Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randell, R. J. (1951) *J. Biol. Chem. 193*, 265.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149.
McCammon, J. A., & Karplus, M. (1983) *Acc. Chem. Res. 16*, 187.
Moonen, C. T. W., Scheek, R. M., Boelens, R., & Müller, F. (1984) *Eur. J. Biochem. 141*, 323.
Pardi, A., Wagner, G., & Wüthrich, K. (1983) *Eur. J. Biochem. 137*, 445.
Pearson, G. A. (1977) *J. Magn. Reson. 27*, 265.
Reisfeld, R. A., Lewis, U. J., & Williams, D. E. (1962) *Nature (London) 195*, 281.
Rousselet, A., Faure, G., Boulain, J.-C., & Ménez, A. (1984) *Eur. J. Biochem. 140*, 31.
Schmidt, P. G., & Kuntz, I. D. (1984) *Biochemistry 23*, 4261.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.
Stassinopoulou, C. I., Wagner, G., & Wüthrich, K. (1984) *Eur. J. Biochem. 145*, 423.
Vincent, J.-P., & Lazdunski, M. (1973) *Eur. J. Biochem. 38*, 365.
Wagner, G., & Wüthrich, K. (1982a) *J. Mol. Biol. 155*, 347.
Wagner, G., & Wüthrich, K. (1982b) *J. Mol. Biol. 160*, 343.
Weiss, M. A., Eliason, J. L., & States, D. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6019.
Wien, R. W., Morrisett, J. D., & McConnell, H. M. (1972) *Biochemistry 11*, 3707.
Williamson, M. P., Marion, D., & Wüthrich, K. (1984) *J. Mol. Biol. 173*, 341.
Williamson, M. P., Havel, T. F., & Wüthrich, K. (1985) *J. Mol. Biol. 182*, 295.
Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311.
Yamada, S., & Itano, H. A. (1966) *Biochim. Biophys. Acta 130*, 538.

Proton NMR Studies of Plastocyanin: Assignment of Aromatic and Methyl Group Resonances from Two-Dimensional Spectra†

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Received November 14, 1985

ABSTRACT: Two-dimensional NMR methods have been used to assign aromatic and methyl group resonances in the ^{1}H NMR spectra of French bean and poplar plastocyanins. Specific assignments are presented for all 11 of the aromatic spin systems and 32 methyl group resonances of French bean plastocyanin. A further nine methyl group resonances are attributed to individual spin systems but have not yet been specifically assigned. For poplar plastocyanin, 10 aromatic spin systems and 20 methyl group resonances are specifically assigned. Assignments to specific amino acids are based on the X-ray structure of poplar plastocyanin. The assignment problem has been simplified by using the two-dimensional spectra of oxidized plastocyanin as subspectra of protons distant from the copper center. Two-dimensional relayed coherence transfer spectra were particularly useful for making unambiguous assignments of Ala, Thr, and aromatic spin systems. A discrepancy between the NMR spectra and amino acid sequence of poplar Pc at residue 39 is observed.

Plastocyanin (Pc)[1] is a blue copper protein (M_r 10600) that functions as an essential component of the photosynthetic electron transport chain (Boulter et al., 1977). Many types of physicochemical studies have been performed on Pc, placing it among the best characterized of the electron transfer proteins. High-resolution crystal structures have been determined for both oxidation states of poplar plastocyanin under a variety of crystallization conditions (Colman et al., 1978; Freeman, 1981; Guss & Freeman, 1983). A structure of the apoprotein, which differs very little from that of the holoprotein, has also been reported (Garrett et al., 1984). This wealth of crystallographic information on Pc has not been matched by detailed data on its solution conformation and dynamics, which are potentially obtainable from ^{1}H NMR experiments. High-resolution ^{1}H NMR has been previously applied to Pc for an investigation of the electron self-exchange reaction (Beattie et al., 1975), identification of resonances from groups near the copper center (Markley et al., 1975; Ulrich & Markley, 1978; Hill & Smith, 1978; Kojiro & Markley, 1983), an examination of structural conservation between species by overall spectral comparison (Freeman et al., 1978a,b), and location of protein binding sites for inorganic electron transfer reagents (Cookson et al., 1980a,b; Handford et al., 1980). These earlier studies have produced only a few specific resonance assignments; most were carried out in the absence of a crystal structure and before recent improvements in NMR methodology became commonly available. Remarkably, as

† This work was supported by the Australian Research Grants Scheme (Grant D2 82/15284). G.C.K. acknowledges the receipt of a Commonwealth Postgraduate Research Award.
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[1] Abbreviations: Pc, plastocyanin; 2D, two-dimensional; 2D*J*, two-dimensional *J*-resolved spectroscopy; COSY, two-dimensional scalar correlated spectroscopy; RCT, two-dimensional relayed coherence transfer spectroscopy; NOESY, two-dimensional dipolar correlated spectroscopy; NOE, nuclear Overhauser effect; FID, free induction decay; pt, point.

0006-2960/86/0425-2364$01.50/0

yet there are no published assignments for the ^1H NMR spectrum of poplar Pc.

This paper is addressed to the determination of a substantial body of systematic assignment data for the plastocyanins from French bean and poplar. Two-dimensional (2D) NMR techniques are used as the primary source of spectral information. To date, spectra of several small proteins have been almost completely assigned by using 2D NMR methods and the sequential assignment procedures of Wüthrich and coworkers (Billeter et al., 1982; Wagner & Wüthrich, 1982). The present work takes a more traditional approach, using the structural information provided by X-ray crystallography. A similar approach has been used to assign resonances for the related blue copper protein azurin (Canters et al., 1984). The majority of the plastocyanin assignments reported here are for the French bean protein, which has been the subject of more detailed studies and yields better quality NMR spectra in our hands than does poplar Pc. The use of structural data for poplar Pc to assign resonances for French bean Pc relies on the assumption that the conformations of these species are very similar. There is considerable evidence in support of this assumption. Amino acid sequences are highly conserved among plastocyanins from a wide variety of higher plant families (Boulter et al., 1977; Ramshaw, 1982), which suggests that the structure is also highly conserved. The sequences of poplar Pc [given in Guss and Freeman (1983)] and French bean Pc (Milne et al., 1974) differ in 21 out of 99 residues. However, only three of these substitutions involve buried residues (Chothia & Lesk, 1982). The majority of substitutions involve surface side chains and are therefore expected to have little effect on the protein folding. Further, each of the three substitutions of buried residues (Ala-7, Ile-21, and Ile-39 in poplar to Ser-7, Val-21, and Val-39 in French bean) can be considered to be conservative. The peptide backbone of poplar Pc is highly hydrogen bonded (Colman et al., 1978; Guss & Freeman, 1983; Garrett et al., 1984) and would be expected to show little overall change in structure on conservative substitution of such a small number of internal side chains. Indeed, the similarity of the ^1H NMR spectra of several Pc's from higher plant species (Freeman et al., 1978a,b) provides evidence for conservation of overall structure.

The assignments described in this paper are being used in studies of the solution structure and properties of Pc, including its interactions with other electron transfer proteins and inorganic electron transfer reagents.

Materials and Methods

NMR Sample Preparation and Instrumentation. Plastocyanin was isolated from the leaves of French bean (*Phaseolus vulgaris*) by a modification of the method of Ramshaw et al. (1973). Protein with an A_{278}/A_{597} ratio of 1.1–1.2 was used at all times. Poplar (*Populus nigra*) Pc was kindly provided by Drs. M. Murata and H. C. Freeman and was used without further purification. NMR samples of 3–10 mM final concentration were prepared by Amicon ultrafiltration with deuterated phosphate buffer (100 mM, pH 7.5) or by solvent exchange on a 1 × 10 cm Sephadex G-15 column. Copper(I) Pc was prepared by addition of a minimum quantity of solid sodium dithionite to the NMR tube, which was then sealed with a septum cap. When necessary, amide protons were exchanged for deuterons by heating samples in deuterated phosphate buffer at 338 K for 30 min. All spectra of French bean Pc were recorded at 313 K and those of poplar Pc at 308 K.

Lyophilization of French bean Pc was found to cause the appearance of three extra cross-peaks in the aromatic region of the COSY spectrum, caused by coupling of resonances at 7.23 and 6.93, 7.14 and 6.85, and 7.43 and 7.35 ppm. These cross-peaks can appear at high intensity [see Figure 1 of King and Wright (1982)] despite the relatively low intensities of the contributing peaks in the 1D spectrum. Extra peaks could not be detected in other spectral regions. The specific appearance of a few new peaks after lyophilization suggests the occurrence of localized unfolding in a small fraction (<5%) of the protein. A single lyophilization was found to be sufficient to cause these spectral changes.

NMR spectra were acquired with a Bruker WM-400 spectrometer equipped with an Aspect 2000 computer. All 2D spectra were processed with Bruker software and are presented in absolute value mode. Dioxane was used as an internal standard, but all chemical shifts are referred to (trimethylsilyl)propanesulfonic acid (TSS). Quoted pH values are uncorrected meter readings.

J-Resolved Spectra. 2D *J*-resolved (2D*J*) spectra were acquired with the standard $(T_w\text{–}90°\text{–}t_1/2\text{–}180°\text{–}t_1/2\text{–}t_2\text{–})_n$ pulse sequence (Aue et al., 1976b; Nagayama et al., 1978) except that the 180° pulse was replaced by a composite pulse (Levitt & Freeman, 1981) to obtain maximum compensation of pulse length errors. Phase cycling based on the "exorcycle" routine (Bodenhausen et al., 1977) was used for maximum suppression of artifacts in quadrature detection mode. Several spectra were recorded with between 64 and 256 values of t_1 and with FIDs of 8K–16K points. A total of 512 scans were collected for each value of t_1. Resolution enhancement was performed with phase-shifted sine-bell window functions. Final spectra were 128–256 (F_1) × 8K – 16K (F_2) points with 0.6 Hz/pt resolution or better on each axis. Spectra were tilted to give the familiar (J, δ) presentation (Nagayama et al., 1978).

2D Scalar Correlated Spectra. Scalar correlated (COSY) spectra were acquired with the $(T_w\text{–}90°\text{–}t_1\text{–}90°\text{–}t_2\text{–})_n$ pulse sequence (Aue et al., 1976a; Bax & Freeman, 1981; Wagner et al., 1981). Phase cycling for quadrature detection in both F_1 and F_2 was used (Bax et al., 1981). Both P- and N-type spectra were recorded. In some cases, P-type spectra were given the appearance of the more commonly used N-type spectra by F_1 reversal with the Pascal program REVERSE, written by Dr. W. Barton for the Aspect 2000. In a typical experiment on a 10 mM protein sample, data were collected for 512 values of t_1. A total of 64 scans were collected for each value of t_1, and 2048 points were collected in t_2. When T_w was 1.0 s, total experimental time was 13 h. Sine-bell window functions were used in both dimensions for maximum resolution enhancement. Final spectra were 1024 × 1024 points, with a digital resolution of 4.9 Hz/pt on each axis.

2D Dipolar Correlated Spectra. Dipolar correlated (NOESY) spectra were acquired with the $(T_w\text{–}90°\text{–}t_1\text{–}90°\text{–}\tau_m\text{–}90°\text{–}t_2\text{–})_n$ pulse sequence (Jeener et al., 1979; Kumar et al., 1980), where τ_m is the mixing time over which the NOE is built up. J-coupling cross-peaks were eliminated by phase cycling and random variation of τ_m (Macura et al., 1981). Acquisition and processing parameters were identical with those given for COSY with the exception that some data were collected over 256 values of t_1 and T_w was always 1.0 s. NOESY spectra were usually symmetrized (Baumann et al., 1981).

2D Relayed Coherence Transfer Spectra. 2D relayed coherence transfer (RCT) spectra were acquired with the $(T_w\text{–}90°\text{–}t_1\text{–}90°\text{–}\tau/2\text{–}180°\text{–}\tau/2\text{–}90°\text{–}t_2\text{–})_n$ pulse sequence (Eich et al., 1982).

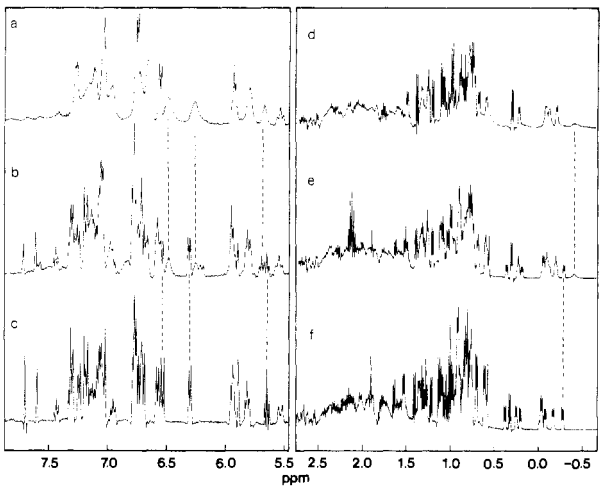

FIGURE 1: Low-field (a–c) and high-field (d–f) regions of ^{1}H NMR spectra of French bean Pc at 313 K (100 mM phosphate, pH 7.5): (a, d) oxidized protein; (b, e) equimolar mixture of oxidized and reduced proteins; (c, f) reduced protein. Resonances with significantly different chemical shifts (>0.03 ppm) in each redox state are indicated.

The mixing time τ was set in the range 60–80 ms. NOE cross-peaks (King & Wright, 1983) were eliminated with a phase cycling scheme suggested by Dr. Geoffrey Bodenhausen:

90°	90°	180°	90°	acquisition
X	X	X	X	+
X	X	Y	X	-

Addition of phase cycling for N-type peak selection and quadrature detection gave a minimum 32-step phase cycle. Acquisition and processing parameters were identical with those given for COSY experiments except that some spectra were collected over 256 values of t_1.

Structural Calculations. Coordinates from the refined crystal structures of oxidized poplar Pc at pH 6 (1.6 Å) and reduced poplar Pc at pH 7 (1.8 Å) were used for structural calculations. The coordinates were kindly provided by Drs. J. M. Guss and H. C. Freeman. The program PROTON was used to generate proton coordinates from the protein crystal coordinates by using standard bond lengths and angles. Proton–proton and copper–proton distances were calculated with the program HHDIST. Ring current shifts were calculated with the program RCSHIF using the semi-classical Johnson–Bovey model (Johnson & Bovey, 1958) and the parameterization of Perkins (1980). PROTON and RCSHIF were written in FORTRAN by Dr. W. Barton for the CDC Cyber 760 at the University of Sydney.

Results and Discussion

Figure 1 shows the low- and high-field regions of the ^{1}H NMR spectra of both redox forms of French bean Pc, individually and together in an equimolar mixture. Many of the resonances in the spectrum of the oxidized protein are broadened due to relaxation by the Cu(II) atom, some beyond detection. It is clear from Figure 1 that most resonances occur at identical (within 0.02 ppm) chemical shifts in the spectra of both redox forms, with a few obvious exceptions indicated by dashed lines. The 2D spectra of oxidized Pc can thus be used as subspectra of protons more distant than approximately 15 Å from the copper center.

Assignment Strategy. The procedure used in this study involves the unequivocal determination of a small core of specific assignments, which is then extended to form an internally consistent network. Spin systems were assigned primarily from COSY and RCT spectra. Specific assignments for the aromatic residues were made first since several occupy

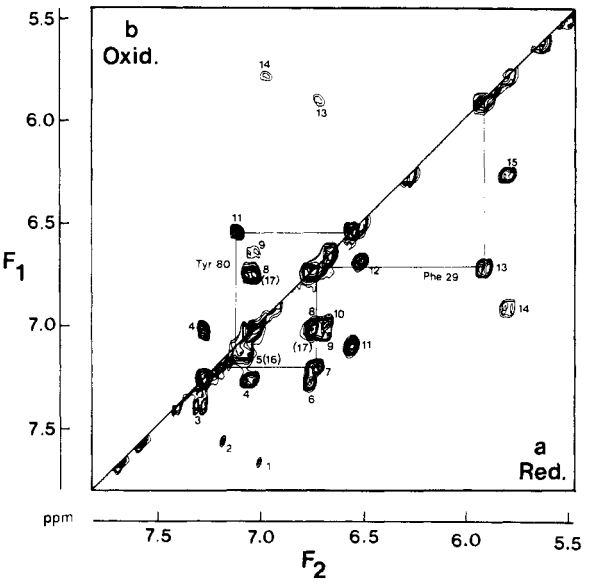

FIGURE 2: Aromatic region of a combined COSY connectivity diagram for both redox forms of French bean Pc at 313 K: (a) reduced protein; (b) oxidized protein. Cross-peaks are arbitrarily numbered. Numbers in parentheses indicate overlapping cross-peaks that are resolved under different conditions or are inferred from other spectra. Connectivities for Phe-29 and Tyr-80 are indicated.

unique positions in the structure and could be readily assigned. Assignments were then extended to the methyl region of the spectrum on the basis of NOEs from aromatic proton resonances. Interproton distances calculated from the crystal structure were used to interpret the NOE data and obtain resonance assignments. The resulting assignments were checked for consistency with NOEs involving nonaromatic resonances, the distance of protons from the copper center, and calculated ring current shifts. This requirement for internal consistency of several parameters minimizes the possibility of incorrect assignments in cases where small structural differences may exist between French bean and poplar Pc. It is apparent from the above that a large amount of data has been used to deduce the assignments presented in this paper. In order to conserve space and reduce redundancy, not all of the evidence for a particular assignment is always described in the text.

Aromatic Residues. French bean Pc contains 2 His, 3 Tyr, and 6 Phe residues (Milne et al., 1974) while the amino acid sequence of poplar Pc shows 2 His, 2 Tyr, and 7 Phe residues (Guss & Freeman, 1983). The 2D*J* spectrum (not shown) of reduced French bean Pc resolves 4/4 singlets, 10/12 doublets, and 11/12 triplets expected to appear in the aromatic region of the spectrum on the basis of the amino acid composition (assuming rapid flipping of Tyr and Phe rings). The COSY spectrum of reduced French bean Pc is expected to contain 17 cross-peaks; those numbered 1–15 are clearly resolved in Figure 2a. COSY spectra of oxidized French bean Pc contain six cross-peaks in this region, three of high intensity and three weaker ones (Figure 2b). The aromatic proton resonances of reduced poplar Pc are somewhat better dispersed than those of the French bean protein (Figure 3a). The COSY spectrum (Figure 3b) contains 16 resolved cross-peaks out of the 18 expected. These correlations have been numbered to coincide with the scheme used for the French bean protein. Specific assignments for the aromatic residues of both species are summarized in Table I. The COSY correlations produced by pairs of aromatic resonances are included in the table preceded by the identifier "A". The evidence for these assignments will now be described according to residue type.

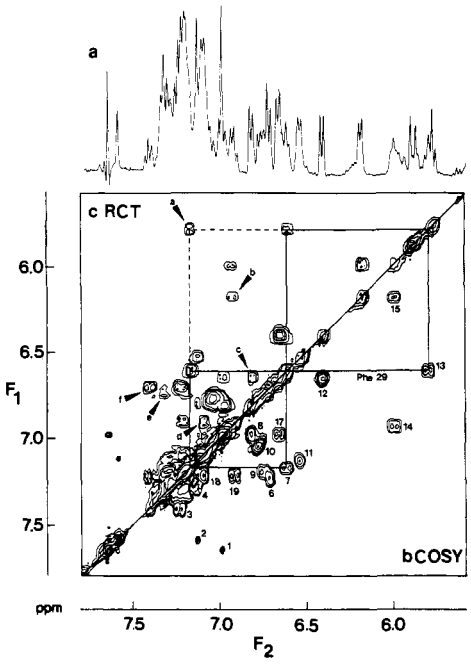

FIGURE 3: Aromatic region of the NMR spectrum of reduced poplar Pc at 308 K (50 mM phosphate, pH 7.5). (a) 1D spectrum. (b) COSY spectrum. Cross-peaks are numbered to correspond as closely as possible to Figure 2a. (c) RCT spectrum. Mixing time τ = 60 ms. Relayed magnetization cross-peaks are designated a–f. Connectivities for Phe-29 are indicated.

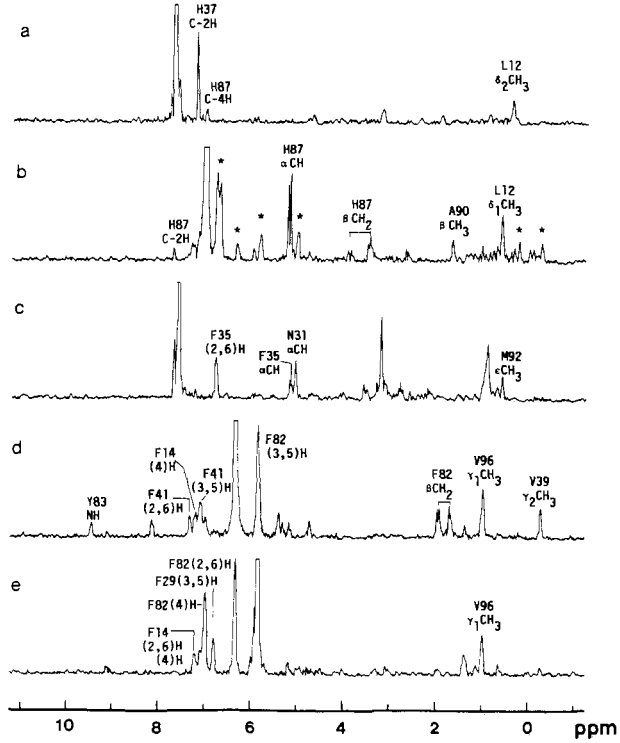

FIGURE 4: Cross sections from NOESY spectra of reduced French bean Pc at 313 K acquired with τ_m = 300 ms. The cross sections show NOEs from the following resonances: (a) His-87 C-2H at 7.69 ppm; (b) His-87 C-4H at 7.02 ppm; (c) His-37 C-4H at 7.60 ppm; (d) Phe-82 C(2,6)H at 6.30 ppm; (e) Phe-82 C(3,5)H at 5.81 ppm. The asterisks denote NOEs from other resonances overlapped with the C-4H resonances of His-87.

(*A*) *Histidine.* Singlet resonances at 7.69 and 7.02 ppm in the spectrum of reduced French bean Pc were previously assigned to the C-2H and C-4H protons of the solvent-exposed His-87 (Cookson et al., 1980a,b). Other singlets at 7.60 and 7.20 ppm were assigned to the C-2H and C-4H resonances of His-37 by elimination (Cookson et al., 1980a). The presence of COSY cross-peaks at 7.02/7.70 (A1) and 7.20/7.60 ppm (A2) confirms that these pairs of resonances derive from individual His residues (King & Wright, 1982). However, the NOESY spectra indicate that the order of the C-2H and C-4H assignments for His-37 should be reversed. The resolved His-87 C-2H resonance shows an intense NOE onto the His-37 resonance at 7.20 ppm (Figure 4a), which the crystal structure indicates can only arise from the C-2H group of His-37. Similar NOEs are observed between the His singlet resonances of reduced poplar Pc. The resulting order of shifts for His-37 is the reverse of that normally encountered (Bundi & Wüthrich, 1979; Markley, 1975). Reversal of the His-37 assignments is in agreement with results reported for *Anabaena variabilis* Pc on the basis of ^{13}C–^{1}H chemical shift correlation spectra (Kojiro & Markley, 1983).

The αCH resonance of His-87 is located for both species by the large NOE from the C-4H resonance (shown for French bean Pc in Figure 4b). The ABX fragment of the spin system of His-87 can also be identified in COSY spectra of reduced French bean Pc; the assignment is confirmed by the presence of small NOEs between the C-4H and the inequivalent βCH proton resonances (Figure 4b). It has not yet been possible to assign the αCH or βCH resonances of His-37.

(*B*) *Tyrosine.* Three AA′XX′ spin systems of tyrosine residues were previously assigned in the spectrum of French bean Pc by spin decoupling (Freeman et al., 1978a; Cookson et al., 1980b). The assignments of Tyr-80 and Tyr-83 are confirmed in the present work. Symmetrical cross-peaks characteristic of Tyr occur in the COSY spectrum of reduced Pc at the chemical shifts previously assigned to Tyr-80 (A11) and Tyr-83 (A12) (Figure 2a). Only the Tyr-80 correlation is present in the COSY spectrum of the oxidized form (Figure 2b); this residue lies some 18 Å from the copper. The previous assignment for Tyr-70 was found to be incorrect: the resolved doublet at 7.24 ppm that was ascribed to this residue is actually part of a Phe spin system. Cross-peaks characteristic of Tyr are found at 6.69/7.02 ppm (A10) in the COSY spectrum and are assigned to Tyr-70.

Resonances of Tyr-80 and Tyr-83 occur at very similar chemical shifts in the spectrum of poplar Pc (Figure 3 and Table I). The amino acid sequence shows only two tyrosines in poplar Pc; phenylalanine occurs at position 70. Unexpectedly, the COSY spectrum also contains symmetrical cross-peaks at 6.78/7.05 ppm (A10) that appear to arise from a tyrosine residue. The corresponding peaks in the 1D spectrum seem to be of less than 2 protons intensity (Figure 3a), which suggests that they may arise either from amino acid sequence heterogeneity, i.e., a mixture of proteins with Phe and Tyr at position 70, or from partial protein denaturation producing resonances with shifts near those of the "random coil" values for tyrosine (Bundi & Wüthrich, 1979).

(*C*) *Phenylalanine.* AA′BB′C spin systems of Phe residues are readily identified in crowded spectra by using a combination of COSY and RCT spectra (King & Wright, 1983) (shown in Figure 3b,c for poplar Pc). In the absence of resonance overlap, COSY correlations from Phe can often be differentiated from those of Tyr by differences in cross-peak fine structure. However, the COSY spectrum of reduced French bean Pc allows unambiguous assignment of only two Phe spin systems (A14 + A15, A3 + A6). The resonances of two more Phe spin systems (A7 + A13, A4 + A9) can be identified from RCT spectra. These spin systems can be assigned to specific phenylalanine residues on the basis of ring current shifts and NOEs.

Table I: Specific Assignments for the Aromatic Side Chains of French Bean and Poplar Plastocyanins[a]

assignment		cross-peak[c]	French bean Pc		Poplar Pc	
			Cu(I)	Cu(II)	Cu(I)	Cu(II)
His-37	C-2H	A2	7.20	*b*	7.14	*b*
	C-4H		7.60	*b*	7.61	*b*
His-87	C-2H	A1	7.69	*b*	7.66	*b*
	C-4H		7.02	*b*	7.00	*b*
	βCH		3.90	*b*		
	βCH		3.44	*b*		
	αCH		5.19	*b*	5.16	*b*
Tyr-70[d]	C(2,6)H	A10	7.02	*b*	(7.05)[e]	
	C(3,5)H		6.69	6.64	(6.78)[e]	
Tyr-80	C(2,6)H	A11	7.13	7.10	7.14	
	C(3,5)H		6.57	6.55	6.55	6.55
Tyr-83	C(2,6)H	A12	6.72	*b*	6.66	
	C(3,5)H		6.52	6.48	6.42	6.35
Phe-14	C(2,6)H	A5,A16	7.17	*b*		
	C(3,5)H		7.09	*b*		
	C(4)H		7.17	*b*		
Phe-19	C(2,6)H	A8,A17	6.77	6.77	6.82	
	C(3,5)H		7.05	7.03	6.97	
	C(4)H		6.77	6.77	6.65	
	βCH		3.15	3.14	3.16	
	βCH		3.32	3.32	3.36	
	αCH		5.14	5.14	5.13	
Phe-29	C(2,6)H	A7	7.24	*b*	7.18	
	C(3,5)H	A13	6.75	6.69	6.63	
	C(4)H		5.92	5.90	5.80	
Phe-35	C(2,6)H	A6	6.78	*b*	6.72	
	C(3,5)H	A3	7.33	*b*	7.24	
	C(4)H		7.44	*b*	7.42	
Phe-41	C(2,6)H	A4	7.30	7.32	7.31	
	C(3,5)H	A9	7.06	7.08	7.21	
	C(4)H		6.71	6.65	6.74	
Phe-70[d,e]	C(2,6)H	A18			7.09	
	C(3,5)H	A19			7.22	
	C(4)H				6.92	
Phe-82	C(2,6)H	A15	6.30	6.25	6.21	6.17
	C(3,5)H	A14	5.81	5.79	5.99	5.99
	C(4)H		6.95	6.98	6.93	6.93
	βCH		1.95		1.94	
	βCH		1.69		(1.39)	

[a] Assignments in parentheses are regarded as tentative. [b] Indicates resonances broadened beyond detection in 1D or COSY spectra. [c] Cross-peaks are numbered according to Figures 2 and 3. "A" denotes aromatic cross-peaks. [d] Residues in italics are not conserved between poplar and French bean plastocyanins. [e] The amino acid sequence of poplar Pc [reported in Guss and Freeman (1983)] suggests that residue 70 is Phe in this species. The origin of the signals giving rise to cross-peak A10 in the poplar Pc COSY spectrum is unclear (see text).

The spin system giving rise to cross-peaks A14 + A15 is assigned to Phe-82 on the basis of the agreement between the large upfield shifts (0.99 and 1.58 ppm) observed for the C(2,6)H and C(3,5)H resonances and the ring current shifts calculated from the X-ray structure of poplar Pc. Details of the ring current shift calculations will be given in a later paper. Similarly, the large upfield shift (1.42 ppm) observed for the C(4)H resonance of the A7 + A13 spin system assigns it to Phe-29. The calculations show that no other phenylalanine proton resonances are subject to such large ring current shifts. It should be noted that ring current effects would ordinarily be unsatisfactory for assignment of aromatic proton resonances (Redfield et al., 1983), but in this case the shifts are large and the assignments are corroborated by other evidence. The A3 + A6 spin system is assigned to Phe-35 on the basis of an NOE from the resolved C-4H resonance of His-37 to the C(2,6)H resonance (Figure 4c). The fourth resolved phenylalanine spin system (cross-peaks A4 + A9) is assigned to Phe-41 on the basis of small NOEs between its C(3,5)H and C(2,6)H resonances and the C(2,6)H resonance of Phe-82 (Figure 4d). This assignment is consistent with its appearance in COSY spectra of oxidized French bean Pc (Figure 2b).

The two remaining Phe spin systems of French bean Pc do not give rise to obvious relayed cross-peaks in RCT spectra due to spectral overlap. They are identified by reference to the peaks in the 2D*J* spectrum that remain unassigned. Overlapping COSY cross-peaks A5 + A16 are due to coincident resonances at 7.17 ppm which are coupled to a C(3,5)H triplet resonance at 7.09 ppm. This spin system is assigned to Phe-14 on the basis of NOEs from the C(2,6)H resonance of Phe-82 to the C(4)H resonance and from the C(3,5)H resonance of Phe-82 to the C(3,5)H and C(4)H resonances (Figure 4d,e). All of these NOEs are predicted from the X-ray structure. The remaining spin system is defined by cross-peaks A8 + A17, which are overlapped at 313 K but appear as separate cross-peaks in COSY spectra acquired at 298 K. The prominence of cross-peaks A8 + A17 in COSY spectra of the oxidized protein (Figure 2b) confirms their assignment to Phe-19, as this residue is the furthest phenylalanine from the copper. The above aromatic assignments for French bean Pc are consistent with all available evidence, including the relative intensities of the resonances in the 1D spectrum.

The aromatic region of the RCT spectrum of reduced poplar Pc contains six relayed cross-peaks arising from Phe spin systems (Figure 3c). Specific assignments for Phe-82, Phe-29, and Phe-35 are made with the same evidence as used for the French bean protein. The spin systems of Phe-41 and Phe-19 were assigned on the basis of the similarity of their chemical shifts to those determined for French bean Pc. Both assignments are confirmed by NOEs, as described for the French

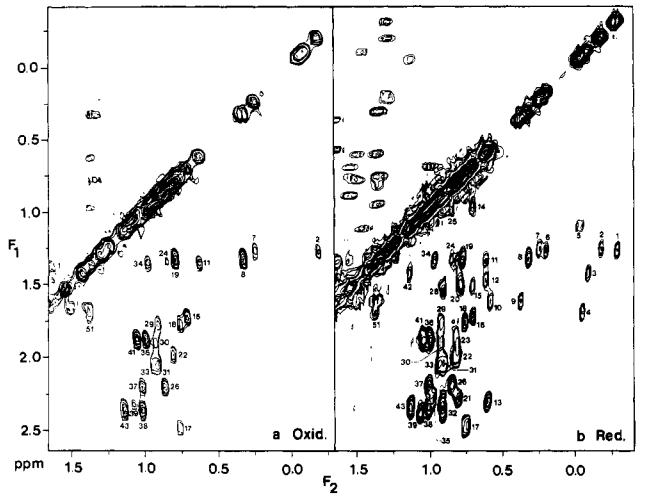

FIGURE 5: Val/Leu/Ile methyl group correlations in COSY spectra of French bean Pc at 313 K: (a) oxidized protein; (b) reduced protein. Resolved cross-peaks are arbitrarily numbered.

bean protein. The sixth resolved Phe spin system of poplar Pc (A18 + A19) is assigned to Phe-70 since the chemical shift of its C(4)H resonance is quite dissimilar to that of Phe-14 in French bean Pc. This assignment is corroborated by the similarity of NOEs to methyl resonances shown by this spin system and that of Tyr-70 in French bean Pc. It is unclear whether these resonances appear at partial intensities, as signal overlap prevents intensity measurements in the 1D spectrum. The Phe-14 spin system of poplar Pc is not resolved in COSY or RCT spectra, but unassigned intensity remains in the region between 7.0 and 7.3 ppm that may belong to a tightly coupled spin system of Phe-14.

Spectral overlap makes unequivocal identification of the βCH_2 resonances of most of the phenylalanines somewhat difficult at present. However, the βCH and αCH resonances of Phe-19 are readily assigned from the NOESY and COSY spectra of oxidized French bean Pc. The βCH resonances of Phe-82, which are expected to receive large upfield ring current shifts from Phe-41, are obvious in NOESY spectra (Figure 4d), but the αCH resonance of this residue has not yet been assigned.

Methyl-Containing Residues. French bean Pc contains 60 methyl groups that derive from 2 Met, 5 Thr, 5 Ala, 14 Val, 7 Leu, and 3 Ile residues. Of the methyl-containing residues of poplar Pc, both Met, both Thr, 4/7 Ala, all 9 Val, all 6 Leu, and 3/6 Ile occur at identical positions in the French bean sequence. Figure 5 shows the Val/Leu/Ile methyl correlation region from COSY spectra of both redox forms of French bean Pc. The Thr/Ala correlation region of the reduced protein is shown in Figure 6a. A total of 51 from an expected 58 methyl correlations are readily resolved in the COSY spectrum of the reduced plastocyanin. The COSY spectrum of oxidized French bean Pc is considerably simplified and contains only 28 methyl group correlations. The number of COSY cross-peaks could not be accurately determined for reduced poplar Pc due to the lower quality of its spectrum. Specific assignments for methyl-containing residues of both species are summarized in Table II. Figure 7 shows the aromatic–methyl correlation regions of representative NOESY spectra of French bean Pc that were used to obtain specific assignments, some of which are indicated in the figure. Spin systems that have been identified but not yet assigned to specific amino acid residues are summarized in Table III. Methyl group correlations in Tables II and III are designated "M", followed by the number of the cross-peak from Figures 5 and 6.

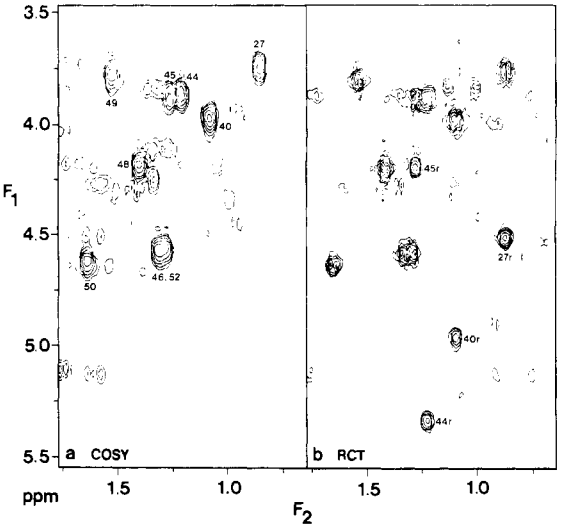

FIGURE 6: Thr/Ala methyl group connectivities for reduced French bean Pc at 313 K: (a) COSY spectrum; (b) RCT spectrum, τ = 60 ms. Cross-peaks are arbitrarily numbered. Relayed cross peaks in the RCT spectrum are designated by the suffix "r".

(*A*) *Methionine.* The three-proton singlet resonances of the ϵ-methyl groups of Met-57 and Met-92 have been assigned previously in the spectrum of reduced French bean Pc (Freeman et al., 1978a; Cookson et al., 1980b). The Met-92 ϵ-methyl resonance of reduced poplar Pc is completely resolved at 0.52 ppm (Figure 8). Assignment to Met-92 is confirmed by the presence of NOEs from the His-37 C-2H and C-4H resonances. The other resonances of the Met spin systems have not yet been identified.

(*B*) *Threonine and Alanine.* Nine methyl group correlations from the AX_3 and AMX_3 spin systems of Ala and Thr are evident in the COSY spectrum of reduced French bean Pc (Figure 6a). Correlations due to Thr can usually be readily differentiated from those of Ala in RCT spectra (Figure 6b), where extra cross-peaks appear from Thr residues due to A $\rightarrow$ M $\rightarrow$ X_3 relayed coherence transfer (King & Wright, 1983). Four of the five expected Thr spin systems are identified in this manner. A relayed cross-peak characteristic of a fifth Thr could not be detected, perhaps as a consequence of a very small $^3J_{\alpha\beta}$ coupling constant.

The spin system of Thr-79 is assigned on the basis of an NOE from the Tyr-80 C(2,6)H resonance to the αCH resonance of a threonine at 5.34 ppm. From the crystal structure of poplar Pc, the αCH proton closest to the Tyr-80 C(2,6)H is that of residue 79 (Glu for poplar, Thr for French bean plastocyanin). The assignment assumes that the local structure is conserved upon substitution of Thr for Glu; this assumption appears to be justified since additional NOEs are observed between the Thr-79 αCH resonance and the αCH and βCH_3 resonances (at 5.00 and 1.10 ppm, respectively) of another Thr spin system. Only Thr-79 and Thr-97 are close enough to give rise to such NOEs.

Two Thr spin systems are observed in an RCT spectrum of reduced poplar Pc with resonances at 5.10, 3.92, and 1.26 and 4.28, 4.12, and 1.12 ppm for the αCH, βCH, and γCH_3 protons of each spin system, respectively. Poplar Pc contains only two threonine residues, Thr-97 and Thr-69. The former is assigned from the similarity of its chemical shifts to those of Thr-97 of French bean Pc, and the latter is assigned by elimination. Comparison of the chemical shifts for Thr-69 in poplar Pc with those of the unassigned Thr spin systems in French bean Pc allows the assignment of correlation M45 to Thr-69. The absence of the resonances assigned to Thr-79 for

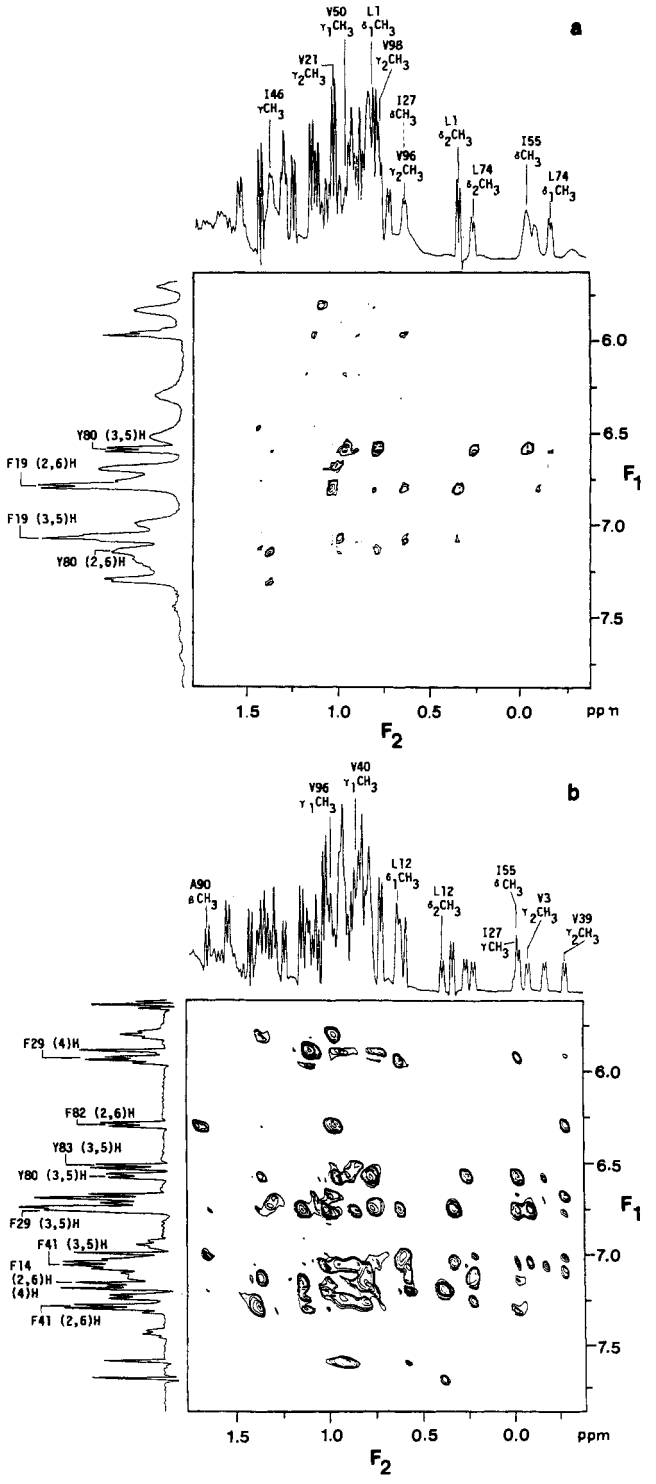

FIGURE 7: Regions from NOESY spectra of French bean Pc showing aromatic to methyl correlations: (a) oxidized protein, τ_m = 250 ms; (b) reduced protein, τ_m = 300 ms. Some assignments are indicated.

French bean Pc from the spectrum of poplar Pc lends further support to the Thr-79 assignment.

Cross-peak M50, which appears to arise from an alanine spin system, is observed only in COSY spectra of the reduced form and must therefore be due to Ala-33 or Ala-90, both of which are less than 10 Å from the copper atom. This spin system is assigned to Ala-90 on the basis of an NOE from the His-87 C-4H resonance to the methyl doublet at 1.65 ppm (Figure 4b). One of the overlapping cross-peaks M46 and M52 disappears from the COSY spectrum of oxidized Pc and is tentatively assigned to Ala-33.

Cross-peaks M48, M49, and either M46 or M52, all of

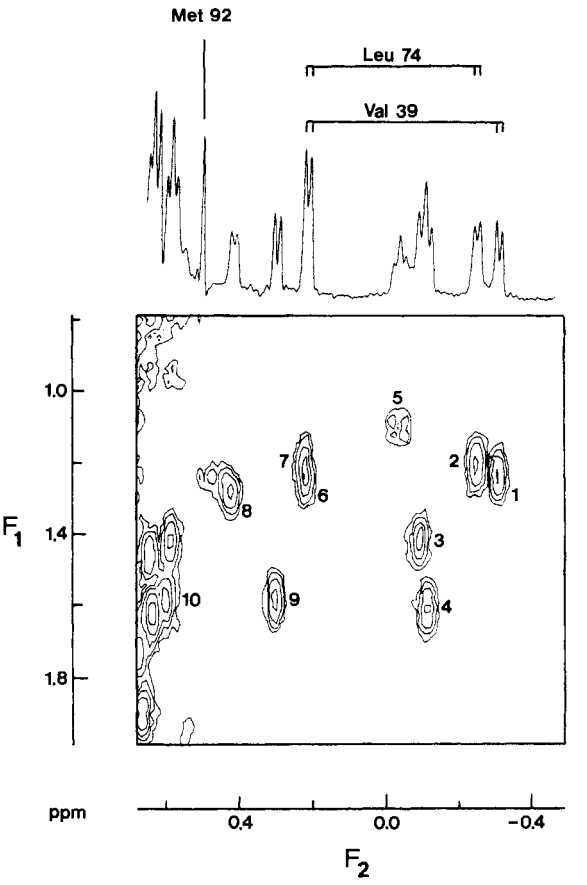

FIGURE 8: Region from the COSY spectrum of reduced poplar Pc at 308 K, showing cross-peaks from ring current shifted methyl resonances. Cross-peaks are numbered to correspond as closely as possible to Figure 5b.

which appear to arise from alanine spin systems, remain in COSY spectra of oxidized French bean Pc. These are expected to arise from alanines-48, -52, and -65, which are more than 14 Å from the copper atom. The Ala-52 spin system is the only one of these that can be specifically assigned, on the basis of NOEs from the Ile-55 δCH_3 and γCH_3 resonances (see below) to the Ala αCH resonance at 3.83 ppm.

Correlations for several Ala spin systems are observed in the COSY spectrum of reduced poplar Pc, but most have not been specifically assigned. Assignments for Ala-33 and Ala-90 have been made on the basis of similarity of chemical shifts in the spectra of the two proteins.

(C) Valine and Leucine. Complete connectivities for the A_3B_3M portions of 8 out of 21 Val and Leu residues can be identified in the COSY spectrum of oxidized French bean Pc (Figure 5a). Many additional connectivities are evident in spectra of the reduced protein. Few spin systems have been identified for reduced poplar Pc due to the poorer quality of the COSY spectrum, but correlations from the upfield ring current shifted methyl group resonances are shown in Figure 8.

The simplified COSY spectra of the oxidized French bean Pc are particularly useful for assignment of the Val and Leu resonances and will be considered first. From the crystal structure, the Tyr-80 C(3,5)H group is close to the Ile-55 δCH_3, Leu-74 δ_2CH_3, Val-98 γ_2CH_3, and Val-50 γ_1CH_3 groups. NOEs between the Tyr-80 C(3,5)H resonance and four methyl group resonances are evident (Figure 7a). One NOE is to a triplet resonace at −0.05 ppm, which can be immediately assigned to Ile-55 (see below). A second NOE is to a resonance at 0.25 ppm, which is assigned to Leu-74

Table II: Specific Assignments for the Aliphatic Residues of French Bean and Reduced Poplar Plastocyanins[a]

assignment		cross-peak[c]		French bean Pc		poplar Pc
				Cu(I)	Cu(II)	Cu(I)
Met-57	ϵCH_3			1.91	*b*	1.86
Met-92	ϵCH_3			0.58	*b*	0.52
Thr-69	γCH_3	M45		1.28	1.28	1.12
	βCH			3.92	3.92	4.12
	αCH			4.22	4.22	4.28
Thr-79	γCH_3	M44		1.23	1.23	
	βCH			3.92	3.92	
	αCH			5.34	5.34	
Thr-97	γCH_3	M40		1.10	1.10	1.26
	βCH			4.02	4.02	3.92
	αCH			5.00	5.00	5.10
Ala-33	βCH_3	M46/M52		(1.32)	*b*	(1.31)
	αCH			(4.61)	*b*	(4.68)
Ala-52	βCH_3	M49		1.54	1.54	
	αCH			3.83	3.83	
Ala-90	βCH_3	M50		1.65	*b*	1.60
	αCH			4.68	*b*	4.58
Val-3	$\gamma_1 CH_3$		M42	1.14	*b*	1.11
	$\gamma_2 CH_3$	M3		−0.09	*b*	−0.08
	βCH			1.45	*b*	1.42
	αCH			4.31		
Val-21[d]	$\gamma_2 CH_3$			1.01	1.01	
Val-39[e]	$\gamma_1 CH_3$		M6	0.21	*b*	0.24
	$\gamma_2 CH_3$	M1		−0.28	−0.39	−0.28
	βCH			1.30	*b*	1.27
	αCH			4.13		4.19
Val-40	$\gamma_1 CH_3$		M25	0.85	*b*	0.79
	$\gamma_2 CH_3$	M14		0.71	*b*	0.66
	βCH			0.99	*b*	
Val-50	$\gamma_1 CH_3$		M33	0.95	0.95	0.83
	$\gamma_2 CH_3$	M31		0.92	0.92	
	βCH			2.04	2.05	
Val-96	$\gamma_1 CH_3$		M34	0.98	0.97	
	$\gamma_2 CH_3$	M11		0.62	0.61	
	βCH			1.37	1.37	
	αCH				4.96	
Val-98	$\gamma_1 CH_3$		M29	0.92	0.90	
	$\gamma_2 CH_3$	M18		0.76	0.77	0.72
	βCH			1.78	1.77	
Leu-1[f]	$\delta_1 CH_3$		M19	0.78	0.78	
	$\delta_2 CH_3$	M8		0.33	0.33	
	γCH			1.33	1.33	
Leu-12	$\delta_1 CH_3$		M10	0.59	*b*	0.61
	$\delta_2 CH_3$	M9		0.38	*b*	0.33
	γCH			1.63	*b*	1.59
	αCH			4.22		4.05
Leu-74	$\delta_1 CH_3$		M2	−0.17	−0.17	−0.24
	$\delta_2 CH_3$	M7		0.25	0.25	0.24
	γCH			1.29	1.28	1.21
	αCH			4.52	4.52	4.43
Ile-27	δCH_3			(0.62)	(0.62)	
	γCH_3	M4		−0.06	−0.06	−0.11
	βCH			1.78		1.62
	αCH			4.20		
Ile-46	δCH_3	M24		0.83	0.83	
	γCH_2			1.34	1.34	
	γCH_3	M51		1.36	1.36	
	βCH			1.66	1.66	
Ile-55	δCH_3	M5		−0.03	−0.05	−0.03
	γCH_2			1.13		1.11

[a] Assignments in parentheses are regarded as tentative. [b] Indicates resonances broadened beyond detection in 1D or COSY spectra. [c] Cross-peaks are numbered according to Figures 5 and 6. "M" denotes methyl cross-peaks. [d] Residue 21 in poplar Pc is Ile. [e] The amino acid sequence of poplar Pc suggests that residue 39 is Ile, but the NMR spectrum indicates Val. [f] Residue 1 in poplar Pc is Ile.

$\delta_2 CH_3$. From the COSY spectrum (Figure 5a), the $\delta_1 CH_3$ resonance of this residue occurs at −0.17 ppm (M2). The assignment is confirmed by NOEs from the $\delta_1 CH_3$ resonance to the Phe-41 C(3,5)H and C(4)H resonances of reduced plastocyanin (Figure 7b). Ring current shift calculations are in good agreement with these assignments. The αCH resonance of Leu-74 is located through an intense NOE from the $\delta_2 CH_3$ resonance. One of the two remaining NOEs from the Tyr-80 C(3,5)H resonance is to a methyl group resonance at 0.77 ppm (M18), which also receives an NOE from the Tyr-80 C(2,6)H resonance, identifying it as the Val-98 $\gamma_2 CH_3$. The fourth resonance at 0.95 ppm is assigned to Val-50 $\gamma_1 CH_3$ by elimination. Inspection of the COSY spectrum indicates that M18 + M29 be assigned to Val-98 and M31 + M33 to Val-50.

Three large NOEs are observed between the Phe-19 C-(2,6)H resonance and methyl group resonances (Figure 7a). The crystal structure shows that the Ile-21 $\gamma_2 CH_3$, Ile-1 δCH_3 and $\gamma_2 CH_3$, and Val-96 $\gamma_2 CH_3$ groups are close to the Phe-19

Table III: Spin Systems Identified in the Spectrum of Reduced French Bean Plastocyanin but Not Specifically Assigned

residues		chemical shifts											
Thr-73, -76	γCH_3	0.88	] M27										
	βCH	3.78											
	αCH	4.54											
Ala-48, -65[a]	βCH_3	1.32	] M46/M52			1.42	] M48						
	αCH	4.61				4.23							
Val-21, -28, -53, -71, -72	γCH_3	1.01		] M38		0.86		] M26	0.99		] M36		
	γCH_3	1.14	] M43			1.01	] M37		1.04	] M41			
	βCH	2.35				2.19			1.88				
	αCH	3.86											

[a] One of these spin systems could arise from a threonine with a very small $^3J_{\alpha\beta}$ coupling constant.

C(2,6)H protons. Residues 1 and 21 are Leu and Val in French bean Pc. NOEs between the resonance at 0.61 ppm, at which chemical shift only correlation M11 is observed in COSY spectra of copper(II) Pc, and the C(2,6)H and C(3,5)H resonances of Phe-19 identify the Val-96 γ_2CH_3. Cross-peaks M11 and M34 in the COSY spectrum allow assignment of the βCH and γ_1CH_3 resonances. These assignments are confirmed by observation of an intense NOE from the Phe-82 C(2,6)H resonance to the γ_1CH_3 resonance in the NOESY spectrum of reduced French bean Pc (Figures 4d and 7b). This NOE is attenuated in the NOESY spectrum of the oxidized protein (Figure 7a) by the relaxation effects of the Cu(II). A second NOE from the Phe-19 C(2,6)H resonance is to a sharp methyl doublet (M8) at 0.33 ppm, which is assigned to the Leu-1 δ_2CH_3. This assignment is supported by NOEs to the C(2,6)H and C(3,5)H resonances of Phe-19 in spectra of the reduced protein. The δ_1CH_3 resonance of this residue (M19) at 0.78 ppm receives a small NOE from the Phe-19 C(2,6)H resonance (Figure 7a). A doublet resonance similar to M8 appears in the spectrum of the poplar protein and is tentatively assigned to the Ile-1 γ_2CH_3. The third large NOE from the Phe-19 C(2,6)H resonance (presumably to the Val-21 γ_2CH_3) falls at a chemical shift that contains two possible Val spin systems which cannot be distinguished at this stage.

The three unassigned Val/Leu spin systems (M36 + M41; M26 + M37; M38 + M43) which appear at high intensity in COSY spectra of the oxidized Pc (Figure 5a) should derive from three of the group Val-21, -53, -28, -71, and -72, each of which lies at least 15 Å from the copper. Neither ring current shift nor NOE information allows the specific assignment of these spin systems at present.

Several Val/Leu spin systems appear only in COSY spectra of the reduced form of French bean Pc (Figure 5b). The Leu-12 spin system can be assigned on the basis of a large NOE from the His-37 C-2H resonance and a weaker one from the His-87 C-2H resonance to the resolved δ_2CH_3 resonance at 0.38 ppm (Figure 7b). The Leu-12 δ_1CH_3 resonance occurs at 0.59 ppm (M10), and the αCH resonance is located by a strong NOE from the δ_2CH_3. This spin system has very similar chemical shifts in the spectrum of poplar Pc.

The crystal structure of poplar Pc shows that the methyl groups of Ile-39 are close to the aromatic rings of Phe-29, Phe-41, and Phe-82 (Guss & Freeman, 1983), such that the Ile-39 γCH_2 and γCH_3 resonances would experience substantial upfield ring current shifts. Ile-39 is replaced by valine in French bean Pc (Milne et al., 1974). NOEs are observed from the highest field methyl group resonance of French bean Pc (M1) onto several aromatic proton resonances, the most intense of which involves the Phe-82 C(2,6)H (Figure 7b). Additional NOEs are to the Phe-29 C(3,5)H, Phe-41 C(4)H, and Phe-41 C(3,5)H resonances. Cross-peaks M1 and M6 clearly arise from the A_3B_3M portion of a Val or Leu spin system. NOEs are observed between the methyl resonance at 0.21 ppm (M6) and the Phe-29 C(2,6)H and the Phe-14 C(3,5)H and C(4)H resonances. Val-39 is the only residue close enough to all of these phenylalanines to give rise to these NOEs. Cross-peaks M1 and M6 in the COSY spectrum of French bean Pc are thus firmly assigned to the Val-39 γ_2CH_3 and γ_1CH_3 groups, respectively. Examination of the COSY spectrum of reduced poplar Pc strongly suggests that residue 39 in this species is not Ile as in the amino acid sequence but is Val or Leu. Figure 8 shows that M1 and M6 clearly form part of an A_3B_3M spin system. Since residue 39 in higher plant plastocyanins is usually valine (Boulter et al., 1977; Guss & Freeman, 1983; Ramshaw, 1982), the assignment is tentatively made to Val rather than Leu. Our observation is not consistent with an examination of the crystallographic electron density maps, which agree with the presence of an Ile residue at position 39 (J. M. Guss, personal communication). It is possible that there is a real difference between the protein samples used in the two studies, since they were extracted from relatively uncontrolled sources some years apart.

One Val/Leu spin system evident in the COSY spectrum of reduced French bean Pc has methyl resonances with a uniquely large chemical shift difference (cross-peaks M3 and M42). The resonance at −0.09 ppm shows NOEs to the C(3,5)H resonance of Phe-19 and to the C(4)H resonance of Phe-19 and/or the C(3,5)H resonance of Phe-29 and is assigned to the γ_2CH_3 of Val-3. This assignment is supported by ring current shift calculations. Cross-peaks M3 and M42 occur at very similar chemical shifts in the spectrum of reduced poplar Pc.

The resolved Tyr-83 C(3,5)H resonance shows an NOE onto a signal at 0.85 ppm (Figure 7b), which can only derived from Val-40. The COSY cross-peak, M25, is almost buried in the diagonal of Figure 5b but is better resolved in other COSY spectra. Calculations show that the βCH resonance of Val-40 should receive a large upfield ring current shift from Tyr-83, in agreement with the observed shift.

Of the valine and leucine residues that are relatively close to the copper (nearer than approximately 15 Å), the methyl resonances from Leu-4, Leu-5, Val-13, Val-15, Leu-62, Leu-63, and Val-93 remain unassigned. Ring current shift calculations are of little assistance as none of their methyl group resonances are predicted to possess shifts significantly different from the random coil values.

(D) Isoleucine. Two methyl group correlations in COSY spectra of reduced French bean Pc (M5 and M24) are readily assignable to Ile δCH_3 resonances by virtue of their fine structure. The triplet at −0.03 ppm shows NOEs to the C(3,5)H resonance of Tyr-80 and the C(2,6)H resonance of Phe-41, allowing assignment to Ile-55. This assignment is supported by ring current shift calculations. The Ile-55 δCH_3 resonance is clearly resolved in the 1D spectrum of reduced poplar Pc (Figure 8).

The NOESY spectrum of oxidized French bean Pc contains cross-peaks between the Tyr-80 C(2,6)H and Phe-41 C(2,6)H resonance and a methyl resonance at 1.36 ppm (Figure 7a), thus identifying the γ_2CH_3 peak of Ile-46 (cross-peak M51).

The final remaining COSY correlation upfield of 0.5 ppm (M4) is assigned to the Ile-27 γ_2CH_3 on the basis of strong NOEs to the Phe-29 C(4)H and Phe-19 C(4)H resonances (Figure 7b). An NOE from the Phe-29 C(4)H resonance to 0.62 ppm allows tentative assignment of the Ile-27 δCH_3 resonance, which does not appear to give rise to a COSY cross-peak of observable intensity. Support for this assignment comes from a strong NOE between the Ile-27 γCH_3 resonance and that at 0.62 ppm. The other resolved Ile $\delta CH_3/\gamma CH_2$ cross-peak (M24) is then assigned to Ile-46 by elimination.

Conclusions

On the basis of the known crystal structure of poplar plastocyanin, we have been able to make extensive assignments in the ^1H NMR spectrum of the protein from French bean. These include complete assignments for the aromatic protons. The assignments were made primarily by comparison of observed nuclear Overhauser effects with proton–proton distances calculated from the X-ray structure. However, the assignments are all supported by other evidence, e.g., ring current shifts, distance from the Cu(II) atom in the oxidized Pc, or broadening by paramagnetic probes that bind at the negative patch near Tyr-83 (Cookson et al., 1980b). In making assignments, the paramagnetic Cu(II) plastocyanin proved particularly useful for providing subspectra of protons distant from the Cu site. A substantial number of resonance assignments have also been made for poplar plastocyanin.

The assignment procedure relied heavily on two-dimensional NMR experiments; it proved difficult to assign more than a few resolved resonances in the spectrum of plastocyanin by one-dimensional methods. Relayed coherence transfer spectroscopy was of particular importance for resolving ambiguities in the connectivities between the highly overlapped resonances of the aromatic side chains and for distinguishing Thr and Ala cross-peaks. We note that the phenylalanine spin system assignments have been confirmed by subsquent triple quantum COSY experiments (Rance & Wright, 1986).

The resonance assignments established in the present work allow a detailed comparison of the structures of the poplar and French bean plastocyanins to be made. This and a comparison of the conformation of plastocyanin in the solution and crystalline states will be described in a later paper. The availability of a large body of resonance assignments has also been invaluable in NMR and kinetic investigations of the site of interaction of plastocyanin with various electron-transfer reagents and electron-transfer proteins (King and Wright, unpublished results).

Acknowledgments

We thank Drs. H. C. Freeman, J. M. Guss, M. Murata, and G. Bodenhausen for helpful discussions and for access to unpublished data and Dr. H. J. Dyson and S. Treat for assistance with preparation of the manuscript. Several of the computer programs used were written by Dr. W. Barton.

References

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976a) *J. Chem. Phys. 64*, 2229–2246.

Aue, W. P., Karhan, J., & Ernst, R. R. (1976b) *J. Chem. Phys. 64*, 4226–4227.

Baumann, R., Wider, G., Ernst, R. R., & Wüthrich, K. (1981) *J. Magn. Reson. 44*, 402–406.

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542–561.

Bax, A., Freeman, R., & Morris, G. (1981) *J. Magn. Reson. 42*, 164–168.

Beattie, J. K., Fensom, D. J., Freeman, H. C., Woodcock, E., Hill, H. A. O., & Stokes, A. M. (1975) *Biochim. Biophys. Acta 405*, 109–114.

Billeter, M., Braun, W., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 321–346.

Bodenhausen, G., Freeman, R., & Turner, D. L. (1977) *J. Magn. Reson. 27*, 511–514.

Boulter, D., Haslett, B. G., Peacock, D., Ramshaw, J. A. M., & Scawen, M. D. (1977) *Int. Rev. Biochem. 13*, 1–40.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–297.

Canters, G. W., Hill, H. A. O., Kitchen, N. A., & Adman, E. (1984) *Eur. J. Biochem. 138*, 141–152.

Chothia, C., & Lesk, A. M. (1982) *J. Mol. Biol. 160*, 309–323.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319–324.

Cookson, D. J., Hayes, M. T., & Wright, P. E. (1980a) *Nature (London) 283*, 682–683.

Cookson, D. J., Hayes, M. T., & Wright, P. E. (1980b) *Biochim. Biophys. Acta 591*, 162–176.

Eich, G., Bodenhausen, G., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 3731–3732.

Freeman, H. C. (1981) in *Coordination Chemistry—21* (Laurent, J. L., Ed.) pp 29–51, Pergamon Press, Oxford.

Freeman, H. C., Norris, V. A., Ramshaw, J. A. M., & Wright, P. E. (1978a) *FEBS Lett. 86*, 131–135.

Freeman, H. C., Norris, V. A., Ramshaw, J. A. M., & Wright, P. E. (1978b) *Proc. Int. Congr. Photosynth., 4th, 1977 (1978)*, 805–809.

Garrett, T. P. J., Clingeleffer, D. J., Guss, J. M., Rogers, S. J., & Freeman, H. C. (1984) *J. Biol. Chem. 259*, 2822–2825.

Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.

Handford, P. M., Hill, H. A. O., Lee, W.-K., Henderson, R. A., & Sykes, A. G. (1980) *J. Inorg. Biochem. 13*, 83–88.

Hill, H. A. O., & Smith, B. E. (1978) *Biochem. Biophys. Res. Commun. 81*, 1201–1208.

Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.

Johnson, C. E., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012–1014.

King, G., & Wright, P. E. (1982) *Biochem. Biophys. Res. Commun. 106*, 559–565.

King, G., & Wright, P. E. (1983) *J. Magn. Reson. 54*, 328–332.

Kojiro, C. L., & Markley, J. L. (1983) *FEBS Lett. 162*, 52–56.

Kumar, A., Ernst, R. R., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1–6.

Levitt, M. H., & Freeman, R. (1981) *J. Magn. Reson. 43*, 65–80.

Macura, S., Huang, Y., Suter, D., & Ernst, R. R. (1981) *J. Magn. Reson. 43*, 259–281.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.

Markley, J. L. Ulrich, E. L., Berg, S. P., & Krogmann, D. W. (1975) *Biochemistry 14*, 4428–4433.

Milne, P. R., Wells, J. R. E., & Ambler, R. P. (1974) *Biochem. J. 143*, 691–701.

Nagayama, K., Bachmann, P., Wüthrich, K., & Ernst, R. R. (1978) *J. Magn. Reson. 31*, 133–148.

Perkins, S. J. (1980) *J. Magn. Reson. 38*, 297–312.

Ramshaw, J. A. M. (1982) *Encycl. Plant Physiol., New Ser. 14A*, 229-240.
Ramshaw, J. A. M., Brown, R. H., Scawen, M. D., & Boulter, D. (1973) *Biochim. Biophys. Acta 303*, 269-273.
Rance, M., & Wright, P. E. (1986) *J. Magn. Reson. 66*, 372-378.
Redfield, C., Hoch, J. C., & Dobson, C. M. (1983) *FEBS Lett. 159*, 132-136.
Ulrich, E. L., & Markley, J. L. (1978) *Coord. Chem. Rev. 27*, 109-140.
Wagner, G., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 347-366.
Wagner, G., Kumar, A., & Wüthrich, K. (1981) *Eur. J. Biochem. 114*, 375-384.
Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311-319.

Resonance Raman Studies of Isotopically Labeled Chloroperoxidase†

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ABSTRACT: Chloroperoxidase (CPO) and cytochrome $P450_{cam}$ have been shown by several techniques to have similar active site properties. Recent resonance Raman investigations using isotopically enriched ^{34}S-labeled samples have demonstrated thiolate ligation in the $P450_{cam}$ system. We report here on a number of parallel studies involving CPO. On the basis of isotopic labeling (^{34}S, ^{13}CO), we assign the Fe–S and Fe–CO stretching frequencies of CPO at 347 ($\bar{\nu}_{Fe-S}$) and 488 cm^{-1} ($\bar{\nu}_{Fe-CO}$). The differences of the $\bar{\nu}_{Fe-S}$ and $\bar{\nu}_{Fe-CO}$ in CPO and $P450_{cam}$ may suggest subtle differences in the thiolate binding in the two systems.

Chloroperoxidase (CPO) is a heme protein of M_r ~42 000 which has been isolated from the mold *Caldariomyces fumago* (Morris & Hager, 1966). It catalyzes the chlorination reactions involved in the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentanedione). In the presence of hydrogen peroxide and a suitable halogen donor (I^-, Br^-, or Cl^-, but not F^-), the enzyme catalyzes the peroxidative formation of a carbon–halogen bond with a suitable halogen acceptor. In addition to the halogenation reaction, chloroperoxidase also catalyzes the peroxidative oxidation of classical peroxidase substrates such as pyrogallol and guaiacol (Thomas et al., 1970), and it catalyzes the decomposition of hydrogen peroxide to give molecular oxygen in a catalase type reaction.

Previous resonance Raman studies of chloroperoxidase have focused primarily on the high-frequency "marker band" region of the spectrum that involves the porphyrin ring stretching modes (Champion et al., 1976; Remba et al., 1979). The relatively low value found for the frequency of the ring-breathing mode (oxidation state marker), ν_4, in CPO is thought to reflect an electron-rich axial ligand that donates electron density into the porphyrin π^* anti-bonding orbitals (Champion et al., 1976; 1978; Remba et al., 1979; Ozaki et al., 1978). Comparisons between the resonance Raman spectra of CPO and cytochrome $P450_{cam}$ have also been stressed in an earlier work (Remba et al., 1979) and are thought to reflect a high degree of similarity in the active sites of these two very different heme proteins. In this respect, the Raman observations are consistent with a variety of other physical–chemical studies including Mössbauer (Champion et al., 1973, 1975), optical (Hollenberg & Hager, 1973), electron spin resonance (Hollenberg et al., 1980), magnetic circular dichroism (Dawson et al., 1983), and extended X-ray fine structure (Cramer et al., 1978) in that they suggest that both proteins utilize the electron-rich mercaptide sulfur of cysteine as the axial ligand to the heme iron.

Recent resonance Raman investigations (Champion et al., 1982) of cytochrome $P450_{cam}$ enriched in ^{34}S and ^{54}Fe have conclusively demonstrated that cytochrome $P450_{cam}$ does indeed possess an Fe–S linkage, and the Fe–S stretching frequency is found at 351 cm^{-1}. The resonance enhancement of the Fe–S mode in $P450_{cam}$ is presumably due to a S → Fe charge-transfer transition underlying the intense Soret band. This is evidenced by the fact that the Raman excitation profile of the 351-cm^{-1} mode peaks nearly 1500 cm^{-1} to the blue of the Soret maximum. (If this mode were coupled to the Soret resonance, it would be expected to peak within ca. 350 cm^{-1} of the Soret maximum.) In addition, the magnitude of the isotopic shifts in the $P450_{cam}$ system are suggestive of a more open Fe–S–C bond angle than would normally be expected for sp^3 sulfur bonding. One possible explanation for this observation is that the sulfur bonding involves an sp^2 hybrid geometry in the P450 system, with the remaining p orbital interacting through a coordinate covalent π-bonding arrangement with one of the $d\pi$ orbitals of iron (Champion et al., 1982). This type of bonding geometry could also explain the highly rhombic electron paramagnetic resonance (EPR) splittings found for the P450 class of heme proteins (Hollenberg et al., 1980; Chevion et al., 1977).

As a result of the success in the definitive assignment of the sulfur ligand in the $P450_{cam}$ system, we decided to investigate isotopically labeled CPO in order to confirm the expected Fe–S linkage and possibly gain additional information about the Fe–S–C bonding geometry. We report below the results of

† This work is supported by grants from the National Institutes of Health (AM35090), The Research Corporation, and the National Science Foundation. P.M.C. is a recipient of NIH Research Center Career Development Award AM01405.

0006-2960/86/0425-2374$01.50/0