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Two-Dimensional ^{1}H NMR Studies of Cytochrome *c*: Assignment of the N-Terminal Helix†

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ABSTRACT: The ^{1}H resonances of 11 sequential amino acids in the N-terminal helix of horse ferrocytochrome *c* were studied by two-dimensional nuclear magnetic resonance techniques. All the main-chain protons from Lys-5 through Ala-15 and many of the side-chain protons were assigned. *J*-Correlated spectroscopy (COSY) was used to distinguish protons on neighboring bonds and to recognize amino acid types. Nuclear Overhauser effect spectroscopy (NOESY) was used to define spatially contiguous protons and to determine amino acid sequence neighbors. The relayed coherence experiment (relay COSY) was used to resolve many ambiguities in intraresidue *J*-coupled connectivities and interresidue NOE connectivities. This required no explicit knowledge of the solution structure. The pattern of NOEs found is consistent with a regular α helix between glycine-6 and lysine-13; H bonding continues at least through alanine-15 [see Wand, A. J., Roder, H., & Englander, S. W. (1986) *Biochemistry* (following paper in this issue)]. Chain disorder occurs at the N-terminus. There is no indication of significant spin diffusion among the backbone amide and α-protons of this 12.4-kilodalton protein even at the longest NOE mixing time used (140 ms).

The primary problem encountered in ^{1}H NMR[1] studies of proteins is that of resolving individual resonances and assigning them to individual protons in the molecule. Even in the most recent past, considerable ingenuity has been required to assign a modest number of resonances in relatively small proteins. The conception (Jeener, 1971) and subsequent development of two-dimensional NMR techniques (Aue et al., 1976; Sorensen et al., 1983; Braunshweiler et al., 1983; Wider et al., 1984) has raised the possibility that ^{1}H NMR spectra of macromolecules may become amenable to complete analysis. The sequential assignment approach (Billeter et al., 1982; Wüthrich, 1983) provides a straightforward proton assignment methodology that has proven successful with several small proteins (Wagner & Wuthrich, 1982; Arseniev et al., 1982; Wemmer & Kallenbach, 1983; Keller et al., 1983; Stropp et al., 1983; Zuiderweg et al., 1983; Stassinopoulou et al., 1984). However, significant difficulties can be expected in the application of these techniques to the assignment of larger proteins (Wand & Englander, 1985). Here we describe an

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

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analysis of two-dimensional ^{1}H NMR spectra leading to the assignment of the proton resonances of 11 amino acids that span the N-terminal helix of horse ferrocytochrome *c*.

Cytochrome *c* is nearly twice as large as the largest protein previously assigned. The attempt to assign its proton resonance spectrum tests the limits of available 2D NMR techniques and the sequential assignment analysis. The results obtained can provide a detailed view of solution structure and internal dynamics in this functionally interesting protein (Wand et al., 1986).

Materials and Methods

Cytochrome *c* from horse heart was obtained from Sigma Chemical Co. in the highest available grade. The lyophilized protein was dissolved in 50 mM potassium phosphate buffer in 99.8% D_2O or 90% H_2O/10% D_2O as required. Samples used in the assignment work were reduced with 3–5 equiv of solid sodium dithionite, adjusted to pH* 5.7 with dilute NaOD or DCl as required, and kept under nitrogen during long data acquisition periods. Protein concentration was 6–8 mM in D_2O solution and 12–14 mM in H_2O. D_2O samples were allowed to exchange with solvent in the oxidized state at pH* 6 and room temperature for 10 min prior to reduction.

Two-dimensional NMR spectra were recorded on Bruker WM 500 spectrometers (Yale University, New Haven, and University of Washington, Seattle). *J*-Correlated (COSY) spectra were recorded with the standard pulse sequence (Aue et al., 1976; Nagayama et al., 1979; Bax & Freeman, 1981)

$$(-t_0\text{-}90°\text{-}t_1\text{-}90°\text{-}t_2\text{-})_n$$

with phase cycling appropriate for suppression of axial peaks and quadrature detection in both dimensions (Wider et al., 1984). The sweep width was 8064 Hz in both dimensions and the recycle delay ($t_0 + t_2$) was 1.5 s including acquisition (t_2 = 0.12 s).

Phase-sensitive NOE spectra were collected with the pulse sequence (Macura & Ernst, 1980)

$$(-t_0\text{-}90°\text{-}t_1\text{-}90°\text{-}t_m\text{-}90°\text{-}t_2\text{-})_n$$

where t_m is the mixing time during which the NOE develops. Quadrature detection in t_1 and elimination of axial peaks were by the method of States et al. (1982). The real and imaginary data sets were collected in tandem at each value of t_1. Initial t_2 and t_1 delays were set at one and two dwell periods, 62 and 124 μs, respectively, to aid phasing of the frequency domain spectra. Time domain data included 1024 complex points in t_2 and between 400 and 600 points in t_1. Quoted NOESY cross-peak intensities refer to integrated volumes.

Relayed coherence (relay COSY) spectra (Eich et al., 1982) were collected with the pulse sequence

$$(-t_0\text{-}90°\text{-}t_1\text{-}90°\text{-}\tau\text{-}180°\text{-}\tau\text{-}90°\text{-}t_2\text{-})_n$$

and used phase cycling appropriate for quadrature detection in t_2, single-channel detection in t_1, and elimination of the axial peaks arising during t_1 and the NOEs arising during the refocusing period (Eich et al., 1982; Wagner, 1984).

For spectra in 90% H_2O, solvent elimination was achieved by direct irradiation at all times except during acquisition. Power levels of ca. 20 dB of 0.2 W applied to the probe provided reasonable solvent suppression without introducing Bloch–Siegert shifts during the incremented time domain (Wider et al., 1984). When quadrature detection was employed, CYCLOPS phase cycling (Hoult & Richards, 1975) of all pulses was applied. Time domain data were transferred to magnetic tape and re-formated to a VAX compatible data set. All further processing, on either a Vax 11/750 or a Vax 11/780, used a FORTRAN program, FTNMR, written by Dennis Hare (Copyright 1984). Chemical shifts are referenced to an external standard (coaxial capillary) of 4,4-dimethyl-4-silapentanesulfonate.

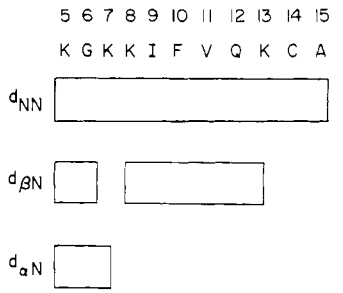

FIGURE 1: Primary sequence of assigned amino acids in horse cytochrome *c*. Bars indicate residues for which an NOE between the designated protons was observed. The distance symbols are $d_{NN} = d(NH_i,NH_{i+1})$, $d_{\alpha N} = d(C_\alpha H_i,NH_{i+1})$, and $d_{\beta N} = d(C_\beta H_i,NH_{i+1})$.

Results

Assignment Strategy. The method of sequential assignment has been described in detail by Wüthrich and co-workers (Billeter et al., 1982; Wüthrich, 1983; Wüthrich et al., 1984). A brief summary is provided here. The first step is the identification of resonances belonging to the same residue, primarily by analysis of *J*-correlated (COSY) spectra. The recognition of a *J*-coupling pattern serves to identify the amino acid type, or at least to classify the spin system as a member of a restricted group of amino acid types. The next step hinges on distance relationships provided by the NOESY experiment. NOE connectivities with the amide proton of any given residue are very much restricted to the same or nearest-neighbor residues; non-nearest-neighbor connectivities are rare (Billeter et al., 1982). A convenient notation introduced by Wüthrich et al. (1984) names the close distances *d* between an amide NH and the protons of neighboring residues in the primary sequence that tend to produce significant NOEs as

$$d_{\alpha N} = d(C_\alpha H_i,NH_{i+1}) \qquad d_{NN} = d(NH_i,NH_{i+1})$$
$$d_{\beta N} = d(C_\beta H_i,NH_{i+1})$$

The matching, with primary sequence information, of defined spin systems (amino acid type) and interresidue NOEs (amino acid neighbors) can lead to the sequence-specific sequential assignment of the protein. A sequential assignment can also provide information about local secondary structure since different types of secondary structure display characteristic patterns of $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ NOEs (Billeter et al., 1982). For example, standard α helices produce a continuous string of d_{NN} and $d_{\beta N}$ NOEs and no $d_{\alpha N}$ NOEs (Billeter et al., 1982), as we find in cytochrome *c* (Figure 1).

The basic problem in analyzing 2D NMR spectra is to distinguish authentic proton connectivities. Cytochrome *c* has over 850 protons (in H_2O) so that several protons may often display the same chemical shift. Many such ambiguities, arising from accidental degeneracy of unrelated protons, are found in spectra correlating chemical shift via *J*-coupling (e.g., COSY) and in spectra correlating chemical shift via the NOE (e.g., NOESY) (Wand & Englander, 1985). Figure 2a displays the extent of ambiguity in the amide NH–C_αH cross-peak region of a COSY spectrum of ferrocytochrome *c* obtained in 90% H_2O. To resolve these, the relayed coherence technique can be helpful. A relayed COSY spectrum (Figure 2b) provides through-bond connectivity information correlating amide NH and C_βH protons of the same amino acid. As illustrated below, this kind of information can also help to unravel inter amino acid NOEs between sequence neighbors.

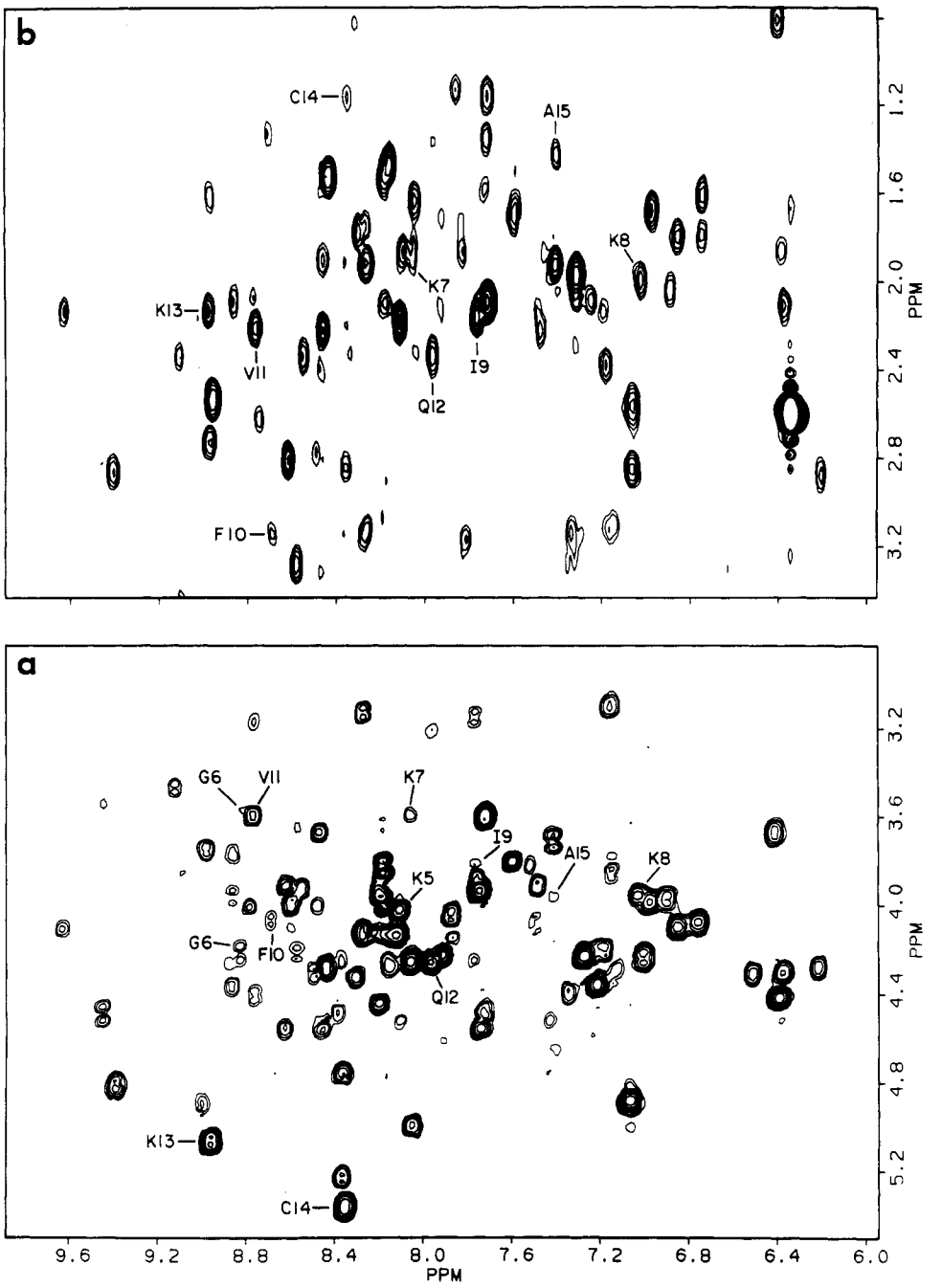

FIGURE 2: (a) Fingerprint region of a COSY spectrum showing predominantly NH–C_αH cross peaks. Cross peaks from amino acids assigned to the N-terminal segment are indicated. Not all NH–C_αH cross peaks observed appear in this expansion. The spectrum was symmetrized. Ferrocytochrome *c* at 14 mM was in 90% H_2O/10% D_2O at pH* 5.7 and 40 °C. (b) Section of a relay COSY spectrum containing cross peaks mostly due to amide to β-proton relayed coherence. The NH–C_βH relays observed for amino acids assigned to the N-terminal helix are indicated. Delay time used (τ) was 28 ms. Conditions were as for panel a, but the spectrum was not symmetrized, and ferrocytochrome *c* was 12 mM. Discrepancies due to slightly different conditions between the two spectra could be checked by mapping cross peaks due to direct three-bond couplings in the relay spectrum with those in the COSY spectrum.

The assignment of proton resonances can be aided by the growing library of shift positions for the various protons of amino acids in polypeptide linkage, provided by Bundi & Wüthrich (1979) and in subsequent literature. In cytochrome *c*, the heme ring current, though helpful in increasing shift dispersion, makes the use of standard shifts dangerous. We have therefore not depended on prior shift information. NH and CH protons were distinguished by their solvent exchangeability (H_2O COSY vs. D_2O COSY) and side chains from amide NH's by their relative exchangeability (40 °C, pH* 5.7) and their connectivities (d_{NN}, $d_{\alpha N}$, $d_{\beta N}$).

Sequential Assignment of the N-Terminal Helix. The degree of complexity present in two-dimensional spectra of cytochrome *c* makes it advantageous to have available a set of assignments that can serve as starting points in the sequential assignment process and as checkpoints along the way. ^1H resonances of four amino acids in the N-terminal helix of ferrocytochrome *c* have been assigned by us and others. By comparing the *J*-correlated spectra of ferrocytochromes *c* from various species, we have assigned the nonexchangeable proton resonances of Ala-15 and have identified two isoleucine spin systems, one of which must correspond to Ile-9 and the other to Ile-95. Assignments for the Cys-14 spin system were obtained on the basis of NOEs with known heme protons (Wand & Englander, 1985). NOE and redox-dependent chemical shift arguments have led to the assignment of a set of aromatic

Table I: Chemical Shifts of Assigned Protons in the N-Terminal Segment of Horse Ferrocytochrome c[a]

	chemical shift (ppm)				
amino acid	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	other
Ala-15	7.42	3.94	1.41		
Cys-14	8.35	5.35	1.15		
Lys-13	8.97	5.07	2.11		
Gln-12	7.95	4.25	2.33	2.71	
Val-11	8.78	3.60	2.21	1.16	
				0.98	
Phe-10	8.68	4.07	3.14		6.98 (ortho)
			3.02		6.12 (ortho)
					6.21 (meta)
					7.17 (meta)
					7.08 (para)
Ile-9	7.77	3.88	2.18	1.29 (CH	
				1.13 (CH_3)	
Lys-8	7.03	3.96	1.98		
Lys-7	8.06	3.58	1.90		
Gly-6	8.84	4.20			
		3.56			
Lys-5	8.09	4.01	1.86		

[a] Chemical shifts are referenced to external sodium 4,4-dimethyl-4-silapentanesulfonate and are quoted to 0.01 ppm since different 2D spectra are, as a rule, consistent to that precision. For reduced cytochrome c in 50 mM sodium phosphate buffer, pH* was 5.7. All chemical shifts are quoted for 40 °C except for the ring protons of Phe-10, which were determined at 20 °C (where the ring protons are an AMXQP system).

ring protons to Phe-10 (Moore & Williams, 1980; Keller & Wüthrich, 1981; Boswell et al., 1980). The Cys-14 and Phe-10 assignments explicitly depended upon crystal structure information.

The amino acid sequence in the N-terminal segment of horse cytochrome c is shown in Figure 1. The sequential assignment starts from the spin systems assigned to Ala-15 and Cys-14 as just indicated and proceeds toward the N-terminus. NOE connectivities obtained are summarized in Figure 1. COSY, relay COSY, and NOESY spectra are shown in Figures 2 and 3. Table I lists resonances assigned in this exercise.

Ala-15 and Cys-14. The Ala-15 CH_3 protons have been assigned by comparing spectra for horse and chicken cytochromes c (to be reported). Protons of the Cys-14 side chain, which bridges to the heme group, have been identified by 1D and 2D NOEs with known heme protons (Wand & Englander, 1985). Extension of the Ala-15 and Cys-14 spin systems to their own amide NH is provided by the COSY spectrum (Figure 2a), which displays the $C_\alpha H$–NH coupling, and the relay COSY spectrum (Figure 2b), which shows the $C_\beta H$–NH pairing (in H_2O). The two amide protons thus identified are NOE-connected (Figure 3), confirming that they are spatially close. This d_{NN}, amide NH to amide NH interaction, is characteristic of (but not limited to) α helices (Billeter et al., 1982; Wüthrich et al., 1984). It terminates a long sequential stretch of α-helix d_{NN} NOEs (see Figure 3). Since the order of Cys-14 and Ala-15 is known, the direction of the connectivity is determined.

The amide proton of Cys-14 gives rise to five significant NOEs. One is the NOE to the amide proton of Ala-15. Two are to its own α- and β-protons. The remaining two are to resonances at 4.29 and 8.97 ppm. These two resonances appear not to be scalar coupled (COSY, Figure 2a); therefore, they are presumed not to represent the $C_\alpha H$ and amide NH protons of the same amino acid. The 4.29 ppm resonance could not be identified at this point in the analysis; it may be due to Cys-14 or arise from an accidental degeneracy of the Cys-14 amide proton with another amide proton. The 8.97 ppm resonance, however, leads to the rest of the helix.

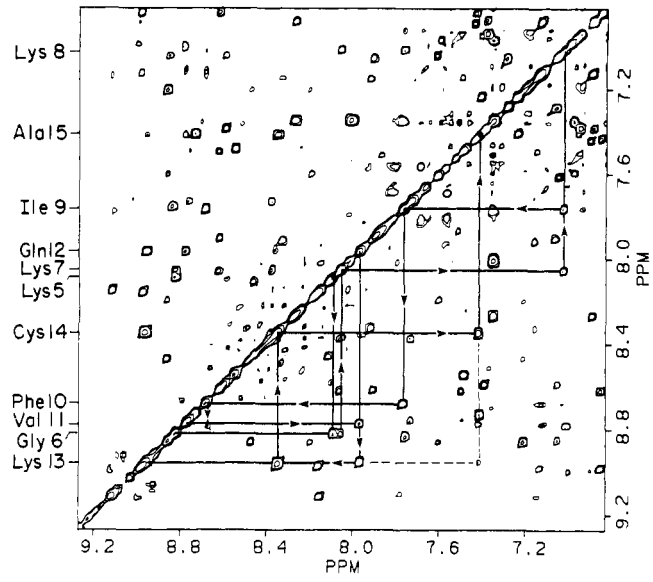

FIGURE 3: Section of a phase-sensitive NOESY spectrum showing NOE cross peaks due to amide–amide connectivities and some aromatic proton connectivities. Solid lines trace the sequential d_{NN} connectivity of the N-terminal segment assigned here, with arrows pointing in the conventional chain direction, although the analysis described ran from Ala-15 to Lys-5. A nonstandard interresidue NOE is indicated by dashed lines. Mixing time was 140 ms, and the spectrum was symmetrized. Conditions were as in Figure 2.

Lys-13. Toward the N-terminus, Lys-13 is next to Cys-14. The amide proton at 8.97 ppm (see just above) is connected via the relay COSY (Figure 2b) to a β-proton at 2.11 ppm, and scalar couplings connect these via an intervening resonance at 5.07 ppm. These cross peaks represent the amide NH, $C_\alpha H$, and $C_\beta H$ of Lys-13. The amide NH shows NOEs to these same α- and β-protons. Although the total Lys-13 spin system could not be unambiguously completed, the absence of cross-peak fine structure and the ability to extend the connectivity past the β-protons are consistent with the assignment of these protons to a complex lysine residue.

The presumed amide proton of Lys-13 at 8.97 ppm shows one very weak and two strong NOEs to other amide NH protons. The weakest of the NOEs (intensity [I] = 0.12 in arbitrary volume units) is a nonstandard NOE reaching back to the Ala-15 NH (dashed connectivity in Figure 3) and suggests a break in helical parameters past Lys-13. The next stronger NOE cross peak (I = 1.14) is the one to the Cys-14 NH. The strongest NOE (I = 1.19) is to a 7.95 ppm resonance, which can be assigned to Gln-12.

Gln-12. The evidence indicates that the 7.95 ppm resonance represents the amide proton of Gln-12. It is the only unassigned amide–amide NOE from the Lys-13 NH. The Lys-13 NH also shows NOEs to its own α- and β-protons *and* to a proton at 2.33 ppm (and no other side-chain protons). COSY and relay COSY spectra document the scalar-coupled string $C_\beta H$ (2.33)–$C_\alpha H$ (4.25)–NH (7.95). This scalar connectivity can be extended to an additional ($C_\gamma H$) proton at 2.71 ppm. The apparently scalar-connected protons are in fact close in space; the α- and β-protons show NOEs to the 7.95 ppm (Gln-12 NH) proton, and the NH and $C_\gamma H$ protons show NOEs to the Lys-13 NH. All these observations indicate the assignment of this spin system to Gln-12.

The relay COSY and NOESY data were combined several times in this way to identify the correct combination of $d_{\beta N}$ and d_{NN} NOEs, as illustrated in Figure 4. Such information can provide a firm basis upon which to extend the sequential assignment. It can be noted that the demonstration of both

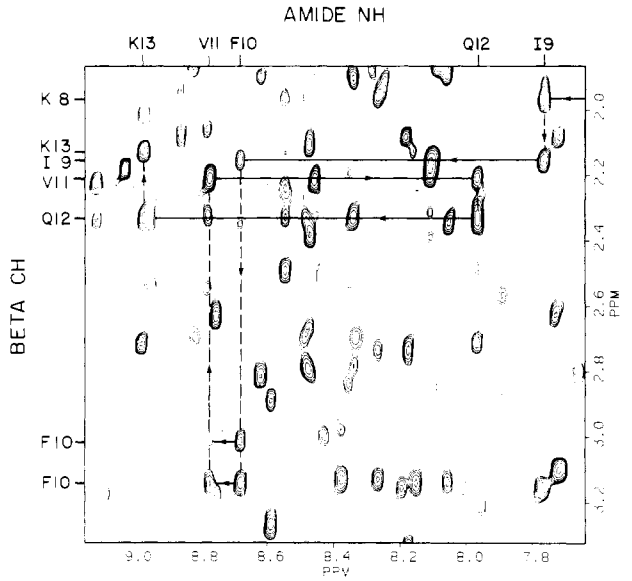

FIGURE 4: Section of a phase-sensitive NOESY spectrum showing cross peaks due to both intra- and interresidue NOEs. Conditions were as in Figure 2. The lines drawn connect ^1H–^1H cross peaks, thus represent second-order connectivities. Each horizontal line (solid) connects an intraresidue NH–$C_\beta H$ cross peak to an interresidue $d_{\beta N}$ cross peak characteristic of α helices; cross peaks in a given pair involve the NH in sequential residues with the same $C_\beta H$ proton. Each vertical line (dashed) extends the connectivity pattern by pairing peaks that involve a given amide NH and sequential $C_\beta H$. Authentic intraresidue NH–$C_\beta H$ cross peaks show up in the relay COSY spectrum (Figure 2b) and can be inferred in addition from COSY NH–$C_\alpha H$–$C_\beta H$ connectivities (partly shown in Figure 2a). The second-order connectivities independently define chemical shift coordinates of sequential amide NHs, which are often ambiguous in the NOESY NH–NH region (Figure 3). The combined use of these several spectra allows ambiguities in the connectivities to be resolved in most cases. The pattern shown here traces out a "snail" pathway, formed by alternating intra- and interresidue cross peaks, that can be diagnostic for standard α helices, though the intraresidue NH–$C_\beta H$ cross peak observed for all the residues in this sequence has not previously been expected.

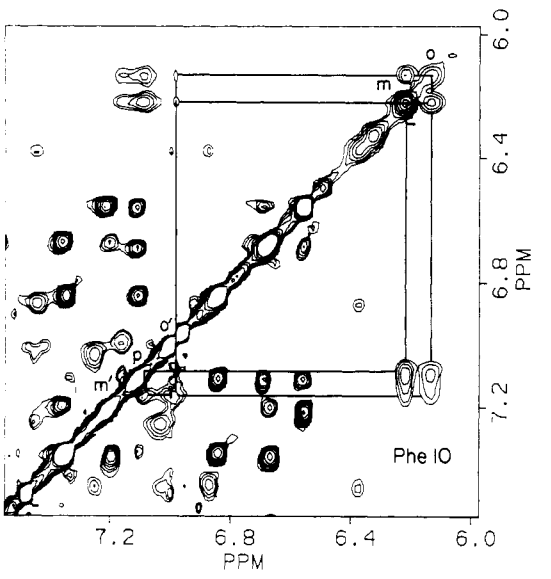

FIGURE 5: Section of a phase-sensitive NOESY spectrum showing the spin system of Phe-10. The cross peaks arise from three conditions: direct through-space interactions (e.g., ortho to meta), physical exchange (e.g., meta to meta'), and NOE + physical exchange (e.g., ortho to meta to meta'). The spectrum is not symmetrized. A 120-ms mixing time was used. Ferrocytochrome *c* at 8 mM was in D_2O at pH* 5.7 and 20 °C.

d_{NN} and $d_{\beta N}$ interactions can document α-helical structure independently of knowledge of the crystal structure.

The amide proton of Gln-12 (7.95 ppm) gives rise to eight apparent NOEs. One is to the Lys-13 NH and three are intraresidue to its own α-, β-, and γ-protons (above). Four others are to Val-11.

Val-11. Only one new amide–amide NOE from Gln-12 is observed, to a proton at 8.78 ppm (Figure 3). A scalar-coupled string connects the 8.78 ppm amide proton to an α-proton at 3.60 ppm (Figure 2a) and thence to a β-proton at 2.21 ppm. A pair of intense cross peaks from 2.21 to 1.16 and 0.98 ppm are observed in the COSY spectrum (not shown). The distinctive pattern, intensity, and absence of resolved fine structure of these cross peaks indicate their assignment to a valine. No relay (τ = 28 ms) is observed between the amide and β-proton, but this is not unexpected in light of the predicted loss of intensity in the relay cross peak (Bax & Drobny, 1985) due to coupling of $C_\beta H$ with the six protons of the γ-methyls. The Gln-12 NH exhibits NOEs to the protons resonating at 2.21 (I = 0.60), 1.16 (I = 0.27), and 0.98 ppm (I = 0.16), representing the Val-11 $C_\beta H$ and two $C_\gamma H_3$ groups (see above). Evidently, this valine spin system represents Val-11. Since valine is a unique spin system, its identification after Gln-12 helps to confirm the string of connectivities from Ala-15 and Cys-14 to this point.

Nine apparent NOEs arise from the Val-11 amide proton. Two are to the neighboring Gln-12 amide NH and γ-protons (I = 2.13), and three are to the α- (I = 1.16) and two γ-methyls (I = 2.85 and 0.58) of Val-11, all described above. The remaining four, to 8.86, 4.07 (I = 0.62), 3.14 (I = 0.5), and 3.02 ppm (I = 0.20), are due to Phe-10.

Phe-10. The d_{NN} NOE between the amide proton of Val-11 and the amide proton of the next amino acid, Phe-10, appears unusually weak. This is an artifact due to distortion of the cross-peak shape and intensity by the filter function used to provide resolution enhancement, as can be seen in 1D cross sections (not shown).

Considerable attention has been devoted to the assignment of the aromatic ring protons of Phe-10 (Moore & Williams, 1980; Keller & Wüthrich, 1981; Boswell et al., 1980). The identification of these resonances with a phenylalanine ring was based upon spin decoupling and intraring NOEs. Their assignment to position 10 of the primary sequence was based on interpretation of the presence or the demonstrable absence of NOEs following saturation of protons of Met-80, Leu-32, and the heme and on the effects of change in oxidation state on chemical shift. Distinction between the ortho, meta, and para positions of the ring was based on ring current effects (Boswell et al., 1980) and on the time order of development of NOEs from Met-80 (Keller & Wüthrich, 1981). These arguments relied on the X-ray structure of the tuna enzyme.

The 2D NOESY spectrum in Figure 5 shows the Phe-10 ring protons. Extensive exchange of magnetization between the previously assigned ring protons, indicating rotation about the C_β–C_γ bond, is observed at 20 °C (Figure 5). Our COSY spectrum fails to resolve all couplings from the diagonal but does reveal a linear network of these protons, confirming their assignment to a phenylalanine ring (the only Trp residue in horse cytochrome *c* has been previously assigned). The ring ortho protons can be recognized by their significant NOE connectivities to main-chain protons. At 20 °C, the apparent ortho protons of Phe-10 display five nonring NOEs. The strongest are to protons at 4.13, 3.07, and 3.58 ppm. The first two of these are scalar coupled (not shown) and represent (from COSY and relay COSY experiments done at 40 °C; see below) the α- and β-proton partners of the Phe-10 amide NH (above). The third NOE cross peak from the Phe-10

ortho protons is to the α-proton of Val-11 (above). These connectivities confirm the Val-11 to apprent Phe-10 assignment.

At 40 °C the three resonances just described shift to 4.07, 3.14 (Phe-10 $C_\alpha H$ and $C_\beta H$), and 3.60 ppm (Val-11 $C_\alpha H$), respectively. The significant temperature sensitivity of the chemical shift of the Phe-10 α- and β-protons correlates with the temperature-dependent change in the rate of Phe-10 ring flipping. At 20 °C, the ring flip rate, estimated from the mixing time dependence and relative intensity of the chemical exchange cross peaks, is about 50 s^{-1}. At 40 °C, all these resonances are significantly broadened, indicating enhancement of the flip rate into the intermediate time regime (not shown).

The amide NH of Phe-10 (8.68 ppm) gives rise to 10 apparent NOEs. One is to the Val-11 amide (8.78 ppm; I = 0.27). The three strongest are to the α- and β-protons of Phe-10 assigned above and an additional proton resonating at 3.02 ppm. The consistent appearance of the 3.02 ppm proton in apparent $d_{\beta N}$ and intra amino acid NOEs strongly suggests that it is the remaining Phe-10 β-proton. No $C_\alpha H$ (4.07 ppm) to $C_\beta H$ (3.02 ppm) COSY cross peak is observed. The absence of a $C_\alpha H$ cross peak to one of the β-protons is not unusual (see below), but this is considered a tentative assignment. Two other apparent NOEs are to unidentified side-chain protons. Other NOEs reach to Ile-9.

Ile-9 to Lys-7. Of the four remaining NOEs from the Phe-10 amide proton, three are to the spin system indicated by a comparison of COSY spectra of different cytochrome species to be either Ile-9 or Ile-95 (to be reported elsewhere). Evidently, these represent Ile-9. The remaining Phe-10 NOE is to a proton at 7.77 ppm (I = 0.95), evidently the Ile-9 amide NH since it is scalar coupled to the α-proton of the presumptive Ile-9 spin system.

Extension of d_{NN} connectivities from Ile-9 through Lys-8 to Lys-7 is unambiguous (Figure 3). COSY and relay COSY spectra lead to the $C_\alpha H$ and $C_\beta H$ protons of both lysines, though again here the complex lysine side chains could not be unambiguously completed. A supportive $d_{\beta N}$ NOE is observed (Figure 4) between Ile-9 and Lys-8. Further confirmation that the correct d_{NN} connectivities have been followed comes from identification of the residue after the suggested Lys-7 as a glycine.

Gly-6 and Lys-5. The amide NH of Lys-7 (8.06 ppm) shows an NOE to an amide proton at 8.83 ppm. The latter is scalar coupled to two α-protons (4.20 and 3.56 ppm, see Figure 2a). The two cross peaks show very different intensity, but both are doublets, and the two α-protons are also J coupled. This is clearly a glycine, consistent with its assignment to Gly-6.

Glycine is the only amino acid that can display coupling from an amide to two α-protons. The two α-protons must also be scalar coupled. Further, due to geminal coupling between the two α-protons of glycine, each of the two amide to $C_\alpha H$ cross peaks appears, under the experimental conditions used here, as a doublet with an apparent coupling constant of ca. 25 Hz. Sometimes, only one of the doublet cross peaks is sufficiently intense to be observed. Since the dihedral angles between the amide proton and the two α-protons are in general different, the two amide to $C_\alpha H$ cross peaks often have different intensity. Ten of the twelve glycines in horse cytochrome c produce paired or single $NH–C_\alpha H_2$ doublet cross peaks in the COSY spectrum with the characteristic glycine splitting.

The amide proton of Gly-6 gives rise to six NOEs. An NOE is seen to one of the Gly-6 $C_\alpha H$ protons but not to the other, consistent with the difference in dihedral angles indicated by the difference in $NH–C_\alpha H$ COSY cross-peak intensity (Figure 2a). The d_{NN} NOE to the amide proton of Lys-7 was mentioned above. Another to the $C_\alpha H$ proton of Lys-7 is a nonstandard NOE for an α helix, suggesting that the helix (confirmed by the string of d_{NN} and $d_{\beta N}$ NOEs to this point) begins to unwind toward the N-terminus. However, a strong d_{NN} NOE is observed between the Gly-6 amide proton and another (Lys-5) amide proton at 8.09 ppm (Figure 3), suggesting that the helix is only slightly perturbed at this point. The latter proton is scalar coupled to an α-proton at 4.01 ppm (Figure 2a) and thence to a β-proton at 1.86 ppm, and both of these show an NOE to the Gly-6 NH. These are assigned to Lys-5. Extension past the Lys-5 β-proton is uncertain due to the many possible cross peaks. The nonstandard $d_{\alpha N}$ NOE between Lys-5 $C_\alpha H$ and the Gly-6 amide (I = 0.28) further indicates distortion of helicity at this point. The assignments could not be extended past Lys-5, since no d_{NN} or $d_{\alpha N}$ NOEs are observed and the $d_{\beta N}$ NOEs are weak and ambiguous.

Discussion

The assignment of 11 amino acids spanning the N-terminal helix of ferrocytochrome c has been presented in some detail. Except for the four lysines, which could only be assigned unambiguously from the amide to β-protons, all other amino acid spin systems in this sequence could be identified in the COSY spectrum. Four amino acids, assigned in other work, were used as reference points in the sequential assignment. The sequential assignment was begun at one of these (Ala-15) and led at residue 14 to the previously assigned Cys-14 resonances, at residue 9 to the isoleucine spin system previously revealed by our cytochrome species comparisons, and at residue 6 to an unmistakable glycine spin system. The sequential assignment also led to the main-chain resonances of Phe-10, and NOEs with the independently identified ring protons were confirmatory of the sequential assignment results. Extensive consistency with the cytochrome c crystal structure was also found.

Methodology. Evidently, the method of sequential assignment, although a complex process, is applicable to proteins the size and complexity of cytochrome c. Earlier studies (Kumar et al., 1981) had suggested that spin diffusion might interfere with the recognition of direct inter amino acid connectivities. In this context, spin diffusion is the chemical shift correlation of nonneighbor protons via cross relaxation with a common neighbor. As outlined in detail above, all but a few of the NOEs involving amide protons could be clearly assigned to intraresidue interactions or to interresidue interactions with next neighbor amino acids in the primary sequence. This is consistent with a basic premise of the sequential assignment approach, that interresidue NOEs arising from amide dipolar interactions in an α helix are overwhelmingly restricted to neighboring amino acids in the primary sequence. More to the point, the data give no indication of spin diffusion. This suggests that the study of even larger proteins, where spin diffusion is enhanced, will not be as difficult as had been anticipated.

Though most spin systems could be well-defined in the COSY spectrum, the extent of spin system definition is less than ideal. Cross peaks do not generally appear between all J-coupled protons. For example, the $C_\alpha H–C_\beta H_2$ J coupling of most amino acids in cytochrome c produces a COSY cross peak to only one of the $C_\beta H_2$ protons. This is as expected. In the absence of internal rotation leading to the averaging of coupling constants, the two β-methylene protons are likely to have different coupling constants to the α-proton. Due to the antiphase character of cross-peak fine structure and the

inherently large line widths of cytochrome *c* resonances, cross peaks originating from weak (less than 3 Hz) *J* coupling are expected to vanish. This effect is advantageous in the sense that the resulting spectrum is considerably simplified, but it does reduce the ability of the COSY spectrum to define *J*-coupled networks.

It is important to note that the use of the relayed coherence experiment was highly effective in resolving ambiguities in both intra amino acid *J*-coupled connectivities and inter amino acid NOE connectivities. The correct NH–C_αH–C_βH connectivities in the COSY spectrum are often obscured by multiple ambiguities, which may be resolved by reference to the relay COSY NH–C_βH cross peak. Similarly, because of the numerous degeneracies in amide NH chemical shift, the d_{NN} cross-peak patterns of different structural elements often overlap. In the α helix, the $d_{\beta N}$ NOE can be used to resolve this ambiguity *if* the β-protons can be unambiguously related to their appropriate amide proton. The information provided by the relayed coherence experiment can be used to tie observed $d_{\beta N}$ NOEs to true d_{NN} NOEs, as outlined in Figure 4. The present results illustrate the value of the combined use of NOESY and relay COSY experiments in the assignment of sizeable proteins.

Structure. Knowledge of the crystal structure was not applied in the assignment procedure. The fact that the NOEs found in horse ferrocytochrome *c* in solution are consistent with crystal structure distances for the tuna protein, including the nonstandard amide–amide NOEs at the C-terminal end of the helix, therefore tends to confirm both data sets and independently indicates the similarity of the horse cytochrome solution structure and the tuna cytochrome crystal structure in this region. The occurrence of d_{NN} and $d_{\beta N}$ NOEs throughout this sequence of amino acids supports the existence of an N-terminal α helix in the solution structure of horse ferrocytochrome *c*. The absence of d_{NN}, $d_{\beta N}$, and $d_{\alpha N}$ NOEs between Glu-4 and Lys-5 suggests that the first four residues of the primary sequence do not assume standard α or β structure parameters. The nonstandard (for an α helix) through-space NOE interactions from Gly-6 NH to both C_αH neighbors further indicate helix unwinding at that point. The nonstandard amide–amide NOE observed between Lys-13 and Ala-15 and the absence of $d_{\beta N}$ NOEs between Lys-13 and Cys-14 and between Cys-14 and Ala-15 define termination of the helix at the C-terminal end. These results place the bounds of standard α-helix parameters at Gly-6 and Lys-13.

Disorder at the helix N-terminus in the sense of dynamic end fraying (Zimm & Bragg, 1959) is manifest in the hydrogen-exchange behavior of the amide protons described in the following paper (Wand et al., 1986).

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