanced over most aromatic proton correlations. For proteins, this enhancement is caused

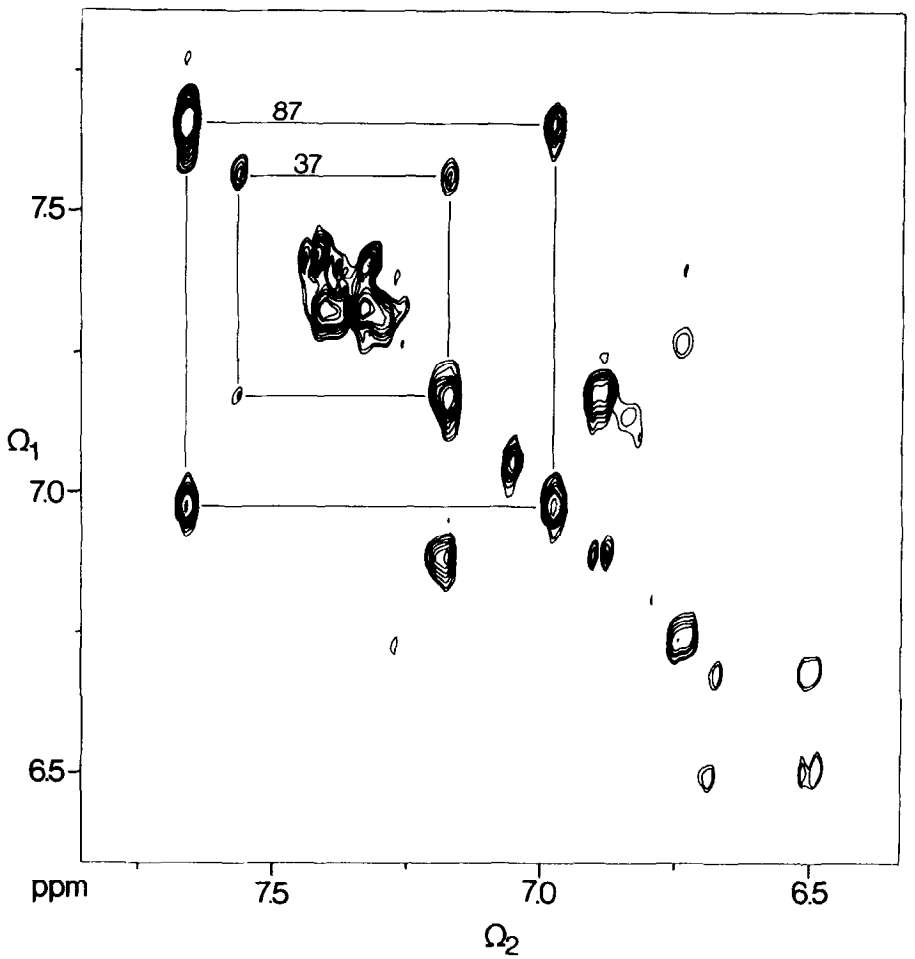

Figure 2. Plastocyanin COSY spectrum from $(T_w\text{-}90^o\text{-}\tau\text{-}t_1\text{-}90^o\text{-}\tau\text{-}t_2\text{-})_n$ pulse sequence, where τ = 100ms. Histidine cross-peaks are enhanced over most others.

by the selection of resonances with relatively long T_2, in the same way as is found for spin-echo methods [2].

The utility of the COSY experiment for the correlation of imidazole proton resonances in proteins containing many histidines is illustrated in Figure 3, where the C-2 and C-4 proton resonances of seven of the titratable histidines of metmyoglobin are correlated. Assignments of the C-2 proton resonances have been made previously by Botelho and Gurd [15]. Our correlations agree with a comparison of pK_as for all the titrating C-2 and C-4 proton resonances of metmyoglobin (P.E. Wright, unpublished results). Correlation using the COSY method eliminates some ambiguous assignments made

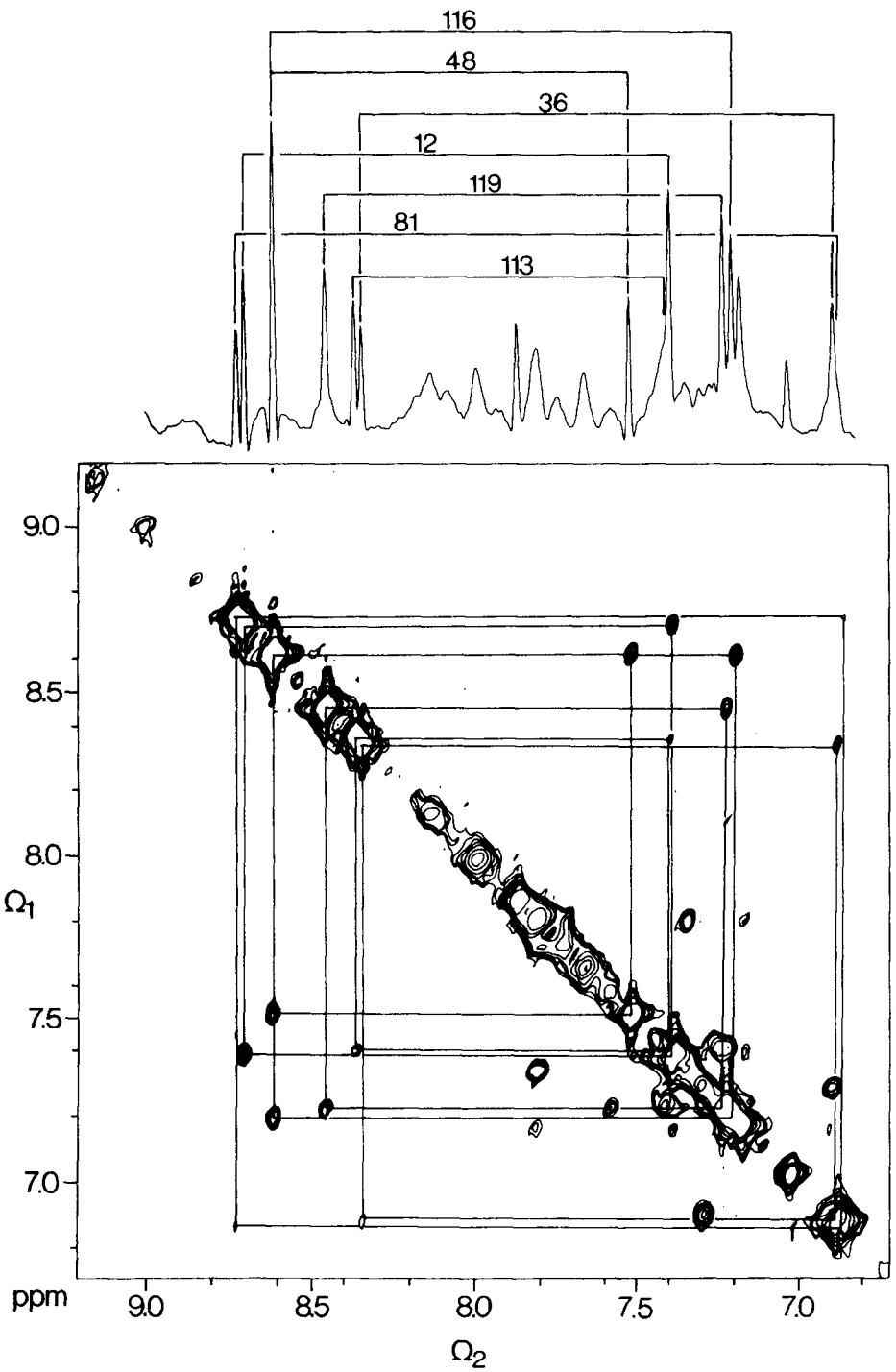

Figure 3. Contour plot of the low-field region of the metmyoglobin COSY spectrum with the one-dimensional spectrum for comparison. Protein concentration was 10mM in 100mM NaCl, pH 5.0, 298K. Pulse sequence: $(T_2\text{-}90^{o}\text{-}t_1\text{-}90^{o}\text{-}t_2\text{-})_n$. Resolution was enhanced by phase-shifted sine-bell multiplication in both domains. The one-dimensional spectrum was resolution enhanced by Lorentzian to Gaussian transformation. Seven histidine correlations are shown. Assignments indicated in the one-dimensional spectrum are based on the C-2 proton resonance assignments of Botelho and Gurd [15].

by pH titration alone. The COSY cross-peak intensities of Figure 3 vary according to the relative intensities of the histidine resonances in the one-dimensional spectrum. These variations may be caused by several factors, including chemical exchange broadening due to mobility or protonation rate, slight paramagnetic relaxation effects due to relative proximity to the ferric heme of metmyoglobin, and cross relaxation from saturation of the residual HDO resonances.

The two-dimensional methods described here for correlation of the C-2 and C-4 proton resonances of individual histidines should be readily applicable to proteins of moderate size. The maximum size of proteins suitable for study by these methods is governed by the requirement that T_2 be large enough to allow significant magnetization exchange during the course of the experiment.

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