Structure and Heme Environment of Ferrocytochrome \(c_{553}\) from \(^1\text{H}\) NMR Studies*

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Cytochrome \(c_{553}\) is a photosynthetic electron transport protein found in algae and cyanobacteria. We have purified cytochromes \(c_{553}\) from five cyanobacteria and studied the structures of the ferrocytochromes by \(^1\text{H}\) NMR spectroscopy at 360 and 470 MHz. Using standard NMR techniques and by comparing the amino acid sequences of four cytochromes \(c_{553}\) with their \(^1\text{H}\) NMR spectra, we have assigned in the spectrum of the Aphaniizonemen flo-s-aque protein 18 resonances to specific amino acid residues and 12 resonances to specific heme protons. Steady state and truncated driven nuclear Overhauser enhancement experiments indicate that a tyrosine and methionine are located near pyrrole ring IV of the heme and that a phenylalanine ring is near the heme \(\alpha\)-mesoproton. The general folding of the cytochrome \(c_{553}\) protein backbone appears to resemble that of Pseudomonas aeruginosa cytochrome \(c_{553}\), but the chirality of the cytochrome \(c_{553}\) axial methine sulfur is \(R\), the same as that of horse heart cytochrome \(c\).

Cytochrome \(c_{553}\) shuttles electrons from cytochrome \(f\) to the photosystem I reaction center, \(P_{680}\), in the photosynthetic electron transport systems of cyanobacteria and algae (1–3). In the past, this small cytochrome (\(M_r \approx 10,000\)) was thought to be the cyanobacterial and algal equivalent of the larger \(c_{553}\) cytochromes in higher plants. True cytochromes \(f\) have now been purified from several cyanobacteria and algae (1, 4−7). Wood (1, 2) and Böhme et al. (3) have shown that the function of cytochrome \(c_{550}\) is the same as that carried out in higher plants by the blue copper protein plastocyanin. Some cyanobacteria have both a plastocyanin and a cytochrome \(c_{550}\), and the relative levels of the two proteins in vivo depend on the metal content of the growth medium (2).

Cytochromes \(c_{550}\) contain 81–89 amino acids and a single heme \(c\) (8). Amino acid sequence homologies between cytochromes \(c_{553}\) and mitochondrial cytochromes \(c\) and other small \(c\)-type cytochromes indicate that the cytochrome \(c_{550}\) peptide backbone is bound to the heme by two cysteine thioether linkages and that histidine and methionine are the fifth and sixth ligands to the iron (9). Keller and co-workers in their x-ray crystallography of \(c_{550}\) from Anacystis nidulans is being determined by x-ray crystallography (14).

We present here \(^1\text{H}\) NMR data at 360 and 470 MHz for cytochromes \(c_{553}\) from the cyanobacteria Aphaniizonemen flo-s-aque, Anabaena variabilis, Spirulina maxima, Microcystis aeruginosa, and an unknown species of Oscillatoria. A number of NMR peak assignments are proposed, and the orientations of the histidine and methionine ligands with respect to the heme are discussed. The overall structure and properties of cytochromes \(c_{553}\) are compared with those of other \(c\)-type cytochromes. A preliminary report of this work has been given (15).

MATERIALS AND METHODS

We used the procedures of Ho et al. (16) to purify cytochromes \(c_{553}\) from \(A.\) flo-s-aque, \(A.\) variabilis, \(S.\) maxima, and \(M.\) aeruginosa. The \(A.\) flo-s-aque, \(S.\) maxima, and \(M.\) aeruginosa cells were harvested from lake algal blooms. The \(A.\) variabilis and Oscillatoria cells were grown in the laboratory in a horse-bred growth chamber using Medium C of Kratz and Myers (17).

\(^1\text{H}\) NMR spectra were obtained using a Nicolet 8.5 T (360 MHz) or 11.1 T (470 MHz) NMR spectrometer equipped with probes that accommodate 5-mm outside diameter sample tubes and operated in the high power, pulse-Fourier transform mode. The probe temperature and other experimental details are given in the figures. Chemical shifts are reported in parts/million from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Protein samples were lyophilized three times from 99.9% isotopic purity \(^2\text{H}_2\text{O}\) (Bio-Rad) to minimize the water signal and to exchange labile \(\text{N—H}\) protons. When completely exchanged protein samples were required, the samples were reduced with a slight excess of sodium dithionite and heated to 50°C for 10 min. The minimal activity of the treated samples was not determined. Except for the absence of \(\text{N—H}\) resonances from slowly exchangeable groups, the spectra of heat-treated and normal protein samples were identical. The pH values determined with a glass electrode standardized by using protic buffers were uncorrected for the deuterium isotope effect; these values are labeled pH*. Peak intensities were measured either by the peak-integrating routine available with the Nicolet NTCTF software package or by cutting out and weighing individual plotted peaks. The low field resonance at ~10 ppm in spectra of ferrocytochromes \(c_{553}\) assigned to a heme ring mesoproton, was used as an intensity of one proton for calibration purposes. NMR titration data were analyzed by a nonlinear least squares computer program written by W. R. Finkenstadt, Jr. (18), and modified for the Nicolet 1180 computer by D. E. Neves.

The J-modulated \(^1\text{H}\) NMR spectra were obtained using a 90°-τ₁-180°-τ₂ pulse sequence with a 90° phase shift on the 180° pulse (19, 20). Truncated driven NOE spectra were collected with a 7, 90°-τ₁ pulse sequence (21). The Met\(^15\) C proton peak was irradiated during the delay, τ₁, which was varied for each spectrum. The delay, τ₀, was set longer than three times the τ₁ of any proton in the sample.

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1 A culture of \(A.\) variabilis was kindly provided by Professor Jack Myers of the University of Texas, Austin.

2 The abbreviation used is: NOE, nuclear Overhauser enhancement.
RESULTS

A complete $^1$H NMR spectrum at 470 MHz of the ferrocytochrome $c_{553}$ from A. flos-aquae is shown in Fig. 1. The numbers in Fig. 1 designate proton resonances that we have assigned to amino acid residues; the letters label peaks that we have assigned to heme ring protons. In Table I are listed the assigned amino acid and heme proton chemical shifts for four of the cytochromes $c_{553}$ we have studied. The cytochrome $c_{553}$ amino acid sequences required to make the assignments are shown in Fig. 2.

Amino Acid Peak Assignments: Tyrosine—The cytochrome $c_{553}$ from M. aeruginosa contains a single tyrosine residue at position 80 (see Fig. 2). We observed two 2-proton intensity doublets at 7.71 and 7.07 ppm at neutral pH which shift upfield in concert with increasing pH (Fig. 3). This behavior identifies the peaks as arising from the single tyrosine (Tyr-80). By homology, we assign the two-proton doublet present near 7.7 ppm in spectra of all the other cytochromes $c_{553}$ we have studied (Fig. 4) to this same residue (Tyr-80). Keller and Wiithrich (13), using the truncated driven NOE technique, have assigned the peaks at 6.94 and 6.32 ppm in the spectrum of E. gracilis cytochrome $c_{552}$ to Tyr-80. According to the somewhat limited pH titration data shown in Fig. 5, all the peaks assigned to Tyr-80 in each respective cytochrome $c_{553}$ titrate with comparable pK', values (10.8 ± 0.5 pH units).

The cytochrome $c_{553}$ from A. flos-aquae has three tyrosine residues, Tyr-39, Tyr-45, and Tyr-80 (Fig. 2). J-modulated decoupling (Fig. 6 E, and F) and pH titration experiments (Fig. 7) indicate that the doublets of two-proton intensity at 7.66 and 6.96 ppm and those at 7.12 and 6.75 ppm at neutral pH are mutually coupled. These peaks are assigned to one of the two remaining tyrosine residues. A third set of coupled two-proton intensity doublets could not be found in the spectrum of A. flos-aquae cytochrome $c_{553}$. The peaks assigned to the second tyrosine residue do not shift at pH values below pH 11, indicating that this tyrosine has a pK', well above 11.

Tryptophan—The cytochrome $c_{553}$ from A. variabilis has only one tryptophan (residue 89). Double resonance and J-modulated decoupling experiments carried out with this protein (Fig. 8) indicate that the one-proton intensity triplets at 6.27 and 6.11 ppm and the one-proton intensity doublets at 7.20 and 7.01 ppm are mutually coupled. This coupling pattern is characteristic of the ring protons of a tryptophan residue; hence, we assign these peaks to Trp-89. A. flos-aquae cytochrome $c_{553}$ also has a single tryptophan residue at position 89. We assign the peaks at 6.38, 6.01, 7.24, and 7.09 ppm in the spectrum of A. flos-aquae ferrocytochrome $c_{553}$ (Fig. 6) to Trp-89 for the same reasons. Spectra of A. variabilis and A.
**TABLE I**

1\(^H\) NMR peak assignments for \(c\)-type ferrocytochromes from *A. flos-aquae*, *A. variabilis*, *M. aeruginosa*, *S. maxima*, *E. gracilis*, *P. aeruginosa*, and horse heart.

The residue numbering system is given in Fig. 2.

<table>
<thead>
<tr>
<th>Ferrocytochrome</th>
<th>Chemical shift ((\text{ppm}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Peak designation</td>
</tr>
<tr>
<td>Heme ring methyl protons</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

**Thioether**

Bridge Protons:

2 methine: 1.07
2 methine: 6.01
4 methine: 2.55
4 methyl: 2.49
4 methyl: 2.44
4 methyl: 2.59

**His 57**

| C6 | C6 | 9 | 6.99 |
| C6 | C6 | 2 | 7.73 |

**Tyr**

| C6 | C6 | 11 | 6.75 | 6.80 | 7.06 |
| C6 | C6 | 5 | 7.12 | 7.14 | 7.20 |

**Tyr 80**

| C6 | C6 | 10 | 6.96 | 6.98 | 7.07 | 6.32 | 6.82 |
| C6 | C6 | 3 | 7.66 | 7.68 | 7.71 | 6.94 | 7.20 |

**Phe 65**

| C6 | C6 | 6 | 7.14 | 8.08 | 8.22 | 7.26 |
| C6 | C6 | 1 | 8.24 | 8.08 | 8.22 | 7.26 |

**Trp 65**

| C6 or C52 | C52 | C52 | C52 | C52 |
| C6 or C52 | C52 | C52 | C52 | C52 | 4 | 7.24 | 7.20 |
| C6 or C52 | C52 | C52 | C52 | C52 | 12 | 6.38 | 6.27 | 6.35 | 6.38 | 6.68 | 7.24 |
| C6 or C52 | C52 | C52 | C52 | C52 | 13 | 6.01 | 6.11 | 5.48 | 5.81 | 5.74 | 7.17 |
| C6 or C52 | C52 | C52 | C52 | C52 | 8 | 7.09 | 7.01 | 6.87 | 7.08 | 7.07 | 7.65 |
| C6 or C52 | C52 | C52 | C52 | C52 | 7 | 7.12 | 7.20 | 7.77 | 7.20 |

**Net 44**

| C6 | C6 | 14 | 2.14 |
| C6 | C6 | 16 | -0.23 |
| C6 | C6 | 17 | -1.14 | -1.28 | -1.14 |
| C6 | C6 | 18 | -3.08 | -3.79 | -3.06 |
| C6 | C6 | 18 | -2.89 | -2.92 | -2.86 |
| C6 | C6 | 15 | 0.21 | 0.17 | 0.22 |
| C6 | C6 | 10 | 0.24 | 0.26 | 0.12 |
| C6 | C6 | 18 | 0.12 | 0.13 | 7.07 |

**References**

| Trp-89d | C6 or C52 | 4 | 7.24 | 7.20 | 7.54 | 7.36 | 7.60 | 7.50 |
| Trp-89d | C6 or C52 | 12 | 6.38 | 6.27 | 6.35 | 6.38 | 6.68 | 7.24 |
| Trp-89d | C6 or C52 | 13 | 6.01 | 6.11 | 5.48 | 5.81 | 5.74 | 7.17 |
| Trp-89d | C6 or C52 | 8 | 7.09 | 7.01 | 6.87 | 7.08 | 7.07 | 7.65 |
| Trp-89d | C6 or C52 | 7 | 7.12 | 7.20 | 7.77 | 7.20 |

**Net 82**

| C4 | C4 | 16 | -0.23 |
| C4 | C4 | 17 | -1.14 | -1.28 | -1.14 |
| C4 | C4 | 18 | -3.08 | -3.79 | -3.06 |
| C4 | C4 | 20 | -3.12 | -3.26 | -3.24 |
| C4 | C4 | 18 | -2.89 | -2.92 | -2.86 |

**Net 18**

| C6 | C6 | 15 | 0.21 | 0.17 | 0.22 |

**References**

*Proton peaks are designated as shown in Fig. 1.

*These residues are unique to the cytochrome from *A. flos-aquae*.

*This residue is unique to the cytochrome from *E. gracilis*.

*This residue is 89 in the amino acid sequences of cytochrome *c*$_2$; 59 in horse heart cytochrome *c*, and 56 in *P. aeruginosa* cytochrome *c*$_{553}$.

*flos-aquae* ferrocytochromes *c*$_{553}$ contain non titrating singlets near 7.12 ppm that are assigned tentatively to the C*\(_H\)* proton of Trp-89.

Keller and Wüthrich (11) have made first order and tentative second order *H NMR peak assignments for the two tryptophan residues in *E. gracilis* ferrocytochrome *c*$_{553}$ of *P. aeruginosa* cytochrome *c*$_{553}$.

Keller and Wüthrich (11) have made first order and tentative second order *H NMR peak assignments for the two tryptophan residues in *E. gracilis* ferrocytochrome *c*$_{553}$. These peaks may be assigned to the specific residues Trp-65 and Trp-89, as shown in Table I, on the basis of the current results.
H NMR of Ferrocytochrome c\textsubscript{553}

Fig. 2. Amino acid sequences of cytochromes c\textsubscript{553} from M. aeruginosa,\textsuperscript{3} A. flos-aquae,\textsuperscript{4} S. maxima (28), A. variabilis (29) and E. gracilis (30). The sequences are aligned as suggested by Dickerson et al. (8). Amino acids in parentheses have not been given a definite order. Dashes indicate gaps in the alignment or, at the ends, missing residues.

Fig. 3. Assignment of peaks to Tyr-80. 360 MHz \textsuperscript{1}H NMR spectra of reduced M. aeruginosa cytochrome c\textsubscript{553} at two pH\textsuperscript{*} values are shown. The peaks assigned to Tyr-80 shift with pH\textsuperscript{*}. The protein concentration was 1 mM in 50 mM deuterated phosphate in \textsuperscript{2}H\textsubscript{2}O, 25 °C. The spectra are the sum of 1024 accumulations at pH\textsuperscript{*} = 10.6 and 512 at pH\textsuperscript{*} = 11.0.

Trp-65 in E. gracilis cytochrome c\textsubscript{553} appears to be located near the heme ring, since one proton peak (the C\textsuperscript{5} or C\textsuperscript{5}') is shifted downfield abnormally to 8.36 ppm.

Phenylalanine—All five cytochromes c\textsubscript{553} studied by \textsuperscript{1}H NMR have an aromatic residue at position 65. In E. gracilis cytochrome c\textsubscript{553}, residue 65 is a tryptophan, but in the four sequenced cytochromes c\textsubscript{553} we have studied, residue 65 is a phenylalanine. The doublet peak at 8.36 ppm in the spectrum of E. gracilis ferrocytochrome c\textsubscript{553} (11) was assigned above to Trp-65. Although the other cytochromes do not have a doublet in this region of their spectra, they do have another peak in the same general region (8.24 to 8.08 ppm) which is not present in spectra of E. gracilis ferrocytochrome c\textsubscript{553}. J-modulated decoupling experiments with A. flos-aquae ferrocytochrome c\textsubscript{553} (see Fig. 6, B and C) indicate that the aromatic protein resonance at 8.24 ppm is not a doublet and is coupled to a single peak, a triplet at 7.14 ppm. These characteristics identify this resonance as arising from either a tryptophan C\textsuperscript{5} or C\textsuperscript{5} proton or a phenylalanine C\textsuperscript{5} or C\textsuperscript{5} proton. Tryptophan can be ruled out since a similar peak is present in spectra of A. variabilis ferrocytochrome c\textsubscript{553} (at 8.08 ppm); A. variabilis has a single tryptophan residue at position 89 all of whose aromatic proton resonances were assigned above. Hence, the peak at 8.24 ppm in spectra of A. flos-aquae and at 8.08 ppm in A. variabilis cytochrome c\textsubscript{553} corresponds to a phenylalanine that is not present in E. gracilis cytochrome c\textsubscript{553}. Only Phe-65 fits this requirement.

Histidine—A. flos-aquae cytochrome c\textsubscript{553} is the only protein studied that contains two histidine residues (Fig. 2), one at position 18 and one at position 57. We observed two singlets in the aromatic region of the A. flos-aquae cytochrome spectrum that shift with pH (Fig. 7), yielding a pK\textsubscript{a} of 6.3 (Fig. 9).
Fig. 5. 'H NMR titration curves for the low field doublet of the conserved Tyr-80 residue in ferrocytochromes c553. A, Oscillatoria; O, A. variabilis; □, A. flos-aquae; ©, M. aeruginosa.

Fig. 6. Assignments of spin-coupled tyrosine resonances in the aromatic region of spectra of A. flos-aquae cytochrome c553. A, aromatic region of a 'H NMR spectrum at 470 MHz of reduced A. flos-aquae cytochrome c553. Cytochrome concentration was 4 mM in 40 mM deuterated phosphate buffer in 'H2O, pH 6.94, 28°C. The spectrum is the sum of 512 accumulations. B, J-modulated spin echo spectrum, T = 60 ms, 1024 accumulations. C-G, same as B except that a saturating frequency was applied at the point indicated by the X.

These peaks which are not present in spectra of the other cytochromes c553 are assigned to the C' and C protons of the nonconserved His-57.

His-18 is conserved in all cytochromes c553 in a Cys-X-Cys-His peptide, which, from sequence homology with c-type cytochromes, is expected to contain the histidine ligated to the heme iron. We assign the singlet resonance of one-proton intensity located at 0.21 ppm (Fig. 1) in the 'H NMR spectrum of A. flos-aquae ferrocytochrome c553 to His-18. This peak could only arise from a histidine C' or C proton or from a very unusual glycine C' proton. A similar proton resonance is observed in spectra of other c-type cytochromes and has been assigned to the histidine ligated to the iron (10, 21, 24). The extreme high field chemical shift of this peak is attributed to the aromatic ring current of the heme.

Methionine—A 695-nm absorbance has been observed in the visible spectrum of ferricytochromes c553 (31, 32) which is characteristic of a methionine sulfur-iron bond (9). Conserved Met-62, therefore, is expected to be ligated to the heme iron. We assign the high field shifted, three-proton intensity peak at -2.89 ppm in Fig. 1 to the methionine C' protons of Met-62. Like the His-18 ring proton, the Met-62 proton resonances are shifted to high field by the σ-electron ring current of the heme. The coupled one-proton intensity resonances at -3.12, -3.08, -1.14, and -0.23 ppm are assigned to the C' and C protons of Met-62.

Spectra of A. flos-aquae ferrocytochrome c553 contain a second resonance of about three-proton intensity at 2.14 ppm. We assign this peak to the C' protons of the nonconserved Met-44 residue. NOE data presented in the following sections indicate that this resonance does not correspond to a heme ring methyl group.

Heme Ring Proton Assignments—The inset in Fig 1 is a sketch of the heme structure found in c-type cytochromes. Distributed around the aromatic porphyrin ring are four mesoprotions (α, β, γ, and δ), four ring methyl groups (positions 1, 3, 5, and 8), two methine protons (positions 2 and 4), two methine methyl groups (positions 2 and 4), and eight propionic acid side chains protons. The rigid structure of the heme ring facilitates dipole coupling between adjacent protons on the ring, and selective irradiation of a heme ring proton resonance is expected to produce an NOE in the resonance arising from an adjacent proton. Because single protons and methyl groups are asymmetrically distributed about the heme ring, NOE experiments can be used to assign many of the heme proton resonances (12, 24, 26). The propionate proton resonances have proved impossible to locate by this technique, because of the complicated spin-spin coupling pattern of these reso-

Fig. 7. The pH-dependent shifts of the histidine peaks (H, His-57) and the tyrosine peaks (Y, Tyr-80) in 'H NMR spectra (360 MHz) of reduced A. flos-aquae cytochrome c553 at several pH* values, 20°C. Protein concentration was 4 mM in 50 mM phosphate buffer in 'H2O. The spectra are the sum of 256 accumulations.
**H NMR of Ferrocytochrome c553**

Assignments of spin-coupled tryptophan resonances in the aromatic region of **H NMR spectra (360 MHz) of A. variabilis cytochrome c553.** The sample contained 3 mM protein in 60 mM deuterated phosphate buffer in H2O, pH 7.5, 20 °C. A, normal single pulse spectrum. The arrow marks the position of a formate impurity. B, J-modulated spin echo spectra, τ = 60 ms. C and D, J-modulated spin echo spectra, τ = 60 ms, in which the peak labeled by X was saturated during the delay between acquisitions.

Fig. 10 contains steady state NOE difference spectra in which the four heme mesoproton resonances and the two free standing heme ring methyl resonances (peaks h and j) were irradiated in turn. The assignments may be made stepwise as follows.

1) When the mesoproton resonance at 9.30 ppm (peak d) is irradiated (Fig. 10 E), two ring methyl resonances at 3.57 and 3.38 ppm (peaks h and j) exhibit a negative NOE. Of the four mesoprotions, only the δ is adjacent to two ring methyl groups. Thus, the resonance at 9.30 ppm is assigned to the δ mesoproton and the peaks h and j to the heme methyls 1 and 8.

2) The mesoproton at 9.71 ppm (peak b) shows the least dipole coupling to the ring methyl resonances (Fig. 10C) and is assigned to the γ mesoproton which has no adjacent methyls.

3) According to the ring geometry, irradiating the ring 3-methyl peak will create an NOE in the α-meso, 4-methylene, and 4-methyl proton peaks. Irradiating the β-mesoproton peak will also create an NOE in the 4-methylene and 4-methyl proton peaks, but will not affect the 3-methyl or α-mesoproton.

**Fig. 9.** Titration curve for the His-57 C' proton peak of reduced A. flos-aquae cytochrome c553. Sample conditions were as in Fig. 7.

**Fig. 10.** Assignment of heme resonances of A. flos-aquae ferrocytochrome c553 by steady state **H NMR (360 MHz) NOE difference spectroscopy.** A is a normal spectrum of the cytochrome. B-F are difference spectra obtained by subtracting a spectrum in which the irradiating frequency was set on the peak indicated by the arrow from a spectrum in which the irradiating frequency was set off resonance. Each spectrum is the sum of 1,024 scans with 16,384 time domain registers and a delay of 3 s between pulses. A normal 90° pulse sequence was used with the irradiating frequency off only during the acquisition. Sample conditions: 4 mM protein, 50 mM deuterated phosphate buffer in H2O, pH 6.5, 25 °C.
peaks. In line with this argument, the 4.00-ppm ring methyl (peak g) and 9.36-ppm meso (peak c) resonances, which are dipole coupled to the same methine proton (peak e) and methine methyl group (peak k) but are not dipole coupled to each other, are assigned to the 3-methyl group and β-mesoproton (Fig. 10, D and F).

4) By difference, the 3.54 (peak i) and 9.98 ppm (peak a) resonances are assigned, respectively, to the heme ring 5-methyl group and the α-mesoproton.

5) In Fig. 10G, when the methyl resonance at 3.38 ppm (peak j) assigned above to either the heme 1-methyl or 8-methyl is irradiated, an NOE is not observed in either the α-mesoproton resonance (peak a) or 2-methine proton resonance (peak f, assigned below), but a strong NOE is observed in the β-mesoproton resonance (peak d), and a small NOE is observed in the γ-mesoproton resonance (peak b). Therefore, the 3.38-ppm heme ring methyl resonance (peak j) is assigned to the 8-methyl group which is nearer the γ-mesoproton than the 1-methyl group.

6) By difference, the 3.57-ppm methyl resonance (peak h) is assigned to the 1-methyl group.

7) Irradiation of the α-mesoproton resonance at 9.98 ppm (peak a) (Fig. 10D) produces an NOE in resonances at 5.38 ppm (peak e), 4.00 ppm (peak g), and 1.07 ppm (peak b). Double resonance experiments (data not shown) indicate that the 1.07-ppm resonance is coupled to the resonance at 5.38 ppm. Based on these results, the 5.38-ppm and 1.07-ppm resonances are assigned, respectively, to the methine proton and methine methyl group at position 2 on the heme.

8) Irradiating either the heme 3-methyl resonance at 4.00 ppm (peak g) (Fig. 10F) or the β-mesoproton resonance at 9.36 ppm (peak c) (Fig. 10D) produces an NOE in both the peaks at 6.01 (peak e) and 2.55 ppm (peak k). Double resonance and J-modulated decoupling experiments (data not shown) indicate that a doublet at 2.55 ppm is coupled to a peak at 6.01 ppm. A single proton coupled to a doublet at higher field is the expected coupling pattern for a methine proton and methine methyl group (or possibly for low-field shifted β- and γ-proton peaks of a threonine residue). The dipolar coupling pattern is consistent with assignment of these peaks to the remaining methine protons. Hence, we assign the peak at 2.55 ppm to the methine 4-methyl and the peak at 6.01 ppm to the 4-methine proton. In the spectrum of A. flos-aquae in Fig. 1, the resonance at 6.01 ppm has an intensity of two protons. One of these protons was assigned above to the ring of Trp-89.

In spectra of A. flos-aquae cytochrome c₅₅₃ at high pH and in spectra of A. variabilis cytochrome c₅₃₃, the Trp-89 ring proton resonance and methine proton resonance do not overlap. NOE experiments carried out with these proteins have shown that the heme mesoproton is dipole coupled to the methine proton and not to the Trp-89 ring proton (data not shown).

A different logical progression could be used to arrive at the heme resonance assignments. What is important is that all of the data in Fig. 10 are consistent with the completed assignments.

Amino Acids in the Heme Environment—Proton peaks assigned to the ring protons of Phe-65 (peaks 1 and 6) and His-18 (peak 15) exhibit an NOE when the α-mesoproton and heme 3-methyl resonances are irradiated (Fig. 10, B and F). Both the Phe-65 and His-18 rings appear to be located near the top edge of the heme. A heme-ring-current effect would explain the abnormally low field shift of the Phe-65 ring proton at 8.24 ppm (see Fig. 1).

In the x-ray crystal structures determined for mitochondrial cytochromes c (34), cytochrome c₅₃₃ (35), and cytochrome c₁ (36), the histidine ring bound to the iron is perpendicular to the plane of the heme and oriented in a plane defined by the α-mesoproton, Fe atom and δ-mesoproton. The Cα proton of the histidine ring, which usually has the higher chemical shift of the two histidine ring protons (25, 27), is located very near the α-mesoproton of the heme ring. The normal chemical shift of the axial histidine proton peak in spectra of A. flos-aquae cytochrome c₅₃₃ (0.21 ppm) and the localization of the histidine near the top edge of the heme ring by NOE experiments indicate that His-18 in cytochrome c₅₃₃ is oriented in the same way as the axial histidine in many c-type cytochromes.

When the heme ring 8-methyl resonance is irradiated (peak j; Fig. 10G), an NOE is observed in the ring proton peaks of a tyrosine, either Tyr-39 or Tyr-45 (peaks 5 and 11), and the methyl proton peak of Met-62 (peak 14). From these data, it appears that Met-44 and a tyrosine residue are located near pyrrole ring IV. The general structural locations discussed above for Phe-65, His-18, and Met-44 in the cytochrome c₅₃₃ structure have been confirmed by x-ray crystallographic studies of A. nidulans cytochrome c₅₅₃ (14).

Fig. 11 contains 'H NMR difference spectra derived from a truncated driven NOE experiment in which the proton peak assigned to the iron ligand Met-62 methyl protons was pre-irradiated for the length of time specified. The shortest pre-irradiation period produced a small but significant NOE in the Phe-65 ring proton peaks. As the pre-irradiation time was lengthened, the NOE in the Phe-65 proton peaks increased, and an NOE could be seen in the heme α- and δ-mesoprotos but not in the heme γ-mesoproton. Preliminary 'H NOE data (not shown) and the similarities between the structures of A. nidulans cytochrome c₅₃₃ and other c-type cytochromes (14) place the Met-62 cα and cγ protons near the β-mesoproton of the heme. These data indicate that the chirality of the Met-62 sulfur bound to the iron is R. If the Met-62 sulfur were S, the Met-62 methyl would be near the heme γ-mesoproton and

\[ \text{NOE differences in the Phe-65 ring proton peaks.} \]
an NOE would be expected in that resonance. An NOE at the position of the \( \gamma \)-mesoproton was not observed.

**DISCUSSION**

**Assignment Techniques**—We have presented \(^{1}H\) NMR peak assignments with four ferrocytochromes \( c_{553} \) (Table I). The first order assignments (to amino acids) were based on chemical shifts, peak intensities, pH titrations, and double resonance and J-modulated decoupling data. The second order assignments (to specific residue positions) were made by comparing \(^{1}H\) NMR data from several cytochromes \( c_{553} \) with the amino acid sequences of these proteins. With the cytochrome \( c_{553} \) from *A. flos-aquae*, we have made second order assignments of 18 amino acid proton resonances and specific assignments of 12 heme proton resonances. Table I contains a comparison of the present \(^{1}H\) NMR peak assignments for cytochromes \( c_{553} \) with analogous assignments for the homologous cytochrome \( c_{553} \) from *E. gracilis* (10–13), cytochrome \( c \) from horse heart (25, 26), and cytochrome \( c_{551} \) from *P. aeruginosa* (22–24).

The heme ring assignments for *A. flos-aquae* ferrocytochrome \( c_{553} \) were obtained from an analysis of steady state NOE experiments in which heme ring proton resonances were selectively irradiated. Adiabatic transfer of the absorbed energy from proton to proton in a steady state NOE experiment, so called “spin diffusion,” can cause a great deal of confusion when assigning proton resonances to adjacent protons in a protein structure. Cytochromes \( c_{553} \) are small proteins and are not expected to exhibit efficient spin diffusion. Fig. 10 shows this to be true; only a limited number of protons in a single experiment experience an NOE. Chemical shifts for the heme methyl and mesoproton peaks are similar for all three cytochromes, \( c_{553}, c_{551} \), and \( c \). For cytochromes \( c_{553} \) and \( c_{551} \), the \( \alpha \)-meso and 8-methyl proton peaks are shifted to low field compared to those for cytochrome \( c \). The low-field shift of the \( \alpha \)-mesoproton is probably caused by the ring currents of Phe-65 in cytochromes \( c_{553} \) and \( c_{551} \) and Trp-27 in *P. aeruginosa* cytochrome \( c_{551} \). The smaller C-type cytochromes, compared to cytochrome \( c \), have fewer aromatic amino acids near the heme pyrrole ring IV. This structural difference may explain the lack of a high field shift for the heme 8-methyl resonance in \(^{1}H\) NMR spectra of cytochromes \( c_{553} \) and \( c_{551} \) as compared with cytochrome \( c \). The proton peaks assigned to the heme \( \gamma \)-mesoproton in spectra of *S. maxima* and *Oscillatoria* are high field shifted compared to those of other cytochromes \( c_{553} \). With the *Oscillatoria* cytochrome \( c_{553} \), a pH titration study indicated that the \( \gamma \)-mesoproton shifted downfield with increasing pH with a \( pK_{\text{mud}} \) of 10.0. At high pH (>10.7), the chemical shift of the \( \gamma \)-mesoproton was equal to that found for other cytochromes \( c_{553} \).

**Heme Environment**—The amino acid residues at positions 10, 18, 65, 80, and 89 are highly conserved in cytochromes \( c_{553} \) (see Fig. 2). We have assigned peaks to four of these residues. The chemical shifts found for the ring proton peaks of Trp-89, Tyr-80, Phe-65, and His-18 are similar in spectra of all the cytochromes \( c_{553} \) studied. His-18 is the fifth iron ligand, and its ring proton resonances are high field shifted by the electron ring current of the heme. Phe-10 and Tyr-80 in cytochromes \( c_{553} \) are homologous with the two aromatic residues that form the “right channel” in mitochondrial cytochromes \( c \). X-ray crystallography and amino acid sequence homologies indicate that these residues are conserved in almost all c-type cytochromes, yet their function in the c-type cytochrome structure has not been determined. Keller and Wuthrich (13) have assigned resonances to Phe-10 and Tyr-80 in the aromatic region of \(^{1}H\) NMR spectra of *E. gracilis* cytochrome \( c_{553} \). The Tyr-80 resonances were found to be high field shifted ~0.7 ppm compared to the Tyr-80 resonances we have assigned in spectra of cytochromes \( c_{553} \). Tyr-80 in *E. gracilis* cytochrome \( c_{553} \) was found to be restricted in its rotation about the \( C'_3-C'_1 \) bond, but this is not the case in the cytochromes \( c_{553} \) that we have studied. These differences between the *E. gracilis* cytochrome \( c_{553} \) and cytochromes \( c_{553} \) may be the result of an additional tyrosine at position 82 in the cytochrome \( c_{553} \). The aromatic residue at position 65 in cytochromes \( c_{553} \) is homologous to a similar residue at position 82 in mitochondrial cytochromes \( c \). From the low field shifts and the dipole coupling between the heme \( \alpha \)-meso and 3-methyl protons found for Phe-65 and Trp-65 in cytochromes \( c_{553} \) and \( c_{551} \), we believe that residue 65 lies near the top of the heme, somewhat like Phe-82 in *E. gracilis* cytochrome \( c \) (34). The Met-44 methyl protons and the ring protons of a tyrosine residue are near the heme 8-methyl group. Whereas the pattern of two doublets indicates that the ring of either Tyr-39 or Tyr-42 flips rapidly about the \( C'_3-C'_1 \) bond, the high \( pK_{\text{mud}} \) for this residue (>11) indicates that it is in a structured area. It may be hydrogen bonded or buried in the protein.

The Trp-89, \( \alpha \), and \( \beta \) proton resonances are shifted to high field by about 1 ppm, and these chemical shifts are conserved in spectra of several cytochromes \( c_{553} \). These shifts indicate that Trp-89 is located in a structured environment in which the \( \alpha \) and \( \beta \) protons are probably near another aromatic ring. It is curious that the chemical shifts of the Trp-89 ring protons of cytochromes \( c_{553} \) resemble those of Trp-56 in spectra of *P. aeruginosa* cytochrome \( c_{553} \) and Trp-59 in spectra of horse heart cytochrome \( c \). In these cytochromes, Trp-56 and Trp-59 are hydrogen bonded to the heme pyrrole ring IV propionate. Nearly every c-type cytochrome contains a conserved tryptophan residue hydrogen bonded in this way. The exceptions have usually been found to be unstable. Apparently Trp-89 does not play such a role since Ludwig *et al.* (14) have shown by x-ray crystallography that Trp-89 in *A. nidulans* cytochrome \( c_{553} \) is not near either heme propionate. Trp-89 is conserved in many cytochromes \( c_{553} \) but is missing from the cytochrome from *Monochrysis lutheri*. Further work is needed to define the hydrogen bonding network for the cytochrome \( c_{553} \) heme propionate groups. Comparison of the structure of cytochrome \( c_{553} \) with those of other c-type cytochromes may help elucidate a structure-function relationship for the cytochrome c tryptophan-propionate hydrogen bond.

**Axial Methionine**—The Met-62 \( \beta \) and His-18 \( \gamma \) proton resonances have high field chemical shifts similar to those of the methionine and histidine residues known to be ligands to the iron in other c-type cytochromes. These data confirm the results of Keller and co-workers (10, 12) obtained with *E. gracilis* cytochrome \( c_{553} \). Moore and Williams (37) have suggested that the length of the iron-sulfur bond may be a significant factor in determining the reduction potential of c-type cytochromes. They proposed that the chemical shift of the methionine ligand methyl resonance is related to the length of the iron-sulfur bond, with a high field shift indicating a short iron-sulfur bond. The ferrocytochrome would be stabilized over the ferrocytochrome by a short iron-sulfur bond, and this would lead to a lower reduction potential. The reduction potentials for *P. aeruginosa* cytochrome \( c_{553} \) (+286 mV) and horse heart cytochrome \( c \) (+255 mV) differ by ~30 mV (38, 39), while the chemical shift of their methionine iron ligand methyl peaks differ by ~0.38 ppm (Table I). Cytochromes \( c_{553} \) have reduction potentials of approximately +350 mV (9, 40, 41) or about 70 mV higher than that of *P. aeruginosa* cytochrome \( c_{553} \), yet the chemical shifts of the methionine iron ligand methyl resonances for the two varieties of c-type cytochromes are nearly identical (Table I). Either the chemical shift of the methionine iron ligand is not directly related to...
the length of the iron-sulfur bond or other factors contribute to the reduction potentials of c-type cytochromes (42).

Dezlocalization of Electron Spin Density in Ferricytochromes c—We have shown, using truncated driven NOE experiments, that the Met-62 sulfur in A. flos-aquae ferricytochrome c(C63) has an R configuration. This is the same configuration found for the Met-80 sulfur of tuna cytochrome c (34), horse heart cytochrome c (43), and the Met-62 sulfur of E. gracilis cytochrome c(C63) (12), whereas the P. ceruginosa cytochrome c(C61) Met-61 sulfur has an S configuration (35, 43). With horse heart ferricytochrome c, Redfield and Gupta (44) found that the unpaired electron spin density is localized more on pyrrole rings II and IV than on pyrrole rings I and III of the heme. Keller and Wüthrich (24) found with P. aeruginosa ferricytochrome c that the unpaired electron spin density is localized more on pyrrole rings I and III. Sen et al. (43) have proposed that the chirality of the sulfur-iron ligand in c-type ferricytochromes determines the distribution of unpaired electron spin density on the heme. According to this hypothesis, the unpaired electron spin density in A. flos-aquae cytochrome c(C63) is localized more on heme pyrrole rings II and IV than on pyrrole rings I and III. Keller et al. (12) have found this to be true for the homologous cytochrome c(C63) from E. gracilis. If the unpaired electron spin density is localized on heme pyrrole rings II and IV, electron exchange reactions between cytochromes c(C63) and other proteins or inorganic redox reagents may occur near the solvent-exposed pyrrole ring II of cytochrome c(C63).

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