Two-Dimensional ^1H NMR Studies of Cytochrome c†

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ABSTRACT: Two-dimensional nuclear magnetic resonance techniques were used to assign the NH, $C_\alpha H$, and $C_\beta H$ protons of over 60 of the 104 amino acid residues in the ^1H NMR spectrum of horse ferrocytochrome c. The majority of these amino acids were completely assigned. Assignments were based on the analysis of two-dimensional J-correlated (COSY), nuclear Overhauser effect (NOESY), and relayed COSY spectra and on comparisons of the J-correlated spectra of various cytochrome c species. Spin diffusion is not a problem with monomeric proteins the size of cytochrome c. Here these advances are illustrated with data that lead to the assignment of the heme-associated residues cysteine-14 and tryptophan-59, the axial ligands methionine-80 and histidine-18, the entire N-terminal helix, and several other amino acid spin systems. With these approaches, structure, structure change, the internal dynamics of cytochrome c, and the interaction of these with function are being studied, especially by observation of the hydrogen exchange behavior of essentially all the H-bonded amides and some side chain protons in both the reduced and oxidized proteins.

Two-dimensional nuclear magnetic resonance (2D NMR)[1] techniques have now led to nearly complete proton resonance assignments in several proteins (Wagner & Wüthrich, 1982; Wüthrich et al., 1984; Zuiderweg et al., 1983; Bystrov et al., 1980; Wemmer & Kallenbach, 1983), and these have been used in some protein chemical studies (Richarz et al., 1979; Wagner & Wüthrich, 1982; Roder et al., 1985). The available techniques have so far been successful only for very small proteins, typically less than 60 amino acids. Here we report some 2D NMR results for cytochrome c, which is almost twice the size of proteins previously solved and has considerable functional interest. The literature records proton assignments for parts of 21 amino acid residues in cytochrome c, largely by 1D NMR methods [e.g., Redfield & Gupta (1971), Stellwagen & Shulman (1973), McDonald & Philips (1973), Moore & Williams (1980a–d), Boswell et al. (1980, 1982), Keller & Wüthrich (1981), Eley et al. (1982), and Robinson et al. (1983)]. By use of two-dimensional NMR methods we have found it possible to confirm, correct, or assign de novo the resonances of the heme protons and over half of the amino acid spin systems. This makes accessible a great deal of information on cytochrome c structure, dynamics, and function.

The assignment of proton resonances in a small protein usually can proceed by a direct sequential assignment method (Wagner & Wüthrich, 1982; Billeter et al., 1982; Wüthrich, 1983). Cytochrome c, due to its size (104 amino acids, over 850 protons in H_2O) and large number of complex spin systems (e.g., 19 lysines), exhibits numerous ambiguities in the J-correlated (COSY) spectrum (Figure 1) and a bewildering array of through-space NOE interactions (2200 cross peaks for protons less than 3.2 Å apart; see, e.g., Figures 2 and 6), all of which makes the direct sequential assignment procedure problematical. Examples of some of the approaches used in overcoming these problems are illustrated here.

Materials and Methods

Cytochrome c (Sigma Chemical Co.) was dissolved in 50 mM potassium phosphate buffer in 99.8% D_2O or 90% H_2O/10% D_2O, reduced with sodium dithionite, adjusted to pH* 5.7 with dilute NaOD or DCl, and kept under nitrogen. The protein concentration was 6–8 mM in D_2O and 12–14 mM in H_2O. Chemical shifts are referenced to an external standard (coaxial capillary) of 4,4-dimethyl-4-silapentane-1-sulfonate.

One-dimensional truncated-driven and steady-state NOE experiments were done on a Bruker WH-360 spectrometer. Two-dimensional NMR spectra were recorded on Bruker WM-500 spectrometers (Yale University, New Haven, CT, and University of Washington, Seattle, WA). J-Correlated (COSY) spectra (Aue et al., 1976; Nagayama et al., 1979; Bax & Freeman, 1981), phase-sensitive nuclear Overhauser effect (NOESY) spectra (Macura & Ernst, 1980), and relayed coherence (relay COSY) spectra (Wagner, 1983) were collected by using established pulse sequences and phase cycling appropriate for quadrature detection and elimination of axial peaks (Wider et al., 1984; States et al., 1982; Hoult & Richards, 1975), as will be detailed in further communications. Data processing was done on a Vax 11/750 using a Fortran program, FTNMR, written by D. Hare (copyright 1984).

Results

Spin Systems in the J-Correlated Spectrum. Unlike the situation with smaller proteins, the COSY spectrum of cytochrome c (Figure 1) allows few spin systems to be directly classified even as to amino acid type. Some residues previously partially assigned by others could be completed. The Leu-32 spin system [$C_\gamma H$ and $C_\delta H_3$ resonances previously identified by Moore & Williams (1980b)] could be directly completed (Figure 1) and was confirmed by intraresidue NOE cross peaks and interresidue NOEs to independently identified neighboring amino acids. Similar results completed the partially known (Moore & Williams, 1980b; Robinson et al., 1983) Ile-57 spin system (Figure 1).

Species Comparisons. Moore, Williams, and co-workers (Moore & Williams, 1980a–d; Boswell et al., 1980, 1982; Eley et al., 1982; Robinson et al., 1983) were able to assign a number of cytochrome c resonances by comparing 1D spectra

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[1] Abbreviations: 2D, two dimensional; 1D, one dimensional; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; COSY, J-correlated spectroscopy; NOESY, NOE correlated spectroscopy; ppm, parts per million.

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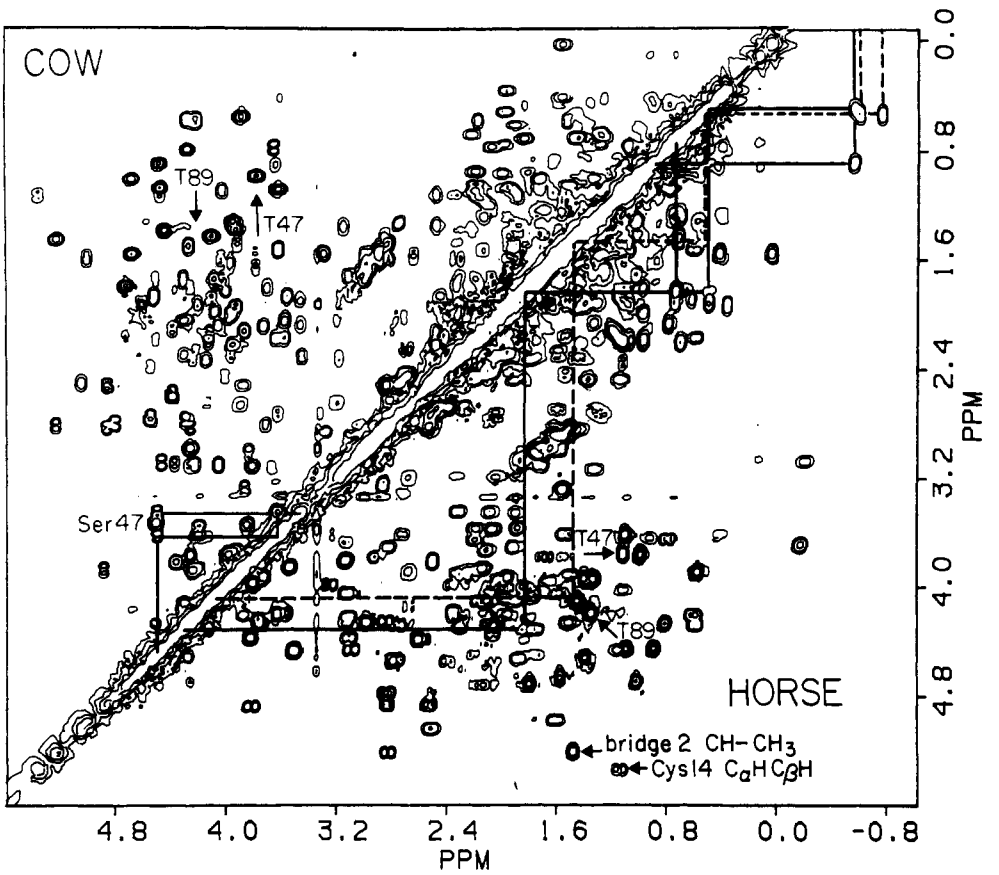

FIGURE 1: Absolute value ^1H *J*-correlated (COSY) spectrum of horse (lower) and cow (upper) ferrocytochrome *c*. Separate spectra for the cow and horse proteins were cut along the diagonal and placed together for this comparison. The diagonal contains the usual ^1H NMR spectrum. Each off-diagonal cross peak indicates a through-bond coupling between two neighboring protons with chemical shifts at the two cross-peak coordinates. The amino acid side chain region shown represents about one-fourth of the total 2D spectrum. In the lower diagonal (horse) the scalar coupling networks of Leu-32 (---) and Ile-57 (—) are shown. Arrows indicate the $C_\alpha H$–$C_\beta H$ cross peak of the heme-linked Cys-14, the heme C2 bridge methine–methyl cross peak, and the $C_\beta H$–$C_\gamma H_3$ cross peaks of the two threonines (T47 and T89) in horse cytochrome *c* that are replaced by Ser-47 and Gly-89 in cow. In the upper diagonal (cow), the absent cross peaks of the two threonines and the scalar coupling network of Ser-47 are indicated. Spectra were taken at 500 MHz on a Bruker WM-500 spectrometer at 40 °C. The total acquisition time was 19 h. The time domain signals were corrected for the effects of nonsimultaneous quadrature detection introduced by the Bruker spectrometer, base line corrected, digitally filtered with an unshifted sine bell in both dimensions, and Fourier transformed, and an absolute magnitude calculation was made. The final data matrix was 1K × 1K after zero filling (7.9 Hz/point). The spectra shown here have been separately symmetrized.

of homologous cytochrome *c* species. With 2D spectra, entire spin systems of residues lost and gained can be followed, and secondary shifts, which often confuse 1D difference spectra, can be effectively interpreted. Comparative COSY spectra obtained for cytochromes from horse, cow, rabbit, dog, chicken, and tuna provided the complete assignment of seven amino acid spin systems in horse ferrocytochrome *c* and another seven spin systems as one of two possible choices (e.g., Thr-28 or Thr-58), which could then be distinguished by NOE data. To illustrate, Figure 1 compares a section of the COSY spectra of cow and horse cytochrome *c*, which differ at three positions. Thr-47, Lys-60, and Thr-89 in horse are replaced with Ser-47, Gly-60, and Gly-89 in cow. Only the $C_\gamma H_3$ protons of Thr-47 and Thr-89 have been identified in one-dimensional difference spectra (Moore & Williams, 1980d). Comparison of the two-dimensional spectra makes the complete spin systems apparent (Figure 1).

Cysteine-14–Heme Bridge. The large porphyrin ring provides a centrally located set of protons that can be extensively used for confirming assignments of neighboring amino acid spin systems. Keller & Wüthrich (1978) have proposed assignments for all the heme methyl, bridge methine, and pyrrole CH protons. Our results support these assignments and provide some additional ones. For example, the X-ray structure predicts that NOE cross peaks should connect the known α-mesoheme proton with the $C_\alpha H$ of Cys-14 (~3-Å separation) as well as with other known heme protons (see Figure 2). Irradiation of the α-meso proton at 9.32 ppm in 1D steady-state difference NOE experiments produces the expected NOEs to the heme protons *and also* a significant NOE to an unidentified proton at 5.35 ppm (Figure 2). Buildup rates in 1D truncated-driven NOE experiments show that the proton at 5.35 ppm is farther from the α-meso proton than is the bridge methine, consistent with its assignment to the Cys-14 $C_\alpha H$. The COSY spectrum (Figure 1) shows that the proton at 5.35 ppm is scalar coupled to a proton resonating at 1.15 ppm. The resolved fine structure of this cross peak (Figure 1) indicates *J* coupling of the 1.15 ppm proton to another proton with apparent coupling constant ca. 20 Hz, consistent with the geminal $C_\beta H_2$ protons in the cysteine side chain. [$C_\beta H$ is an apparent doublet due to loss of the central antiphase triplet component at this line width (~10 Hz), digital resolution (7.9 Hz), and expected coupling constant ($J_{\alpha\beta}$ = 8 Hz, $J_{\beta\beta}$ = 16 Hz; Bundi & Wüthrich, 1979).] The COSY spectrum (Figure 1) also shows that the methine CH (5.22 ppm) of the heme–Cys-14 bridge is scalar coupled to a proton(s) at 1.48 ppm. The intensity and chemical shift of this cross peak and its lack of resolved fine structure (at this digital resolution) suggest that this coupling is to a methyl group, i.e., the expected bridge 2 methyl (Slama et al., 1975).

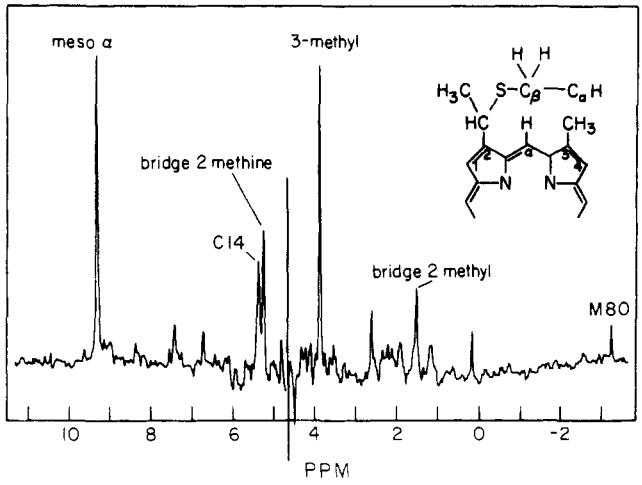

FIGURE 2: Steady-state NOEs resulting from saturation of the heme α-meso proton resonance at 9.32 ppm (horse ferrocytochrome *c*). Saturation was for 2 s. Primary NOEs to the heme C3 methyl (3.83 ppm) and the Cys-14 bridge methine (5.22 ppm) and methyl (1.48 ppm) protons are indicated. A strong NOE is observed between the α-meso proton and a proton resonating at 5.35 ppm. Scalar coupling patterns involving the latter proton and the relative rate of buildup of the NOE from the α-meso proton support its assignment to the $C_\alpha H$ proton of Cys-14. See text for details.

The NOESY spectrum exhibits all the expected cross peaks for these assignments (not shown). Additional confirmation is provided by sequential assignment results, which *independently* place this apparently Cys-14-linked spin system at position 14 in the amino acid sequence (see below). In summary, NOE and scalar coupling patterns confirm the prior assignment (Keller & Wüthrich, 1978) of the methine and methyl protons in the Cys-14–heme bridge, and the heme C_3-methyl protons, and provide new assignments of the Cys-14 $C_\alpha H$ at 5.35 ppm and one of its $C_\beta H_2$ protons at 1.15 ppm.

Heme Axial Ligands. The proton resonances of the heme axial ligands His-18 and Met-80 have been much studied (McDonald & Philips, 1969; Redfield & Gupta, 1971; Moore & Williams, 1980a–d; Keller & Wüthrich, 1981). Assignments have been proposed for most of the nonexchangeable side-chain proton resonances. The COSY spectrum shown in Figure 3 specifies the *J*-coupling network of Met-80, including the $C_\alpha H$ resonance, and identifies the amide proton resonance, which could be confirmed by relay of coherence to the β proton.

Moore & Williams (1980a) have identified the C_2H and C_4H proton resonances of His-18 (now named C_5H and C_2H, as in Figure 4) at 0.13 and 0.50 ppm, respectively. In 90% H_2O an exchangeable proton at 9.63 ppm exhibits *J* coupling to these two protons (Figure 4B). The relayed coherence experiment revealed no additional cross peaks, indicating that the exchangeable proton is not due to an amide or an arginine or lysine side chain; the NOESY spectrum shows it to be a histidine ring NH. His-18 is ligated to the iron via N_τ (see Figure 4 for nomenclature) (Dickerson et al., 1971; Takano & Dickerson, 1977). The His $N_\pi H$ proton (see Figure 4) is expected to show an NOE (Figure 4D) only to the C_2H proton and not to the C_5H proton (Wu et al., 1984), but to *J* couple (Figure 4B) to both. The data in Figure 4 exhibit this pattern and confirm the previous assignments of the nonexchangeable proton resonances of the His-18 ring.

Tryptophan-59. The single tryptophan residue in horse cytochrome *c*, Trp-59, is hydrogen bonded directly to a heme propionic acid side chain. Resonance assignments have been proposed for the indole ring protons of Trp-59, but the connectivity and positioning of these within the benzoid ring have been insecure (Moore & Williams, 1980a; Keller & Wüthrich, 1981; Eley et al., 1982; Robinson et al., 1983). The COSY cross peaks in Figure 5 exhibit the linear coupling pattern expected for the benzoid ring protons of Trp-59. The relayed coherence experiment (Figure 5) provides second nearest-neighbor coupling information, which shows that the apparent pattern does in fact link a linear network of protons, but still does not indicate which resonance represents C_4H and which C_7H (see molecular diagram in Figure 4). However, one expects the exchangeable indole NH to exhibit an NOE (Figure 4C) to both the C_2H and C_7H aromatic protons but to scalar couple (Figure 4A) only to the C_2H proton. These cross peaks are seen in the NOESY and COSY spectra (in H_2O) for the NH proton at 10.05 ppm, thereby defining C_7H and the order of the benzoid ring protons. This order is important in establishing the identity of the $C_\alpha H$ and $C_\beta H_2$ protons by NOE interactions with the C_4H proton (7.55 ppm), thus tying Trp-59 into the main-chain sequence.

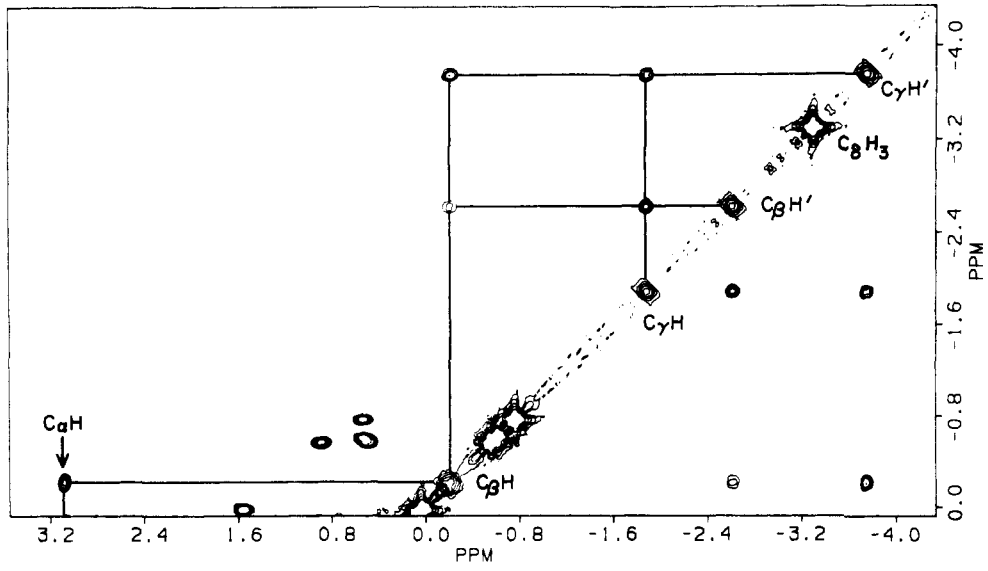

FIGURE 3: Section of an absolute value COSY for the *J*-coupling network assigned to the axial heme ligand Met-80 (horse ferrocytochrome *c* in D_2O at 40 °C). The chemical shifts are $C_\alpha H$ (3.09 ppm), $C_\beta H$ (−0.17, −2.60 ppm), $C_\gamma H$ (−1.85, −3.73 ppm) and $C_\delta H_3$ (−3.25 ppm). COSY and relay COSY spectra obtained in 90% H_2O place the amide proton at 7.16 ppm. (The prominent on-diagonal peaks near −0.8 ppm represent the δ-methyls of Leu-32 and Ile-57 that are off scale in Figure 1.)

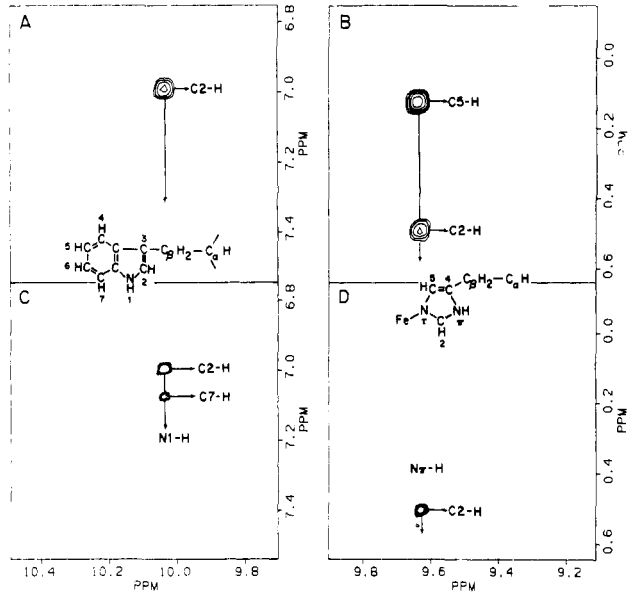

FIGURE 4: Sections of the pure absorption NOESY and COSY of horse ferrocytochrome *c* showing coupling patterns that distinguish histidine and tryptophan residues (obtained in 90% H_2O). The COSY spectrum was processed as in Figure 1 and the NOESY as in Figure 6. Panels A and C display the *J*-coupling and NOE interactions expected for Trp-59. This provides unequivocal support for the assignment of the benzoid ring protons and defines their vectorial order. Panels B and D show the *J*-coupling and NOE interactions between the ring protons of His-18. The molecular diagrams indicate recently recommended IUPAC–IUB (1985) nomenclature.

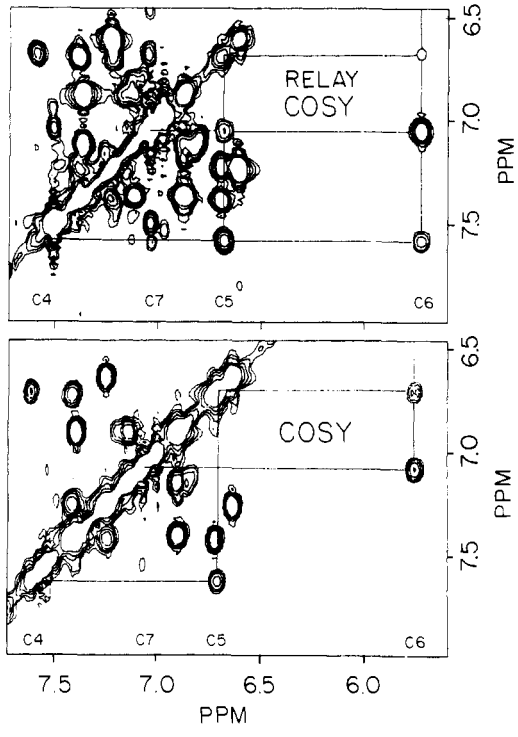

FIGURE 5: Horse ferrocytochrome *c* in the aromatic region. The COSY spectrum shows the linear scalar-coupling network of the Trp-59 ring protons (see molecular structure and nomenclature in Figure 4). The relayed coherence spectrum of the same region provides confirming next neighbor relay cross peaks. The total relay development time (90°–τ–180°–τ–90°) was 60 ms (2τ).

N-Terminal Helix. In smaller proteins, NOE connectivities between amino acids within a common unit of secondary structure (Dubs et al., 1979; Wüthrich, 1983) can identify the amide proton resonances of sequential residues with little ambiguity (Wagner & Wüthrich, 1982; Wüthrich et al., 1984;

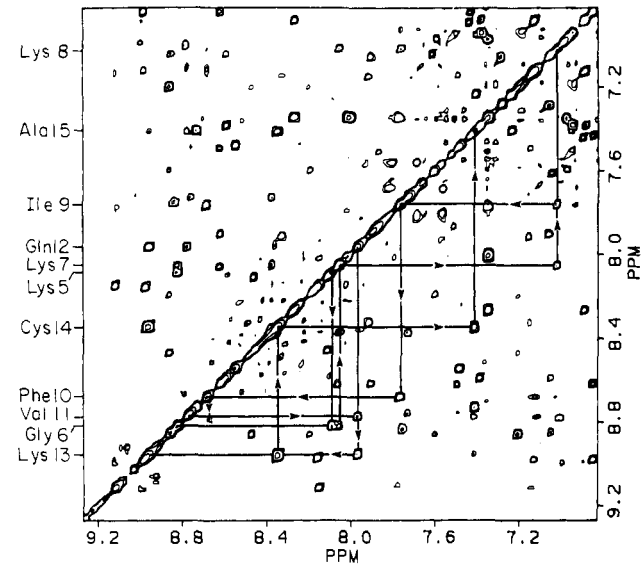

FIGURE 6: Phase-sensitive NOESY spectrum of horse ferrocytochrome *c* in 90% H_2O. Amide–amide NOE connectivities (d_{NN}) for the sequence of amino acids from Lys-5 through Ala-15 are indicated. Most neighboring residues could also be connected via amide NH to β-CH NOEs ($d_{\beta N}$) characteristic of helical sequences. The protein concentration was 12.5 mM, the temperature 40 °C, and the pH 5.7. The mixing time was 140 ms. After zero filling the data set was 2K × 2K (3.9 Hz/point). Symmetrization and the use of a Lorentzian-to-Gaussian digital filter have skewed the near-diagonal Val-11 NH (8.79 ppm) to Phe-10 NH (8.68 ppm) NOE cross peak, as seen most clearly in 1D cross sections.

Zuiderweg et al., 1983; Wemmer & Kallenbach, 1983; Wüthrich, 1983). Figure 6 shows the NOESY spectrum of ferrocytochrome *c* in the amide to amide cross-peak region. A characteristic α helix amide–amide NOE pattern (Billeter et al., 1982) connecting 11 amide NH's of the N-terminal helix is indicated. In most cases at least two interresidue NOEs were required to establish unambiguously the connectivities between these amino acid neighbors. All amino acid spin systems in this segment except for the four lysines could be completed by use of the COSY and relay COSY experiments. Two spin systems, Ala-15 and Ile-9, were verified by species comparisons. The α- and β-carbon protons attributed to Cys-14 through heme connectivities (see above) lead directly to the amide NH that represents residue 14 of this NOE-connected helical segment. The details of these assignments and structural implications of the observed NOEs will be discussed elsewhere.

Discussion

This paper illustrates the capability of already available two-dimensional NMR techniques for solving the assignment problem in proteins of considerable size and functional interest. Earlier work with small proteins (Wagner & Wüthrich, 1979; Kumar et al., 1981) suggested that spin diffusion might pose major difficulties for more sizable proteins. The absence of excess NOEs in our data and results on the time-dependent development of cross-peak intensity in the NOESY spectrum indicate that cross relaxation leading to spin diffusion is not a serious problem for proteins the size of monomeric cytochrome *c*. Earlier indications to the contrary in pancreatic trypsin inhibitor may have reflected protein association into larger aggregates at the NMR concentrations used.

Further advances in 2D NMR technology are continuing to appear, which promises well for the future of these approaches. These NMR methods can provide remarkably detailed information on structure, dynamics, and function. We are especially interested in the motional mechanisms that

govern protein H-exchange behavior (Hvidt & Nielsen, 1966; Woodward & Hilton, 1979; Englander & Kallenbach, 1984) and its exploitation for functional labeling studies of protein structure–function relationships (Englander, 1975; Englander & Englander, 1983; Englander et al., 1983). The NMR approaches used here can display the H-exchange behavior of essentially all the amide NH's in cytochrome *c* and some of the exchangeable side-chain protons (Wand et al., 1985). This is precisely the kind of data needed to resolve remaining uncertainties on the mechanism of protein H exchange (Englander & Kallenbach, 1984) and to make available this powerful methodology for the study of protein dynamics and function. Information on function comes from comparing H-exchange rates in different functional states. For example, this comparison in reduced and oxidized cytochrome *c* highlights those protein segments that interact differentially with the reduced and oxidized heme and therefore help to set its redox potential (Wand et al., 1985). Some of the protons described here exhibit large differences in H-exchange rate with protein redox state, including the Trp-59 ring NH, bonded to the heme propionate, and some of the heme-adjacent amides of the N-terminal helix. Other sites all through the protein can be observed.

These issues will be elaborated elsewhere, along with more detailed descriptions of proton resonance assignments.

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