HIGH RESOLUTION PROTON MAGNETIC RESONANCE STUDIES OF PLASTOCYANIN

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1. Introduction

Plastocyanin is a type I, 'blue' copper protein involved in photosynthetic electron transfer. It has approx. mol. wt 10 500 and contains 1 copper atom/molecule. The copper(II) ion may be used as an intrinsic NMR relaxation probe. The resonances of amino acids close to the copper are broadened in the oxidised protein, the effect decreasing rapidly with increasing distance from the metal. Previous NMR studies of plastocyanin from french bean [1], spinach and anabaena [2] have shown that certain resonances present in the spectrum of the copper (I) protein are broadened beyond detection when the protein is in the copper(II) state. We report here the results of NMR experiments which have led to identification of several amino acid residues near the copper in french bean plastocyanin. Evidence for a high degree of conservation of residues near the copper-binding site in plastocyanins from a wide range of higher plant species is presented.

2. Materials and methods

Plastocyanin was prepared by a modification of the method in [3]. Details of the procedure will be described elsewhere. Plastocyanin solutions for NMR spectroscopy were prepared by dissolving protein samples lyophilized from phosphate buffer (10 mM, pH 7.5) in D_2O.

The NMR spectra were recorded at 40°C using a Bruker HX-270 spectrometer equipped with a Nicolet 1180 computer. The resolution was enhanced by means of the convolution difference technique [4]. The Carr-Purcell method A pulse sequence was used to determine the multiplet structure of resonances [5]. Spin decoupling was carried out using both standard methods [6] and the spin-echo double resonance technique [7]. Dioxane was used as an internal standard but all peaks are referred to TSS (trimethylsilylpropanesulphonic acid). Peak intensities were measured relative to the peaks at 7.66 ppm and 7.57 ppm, both of which have been assigned to single protons. Oxidation of Cu(I)–plastocyanin was achieved by titration with freshly prepared potassium ferricyanide in D_2O.

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3. Results and discussion

Figure 1 shows the resolution enhanced NMR spectra of the fully reduced and oxidized forms of french bean (*Phaseolus vulgaris*) plastocyanin. Some resonances are broadened beyond detection when the protein is oxidized and are seen clearly in the difference spectrum, i.e., the spectrum of the reduced protein minus that of the same solution after oxidation of 16% of the protein (fig.1(c)).

3.1. *Assignment of resonances*

The sharp resonances at 7.66 ppm and 7.57 ppm in the spectrum of reduced french bean plastocyanin (fig.1(a)) have previously been assigned to the C-2 protons of the two histidine residues [1,2]. This assignment is now confirmed as the resonances appear as singlets in the spectra obtained using Carr-Purcell method A pulse sequences (fig.2). In addition two

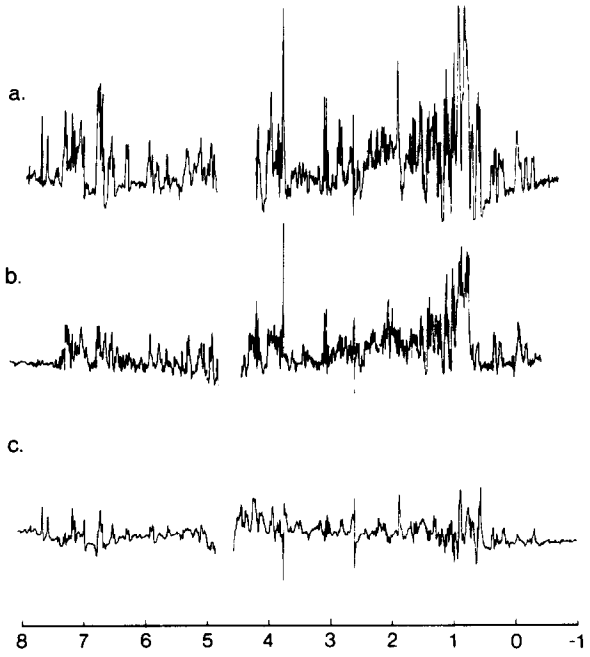

Fig.1. 270 MHz convolution difference NMR spectra of french bean plastocyanin. (a) Reduced protein. (b) Oxidized protein. (c) Spectrum of reduced protein minus spectrum of 16% oxidized protein.

sharp singlets at 7.16 ppm and 6.97 ppm are identified with the histidine C-4 protons. Since tryptophan is absent from french bean plastocyanin, only histidine protons can give rise to singlet resonances in the aromatic region of the spectrum.

Assignments of some phenylalanine and tyrosine resonances have been made by spin decoupling experiments. These were carried out by means of time-shared double resonance, the decoupling being observed either directly or by difference spectroscopy [6], and using the spin-echo double resonance technique [7]. Full details of these experiments will be published separately but assignment of the resonances of one phenylalanine is illustrated in fig.3. Irradiation at the sharp two-proton doublet at 6.27 ppm caused a two-proton triplet at 5.78 ppm to decouple to a doublet. Irradiation at this triplet decoupled the doublet to a singlet and caused another triplet at 6.92 ppm to decouple to a singlet. Confirmation of this coupling pattern was obtained by irradiation at 6.92 ppm which caused the triplet at 5.78 ppm to decouple to a doublet. Similar experiments have so far enabled assignment of the resonances of all three tyrosines (each tyrosine gives rise to a pair of coupled doublets) in french bean plastocyanin and two of the six phenylalanines (table 1).

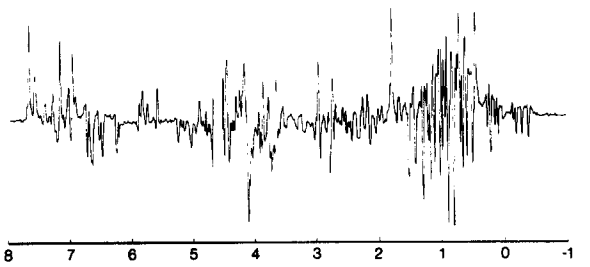

Fig.2. Spectrum of reduced french bean plastocyanin obtained using a $90°-\tau-180°-\tau$ (Carr-Purcell method A) pulse sequence with τ 40 ms. Modulation of the spin coupling causes doublet resonances to be inverted. Singlet and triplet resonances appear as positive peaks.

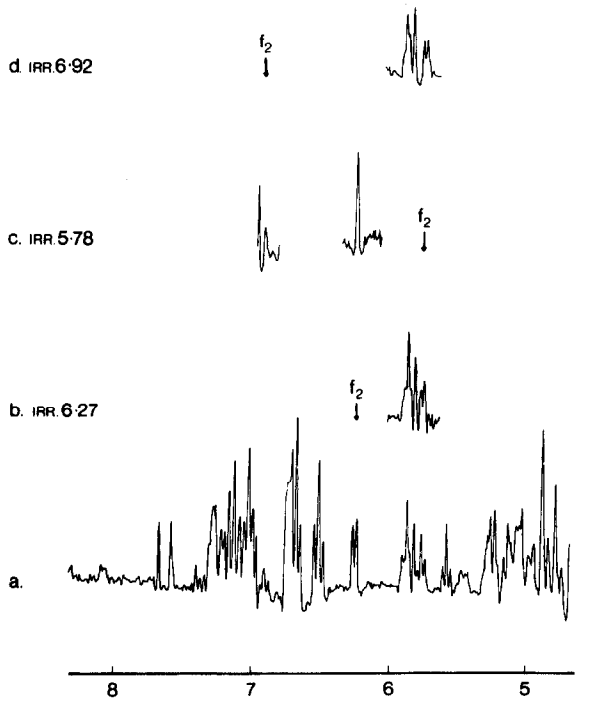

Fig.3. Decoupling of phenylalanine resonances. (a) Aromatic region of the convolution difference spectrum of reduced french-bean plastocyanin. (b) Irradiation at 6.27 ppm causes decoupling at 5.78 ppm. (c) Irradiation at 5.78 ppm causes decoupling at 6.27 and 6.92 ppm. (d) Irradiation at 6.92 ppm causes decoupling at 5.78 ppm.

Table 1
Assignment of resonances in the aromatic region of the ^1H NMR spectrum of reduced french bean plastocyanin

Assignment	Chemical shift (ppm)	Proximity of residue to Cu
His C-2 protons	7.66, 7.57	Ligand
His C-4 protons	7.16, 6.97	Ligand
Tyr-80 or -83	6.51 6.64	Close to Cu
Tyr-83 or -80	6.55 7.11	Remote from Cu
Tyr-80	6.73 7.20	Remote from Cu
Phe *ortho* protons	6.27	Close to Cu
Phe *meta* protons	5.78	Close to Cu
Phe *para* proton	6.92	Close to Cu
Phe *ortho* protons	6.99	Close to Cu
Phe *meta* protons	6.70	Close to Cu
Phe *para* proton	5.88	Close to Cu

The observed coupling pattern for each of the assigned aromatic residues shows that the resonances of the two *ortho* protons on each aromatic ring are equivalent, as are the resonances of the two *meta* protons. This is indicative of rapid rotation of each aromatic residue about the Cβ–Cγ bond. This type of motion has been shown to occur in several other proteins, e.g., cytochrome *c* [8]. The observation that all three tyrosine rings are flipping rapidly is clear indication that tyrosine is not a ligand. Coordination of tyrosine to the copper by way of the phenolate oxygen would restrict rotation of the aromatic ring.

In the spectrum of reduced french-bean plastocyanin several resonances, each with intensity corresponding to three protons, are resolved at fields higher than 0.6 ppm. These are likely to arise from methyl group protons in close proximity to aromatic residues. A sharp singlet at 1.88 ppm in the spectrum obtained using the Carr-Purcell pulse sequence (fig.2) can arise only from the $-CH_3$ protons of one of the two methionines. The methyl group resonance of the second methionine is attributed to a singlet at 0.57 ppm. Only the latter Met resonance is perturbed upon addition of $PtCl_4^{2-}$.

3.2. *Effects of oxidation*

Upon oxidation of Cu(I) plastocyanin resonances of amino acids near the metal are extensively broadened, the effect diminishing rapidly with increasing

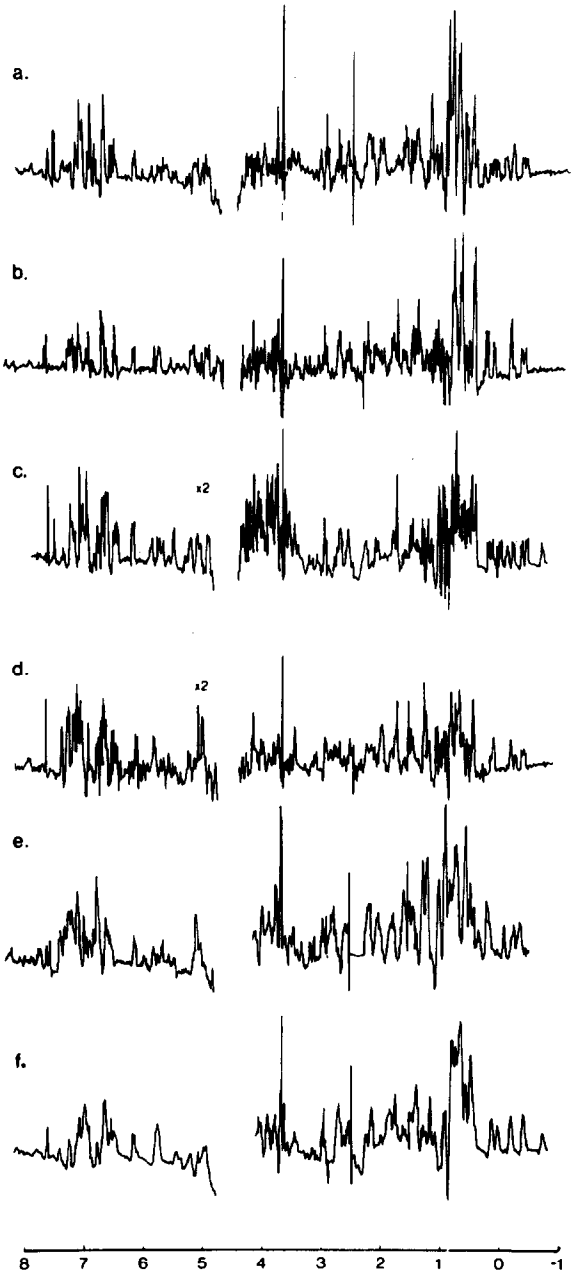

Fig.4. Convolution difference NMR spectra of the reduced plastocyanins from: (a) barley; (b) poplar; (c) cucumber; (d) oleander; (e) carrot; (f) silver beet.

distance from the copper site. Since electron exchange in the presence of ferricyanide is fast on the NMR time scale [1] resonances of amino acids which are very close to the copper will be broadened even upon partial oxidation of the protein. Such resonances are clearly identified in the difference spectrum of fig.1(c). The resonances of protons more distant from the copper are broadened, if at all, only when the protein is fully oxidized.

The histidine proton resonances at 7.66 ppm and 7.57 ppm (C-2 protons) and at 7.16 ppm and 6.97 ppm (C-4 protons) are broadened beyond detection upon partial oxidation of the protein so that both histidine residues must be very near the copper, in accord with [1,2]. The spectra also show several aromatic residues to be close to the copper. These include one of the three tyrosines, with resonances at 6.51 ppm and 6.64 ppm, and at least three phenylalanines. Several of these phenylalanine proton resonances are observed at unusually high fields (viz., the *ortho* and *meta* proton resonances of one Phe at 6.27 ppm and 5.78 ppm, respectively, and the *para* proton resonance of a second Phe residue at 5.88 ppm) which shows that they must be subject to a considerable upfield ring current shift due to neighbouring aromatic residues.

In the aliphatic region the methionine methyl group proton resonances at 1.88 ppm and 0.57 ppm in the spectrum of the Cu(I) protein are broadened beyond detection upon oxidation. A number of other resonances in the aliphatic region of the spectrum are also observed to broaden. These include resonances at 0.35 ppm, 0.18 ppm and –0.30 ppm which clearly arise from methyl group protons in close proximity to aromatic amino acids. In the spectrum of the Cu(II) protein a new broad peak appears at –0.38 ppm.

3.3. *Comparison with plastocyanins from other plant species*

The NMR spectra of the Cu(I) plastocyanins from barley (*Hordeum vulgare*), poplar (*Populus nigra* var. *italica*), cucumber (*Cucumis sativus*), oleander (*Nerim oleander*), carrot (*Dancus carota* var. *sativa*) and silver beet (*Beta vulgaris*) are shown in fig.4. (Independent measurements of the NMR spectrum of spinach plastocyanin have been made [9].) These spectra have several features in common with that of reduced french bean plastocyanin. For each species two sharp resonances which clearly arise from histidine protons are observed between 7.7 ppm and 7.4 ppm. These resonances disappear upon oxidation and intensity changes in the aromatic region are consistent with the presence of several aromatic residues near the copper. A well-resolved doublet invariably occurs at approx. 6.3 ppm and is broadened upon oxidation. This corresponds to a ring-current shifted phenylalanine proton resonance in the spectrum of reduced french bean plastocyanin. The overlapping tyrosine doublets near 6.5 ppm in the spectrum of the french bean protein also appear to be conserved in the spectra of the other plastocyanins. These resonances may be assigned to Tyr-80 and Tyr-83 since only these two tyrosines are always present. Tyr-70 is absent from poplar plastocyanin (Ambler, R. A., personal communication).

Only one of the two methionine methyl group resonances in the spectrum of french bean plastocyanin occurs in the spectra of all the plastocyanins studied. This is the resonance at 0.57 ppm which therefore arises from a methionine which is conserved and close to the copper atom. The unusual position of this resonance probably results from ring current shifts due to neighbouring aromatic residues. The second methionine methyl group resonance is observed near 1.85 ppm in the spectra of all the plastocyanins except those from carrot and barley.

At fields higher than 0.6 ppm several resonances due to aliphatic amino acids in close proximity to aromatic residues are resolved. Species variation in this region clearly reflects differences in sequence or protein conformation. However, upon oxidation peaks with very similar chemical shifts near 0.3 ppm, 0.2 ppm and –0.3 ppm are broadened for all species. Furthermore, a broad peak is observed at approx. –0.4 ppm in the spectrum of each Cu(II) plastocyanin. Since ring-current shifted resonances are highly sensitive to protein conformation these observations suggest conservation of certain aliphatic residues as well as the tertiary protein structure, at least in the vicinity of the copper atom.

4. Conclusions

The present NMR results provide evidence for a high degree of conservation of the protein structure

in the vicinity of the copper centre in plastocyanins from several higher plant species. An earlier proposal [2] that two histidine residues act as copper ligands is supported by the observation that these histidines are present and very close to the copper atom in all the plastocyanins studied. Two histidine ligands have also been proposed in azurin on the basis of NMR studies [10,11]. The NMR spectra of the higher plant plastocyanins show the copper atom to be in close proximity to several hydrophobic aliphatic residues (Ile, Leu, Val and/or Ala residues), one Met and several aromatic residues, including one Tyr and at least one Phe, which remain invariant.

Assignments of the resonances of other amino acids near the copper and quantitative use of the copper ion as an NMR relaxation probe should lead to a more refined model. These studies are being carried out in conjunction with an X-ray crystallographic determination of the structure of poplar plastocyanin [12].

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