Electron–nuclear-double-resonance studies of spinach plastocyanin

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Electron–nuclear-double-resonance (ENDOR) studies of spinach plastocyanin revealed that ^{14}N -hyperfine couplings from its two co-ordinated imidazole groups are characteristically small in anisotropy and relatively high in ENDOR frequencies and that the two imidazole groups are magnetically equivalent. The relatively strong copper–imidazole co-ordinate bonds of plastocyanin are interpreted to be a result of the coexistence of a strong copper–thiol co-ordinate bond.

Several copper proteins have been studied by ENDOR to obtain some structural information on their copper-binding sites (Rist *et al.*, 1970; Roberts *et al.*, 1980; Van Camp *et al.*, 1978, 1982; Stevens *et al.*, 1982), but plastocyanin has not previously been so investigated. Plastocyanin is a well-known 'blue' or 'type 1' copper protein, whose structure is known in full detail by X-ray-diffraction analysis (Fee, 1975; Colman *et al.*, 1978; Murata *et al.*, 1982), and which functions as an electron-transport protein in photosynthesis. We now report the first ENDOR study of spinach plastocyanin in frozen aqueous solutions, in correlation with the structure of its copper-binding site. In addition, we present a discussion of unique ENDOR data on ^{14}N -hyperfine couplings from co-ordinate imidazole groups in copper(II) complexes and copper proteins.

Materials and methods

Plastocyanin was isolated from spinach leaves by the method of Katoh *et al.* (1962) with a slight modification (Takabe & Ishikawa, 1980). The purified oxidized plastocyanin gave an absorbance peak ratio A_{278}/A_{597} of 1.69.

Crystalline powder samples of Cu(II)-doped zinc complexes were isolated from solutions with a copper/zinc ratio of 1:50 (Sandmark & Bränden, 1967; C.-I. Bränden, unpublished work cited by Freeman, 1967; Ashby *et al.*, 1978). These samples are described below as, for example, for hexakis(imidazole)zinc(II) chloride tetrahydrate by $[(\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}})(\text{ImH})_6]\text{Cl}_2 \cdot 4\text{H}_2\text{O}$.

ENDOR experiments were performed in the range

Abbreviation used: ENDOR, electron–nuclear double resonance.

8–30 MHz at temperatures near 5 K with a Varian model E1700 ENDOR spectrometer.

Results and discussion

The observed ENDOR spectra of spinach plastocyanin (Figs. 1*b*, 1*c* and 1*d*) are broad in line shape, as in the case of many other copper proteins and nitrogen-co-ordinated copper(II) complexes in frozen aqueous solutions (Yokoi, 1982). In Fig. 1, ENDOR signals at 10–17 MHz around the free-proton frequency are due to ^1H -couplings. On the other hand, rather broad ENDOR signals at 24–28 MHz are due to ^{14}N -couplings, although there are no resolved nitrogen quadrupole or Zeeman splittings in them. This assignment is verified by the fact that the higher-frequency signal observed at the field setting of D in Fig. 1(*a*) does not remarkably shift to higher frequencies with a change in the e.s.r. microwave frequency from 8.97 to 9.34 GHz, but the lower-frequency signal does by an expected value of 0.65 MHz for ^1H -couplings (Rist *et al.*, 1970). Although the ENDOR frequencies of 24–28 MHz are somewhat high for ^{14}N -couplings in nitrogen-co-ordinated copper(II) complexes, the validity of the above assignment is also supported by consistency with the following facts: plastocyanin contains two co-ordinated nitrogen atoms of imidazole groups with almost usual Cu–N bond lengths (Murata *et al.*, 1982); plastocyanin shows an e.s.r. spectrum of the type indicating that the copper atom is in a cupric state with an unpaired spin density of more than 0.5, in contrast with the case of Cu_A in cytochrome *c* oxidase (Stevens *et al.*, 1982); there are no other ^{14}N -ENDOR signals than the ones at 24–28 MHz in Fig. 1. The observed e.s.r. line shape (Fig. 1*a*) completely excludes the possibility

that another ^{14}N -ENDOR signal might appear at more than 30 MHz. Since there are no indications that the signals at 24–28 MHz consist of two different ^{14}N -couplings in Fig. 1, the two co-ordinating imidazole groups are concluded to be magnetically equivalent.

The ^{14}N -ENDOR signal of plastocyanin progressively shifted the peak position to higher frequencies between 25.7 and 27.5 MHz with changes in the setting field to higher ones between B and D in Fig. 1. The upper and lower limits of such a shifting frequency range are expressed here as A_{max} and A_{min} respectively, and, in all cases in the present work, the A_{max} and A_{min} values could be determined directly from the ^{14}N -ENDOR signals observed by the field settings at the highest-field component of $^{\text{Cu}}A_{\perp}$ and at the lowest-field component of $^{\text{Cu}}A_{\parallel}$ respectively, where $^{\text{Cu}}A_{\perp}$ and $^{\text{Cu}}A_{\parallel}$ are copper hyperfine constants; all the A_{max} and A_{min} values obtained here are listed in Table 1, together with those for related compounds. Hyperfine constants

Fig. 1. E.s.r. and ENDOR spectra of spinach plastocyanin (a) Second derivative e.s.r. spectrum; (b), (c) and (d) ENDOR spectra at the field settings marked by arrows B , C and D in the (a) spectrum respectively. The sample was 2.74 mM in protein, buffered at pH 7.0 [0.05 M 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid], and had a volume of 0.4 ml. Experimental conditions: microwave frequency, 8.97 GHz; microwave power, 5.0 mW; T , approx. 5 K; modulation amplitude, 0.8 mT_{p-p}; sweep rate, 1.25 MHz/min. Abbreviation: DPPH, $\alpha\alpha$ -diphenyl- β -picrylhydrazyl.

due to a co-ordinating nitrogen are generally expressed by two parameters, $A_{\parallel}$ and $A_{\perp}$ ($A_{\parallel} \geq A_{\perp}$) (McGarvey, 1967), which are generally not easy to determine from a frozen-solution type of ^{14}N -ENDOR spectra (Rist & Hyde, 1970). The A_{max} and A_{min} values thus obtained do not always correspond to the $A_{\parallel}$ and $A_{\perp}$ values respectively. From a general consideration of a frozen-solution type of ENDOR and e.s.r. line shapes, it is obvious that a difference between A_{max} and A_{min} proportionally increases with the corresponding difference between $A_{\parallel}$ and $A_{\perp}$. This means that $R = A_{\text{max}}/A_{\text{min}}$ increases with the ratio $A_{\parallel}/A_{\perp}$, as exemplified by the fact that $R > 1.20$ holds as well as $A_{\parallel}/A_{\perp} > 1.20$ for the middle three copper(II) complexes listed in Table 1 (Yokoi, 1982); R is a convenient parameter for anisotropy in ^{14}N -couplings. It is shown by a survey of all nitrogen hyperfine constants so far obtained for nitrogen-co-ordinated copper(II) complexes that the $A_{\parallel}/A_{\perp}$ values for amino nitrogen atoms with near sp^3 hybrid orbitals are 1.5 or so and that those for imino nitrogen atoms with near sp^2 ones are 1.3 or so (Schweiger, 1982).

An inspection of Table 1 clearly indicates that $R < 1.10$ for all the copper(II) complexes of imidazole and/or imidazolate anion and for plastocyanin and many other copper proteins, indicating that ^{14}N -couplings in the imidazole co-ordination are much smaller in anisotropy than those in the other kind of nitrogen co-ordination. Also, Höhn *et al.* (1983) have demonstrated the same fact by detailed ^{14}N -ENDOR experiments on nitrosylhaemoglobin. $R < 1.10$ in the imidazole co-ordination is now regarded as experimentally established, but the reason for this is still somewhat uncertain. From the above dependence of $A_{\parallel}/A_{\perp}$ on the hybridization of nitrogen orbitals a possible explanation might be in terms of a p -character as small as $sp^{0.7}$ for the hybrid orbitals of imidazole nitrogen atoms in the σ -co-ordination bonding (Brown & Hoffman, 1980). In conclusion, however, the R value can now be used as a guide for identification of imidazole co-ordination in copper proteins and copper-substituted metalloenzymes, as has been shown to be applicable to plastocyanin in the present work.

The values of $A = (A_{\text{max}} + 2A_{\text{min}})/3$ for the copper(II) complexes of imidazole and/or imidazolate anion increase with decreasing $g_{\parallel}$ values (Table 1; the $g_{\parallel}$ values in its footnote). $[(\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}})(\text{ImH})_6]\text{Cl}_2 \cdot 4\text{H}_2\text{O}$ has the smallest A and largest $g_{\parallel}$ values, and hence the weakest in-plane co-ordinate bonds (McGarvey, 1967); a weakening of the bonds in this complex is reasonably understood as a result of a strong axial field in its slightly distorted octahedral co-ordination. Accordingly, the magnitude of A in the imidazole co-ordination can be regarded as a measure of the strength of a copper-imidazole co-ordinate bond, as in the case

Table 1. ^{14}N -ENDOR of copper proteins and nitrogen-co-ordinated copper(II) complexes

The samples are frozen aqueous solutions unless otherwise noted (the pH values were adjusted at room temperature). Abbreviations: en, ethylenediamine; gly, glycinate anion; ImH, imidazole. $R = A_{\text{max.}}/A_{\text{min.}}$. Errors in R : ± 0.03 for plastocyanin; ± 0.02 for the copper(II) complexes.

Compound	^{14}N -ENDOR frequency (MHz)		R	Reference
	$A_{\text{max.}}$	$A_{\text{min.}}$		
Plastocyanin	27.5	25.7	1.07	Present work
Stellacyanin	18.0	17.5	1.03	Rist <i>et al.</i> (1970); Yokoi (1982)
	23.5	22.5	1.04	
Cytochrome <i>c</i> oxidase	8.81	8.54	1.03	Van Camp <i>et al.</i> (1978)
Cyanide derivative of bovine erythro- cyte superoxide dismutase	19	18	1.06	Van Camp <i>et al.</i> (1982)
	27.5	23	1.20	
$[\text{Cu}^{\text{II}}(\text{NH}_3)_4]^{2+}$, pH 10	19.6	15.9	1.23	Yokoi (1982)
$[\text{Cu}^{\text{II}}(\text{en})_2]^{2+}$, pH 10	19.7	13.8	1.43	Yokoi (1982)
$[\text{Cu}^{\text{II}}(\text{gly})_2]$, pH 10	17.9	14.7	1.22	Yokoi (1982)
$[\text{Cu}^{\text{II}}(\text{ImH})_4]^{2+}$, pH 6.4–8.0	21.1	20.1	1.05	Yokoi (1982)
$[(\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}})(\text{ImH})_6]\text{Cl}_2 \cdot 4\text{H}_2\text{O}^*$	18.0	16.7	1.08	Present work
$[(\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}})(\text{ImH})_2(\text{Im}^-)]\text{NO}_3^*$	21.4	19.8	1.08	Present work
$[(\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}})(\text{Im}^-)]^*$	21.7	20.8	1.04	Present work

* Crystalline powder samples. The e.s.r. parameters of the three powder samples in the order shown above are as follows: $g_{\parallel} = 2.313$, $g_{\perp} = 2.07$, $10^4 \times A_{\parallel} = 165 \text{ cm}^{-1}$; $g_{\parallel} = 2.268$, $g_{\perp} = 2.06$, $10^4 \times A_{\parallel} = 167 \text{ cm}^{-1}$; $g_{\parallel} = 2.244$, $g_{\perp} = 2.05$, $10^4 \times A_{\parallel} = 194 \text{ cm}^{-1}$ ($g_{\parallel} = 2.268$, $g_{\perp} = 2.06$, $10^4 \times A_{\parallel} = 182 \text{ cm}^{-1}$ for $[\text{Cu}^{\text{II}}(\text{ImH})_4]^{2+}$, pH 6.4–8.0).

of usual super-hyperfine couplings (McGarvey, 1967). Plastocyanin has the largest A value in Table 1, indicating that it contains the strongest copper–imidazole co-ordinate bonds. This result is probably rationalized in terms of effects of the co-existence of an extremely strong copper–thiol bond, because a strong co-ordinate bond in a copper-binding site can make the other co-ordinate bonds stronger, in accordance with the rule of average environment (Jørgensen, 1962; Yokoi *et al.*, 1971). On the other hand, stellacyanin, which is another 'blue' copper protein, of no known function and structure, has also been inferred to contain two imidazole groups as ligands (Hill & Lee, 1979). In addition to the identification of imidazole co-ordination by R , Table 1 indicates that the two co-ordinating imidazole groups are not magnetically equivalent, in contrast with the case of plastocyanin, and that the copper–imidazole co-ordinate bonds of stellacyanin are much weaker than those of plastocyanin. These differences between the two copper proteins may reflect differences in biological functions; no further discussion on this aspect can be given at present, because little is known of stellacyanin.

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