ELECTRON SPIN ECHO SPECTROSCOPIC STUDIES OF TYPE 1 AND TYPE 2 COPPER IN *RHUS VERNICIFERA* LACCASE AND IN *CUCURBITA PEPO MEDULLOSA* ASCORBATE OXIDASE

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1. Introduction

Ascorbate oxidase and laccase are copper proteins that contain metal ions in at least 3 different chemical environments, 2 of which give rise to EPR [1,2]. In the oxidized state, both proteins are intensely blue and the color in each of them arises from a mononuclear CU(II) site, designated type 1 [3] which is believed to be analogous to sites in the low M_r blue copper proteins, stellacyanin, azurin and plastocyanin. In the latter 3 proteins, the copper is ligated by cysteinyl sulfur (which gives rise to an $RS^- \rightarrow Cu(II)$ charge transfer responsible for the intense blue color [4,5]) and by imidazole nitrogen [6–12]. In plastocyanin and azurin, at a distance too far for charge transfer, there is a methionyl sulfur [11,12] which is absent in stellacyanin [13,14].

A second copper site found in ascorbate oxidase and laccase, designated type 2, contains a metal ion in an environment which is not unlike that found in Cu(II) peptide complexes [15]. Except for the presence of an imidazole nitrogen in laccase [16], very little is known about the chemical nature of the type 2 binding site in either protein.

A useful method for probing Cu(II)–imidazole interactions in copper proteins and in models is provided by electron spin echo (ESE) spectroscopy [17,18]. With this technique, one observes periodicities in the electron spin echo decay envelope which arise from the interaction of the electron spin of Cu(II) with the remote ^{14}N of ligated imidazole [19]. From an analysis of the data, one obtains a ^{14}N super-hyperfine spectrum which is characterized by nuclear quadrupolar parameters the same as those observed for protonated nitrogen in imidazole [20].

Here, we compare the super-hyperfine frequencies of the remote ^{14}N in Cu(II) coordinated imidazole found in type 1 and type 2 sites in laccase and in ascorbate oxidase. We find that whereas the ^{14}N frequencies of type 1 Cu(II) are similar to those in stellacyanin and in a variety of imidazole containing models, the frequencies observed for the type 2 sites are different.

2. Materials and methods

Laccase was prepared from *Rhus vernicifera* lac acetone powder (Saito and Co., Tokyo) as in [21]. Type 2 copper was removed from the holoprotein by a modification of the procedure in [22]. Before treatment with dimethylglyoxime, laccase was first passed through a Chelex resin column pre-equilibrated against 0.05 M phosphate buffer (pH 7.0). The final preparation of protein freed of type 2 copper, as well as the holoprotein used in this study, were also passed through Chelex.

Ascorbate oxidase from green zucchini squash was prepared as in [23], and type 2 copper was removed from the preparation as in [24].

Spin echo measurements were made at liquid He

Abbreviations: NQR, nuclear quadrupole resonance; M_r, relative molecular mass

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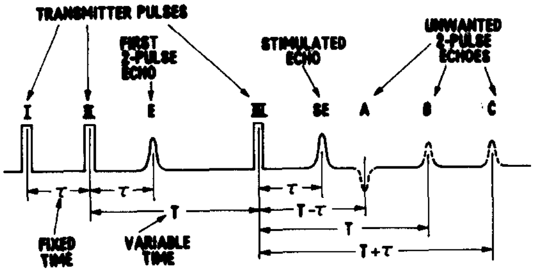

Fig.1. Transmitter pulse sequence and spin echo signals observed in a stimulated echo experiment. In the experiments reported here τ was set to selected fixed values and T was slowly increased. The amplitude of the stimulated echo was measured by sample-hold (boxcar) circuits. The unwanted echoes C, B, A are 2-pulse echoes generated by combinations of pulse III with pulse I, pulse II or the first 2-pulse echo E. An inhomogeneous magnetic field, applied as a short pulse just before pulse III, scrambles the phase relationships underlying the 2-pulse echo generating mechanism and thus eliminates the unwanted echoes. It has no effect on the stimulated echo.

temperatures with an X-band spectrometer [7]. The 3-pulse, or stimulated echo decay envelope, was measured by varying T, the time between transmitter pulses 2 and 3, and it was plotted as a function of the total time $T+\tau$ between pulses 1 and 3 ([25], fig.1). Values of τ, the interval between transmitter pulses 1 and 2, were judiciously chosen to be a whole number multiple of the proton period. This was done to eliminate the proton modulation pattern from the echo envelope. Spurious peaks or 'glitches' which result from overlap of different kinds of spin echo signals in the 3 pulse envelope were eliminated from the data by a method described in the appendix. The spin echo decay envelopes were manually extended to zero time and were Fourier transformed as in [26].

For studies of type 2 copper in holo laccase and ascorbate oxidase, the magnetic field used in the experiments was set at a position in the EPR spectrum where type 1 copper no longer contributes. Measurements of type 1 copper were made near $g_\perp$ on samples from which type 2 copper had been removed. In these experiments it was assumed that the removal of type 2 copper from the protein did not alter the magnetic interactions of type 1 copper.

3. Results and discussion

When imidazole is ligated to Cu(II) the magnetic coupling between the electron spin of the copper and the nuclear spin of the directly coordinated ^{14}N is large and can be studied by conventional, continuous wave EPR, where the super-hyperfine interaction is recognized in the derivative spectrum. The directly coordinated ^{14}N can also be studied by ENDOR [27]. The coupling between the remote ^{14}N of ligated imidazole is weak, however, and cannot be observed as a fine structure in EPR. Moreover, it would appear that, although ENDOR studies of the Cu(II):imidazole system can be used to detect the remote ^{14}N, the ENDOR method yields an incomplete super-hyperfine spectrum [27] and is not well-suited for the determination of ^{14}N nuclear quadrupolar interactions.

When the Cu(II):imidazole system is examined at fields $\simeq$3000 G (as in X-Band EPR experiments) the nuclear Zeeman term $g_N\beta_N H \,.\, I$ is about equal in magnitude to the electron nuclear coupling term $AI \,.\, S$ [19]. Thus, for one electron spin orientation, the nuclear Zeeman term is effectively doubled by the electron nuclear coupling and for the other, it is cancelled out (i.e., the externally applied magnetic field is cancelled by the local field of the nucleus due to the electron). Both of these situations result in the appearance of lines in the echo envelope spectrum (fig.2,3), the case of cancellation being the more interesting one since it yields a set of lines near 0.7 and 1.4 MHz whose frequencies are determined almost entirely by the ^{14}N nuclear quadrupole interaction. These frequencies are close to the zero field quadrupolar frequencies 0.698, 0.719 and 1.417 which have been reported for the protonated ^{14}N of imidazole [20]. The electron spin orientation which corresponds to addition of the nuclear Zeeman term and the electron nuclear coupling term yields a single line at $\simeq$4 MHz. This line corresponds to a transition between the outer two ^{14}N superhyperfine levels (the $\delta M_I = 2$ transition), the remaining 2 transitions giving lines which are generally too broad to detect.

Type 1 copper, as found in laccase and ascorbate oxidase, is coordinated by an imidazole. This assignment is based on the echo envelope spectra shown in fig.2B,3B where the frequencies characteristic of remote protonated ^{14}N of imidazole ligated to Cu(II) are observed. Similar spectra are obtained with stellacyanin [28], imid-Cu(II)–dethylenetriamine [28] and the model Cu(II)–(His)$_2$ [29]. Thus, the type 1 copper sites in ascorbate oxidase and laccase are shown to contain at least one imidazole, and to be in this way structurally similar to type 1 copper in azurin and plastocyanin [11,12].

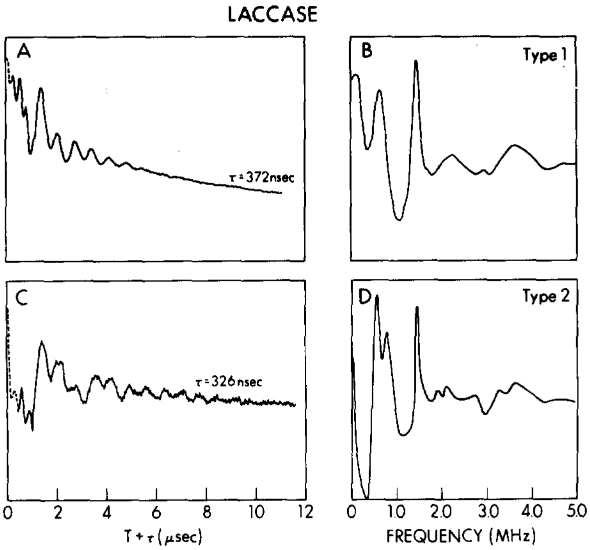

Fig.2. Envelopes of electron spins and echo envelope spectra for type 1 and type 2 Cu(II) in laccase. In A, a spin echo study was made of the type 1 Ci(II) site in a protein sample from which type 2 Cu(II) had been removed. In C, type 2 Cu(II) was studied in the holoprotein at a magnetic field setting at which type 1 Cu(II) did not absorb. Manual extensions of the data to zero time are indicated by the dashed lines and the corresponding Fourier transforms are shown in B and D. The magnetic field settings and spectrometer frequency were 3120 *g* and 8.958 GHz for A and 9263 *g* and 2.879 GHz for C.

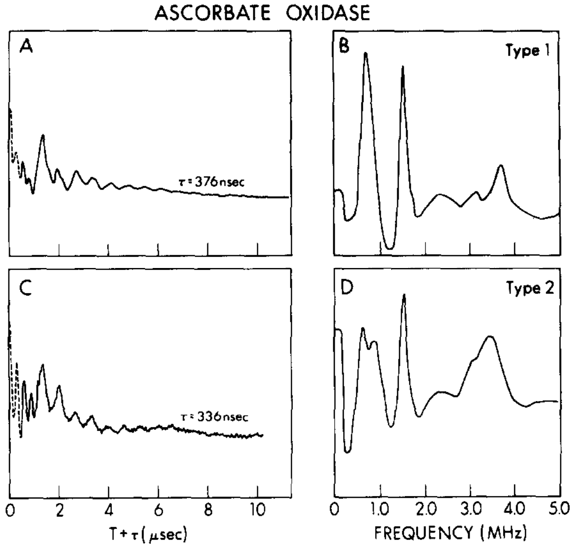

Fig.3. Envelopes of electron spins and echo envelope spectra for type 1 and type 2 Cu(II) in ascorbate oxidase. In A, a spin echo study was made of the type 1 Cu(II) site in a protein sample from which type 2 Cu(II) had been removed. In C, type 2 Cu(II) was studied in the holoprotein at a magnetic field setting at which type 1 Cu(II) did not absorb. Manual extensions of the data to zero time are indicated by the dashed lines and the Fourier transforms are shown in the panels to the right of the echo envelopes. The magnetic field settings and spectrometer frequency were 3100 *g* and 8.850 GHz for A and 2760 *g* and 8.980 GHz for C.

The presence of imidazole at the type 2 copper sites in ascorbate oxidase and laccase is also demonstrated in these studies. Frequencies ~0.7, 1.4 and 4 MHz attributed to imidazole coordination are observed in the spectra (fig.2D,3D). There is a small significant difference, however, in the super-hyperfine frequencies of type 1 copper as compared to those for type 2 copper. In the latter case, the 0.7 MHz line is split resolving the line into 2 frequencies separated by ~0.2 MHz. The splitting of this line was also seen when measurements were made with different values of τ. Splittings of lesser or greater magnitude at this frequency were seen for the type 2 copper sites in galactose oxidase [30], serum amine oxidase [31] and holo superoxide dismutase [32] but were not seen in simple Cu(II) model compounds or in superoxide dismutase from which the Zn(II), normally bound to the remote ^{14}N of imidazole coordinated Cu(II), had been removed.

It is not possible to infer unambiguously from these data the nature of the difference between the imidazole ligands in types 1 and 2 copper in ascorbate oxidase and laccase. However, a number of NQR[1] measurements on imidazole and on related compounds suggest a reason. The splitting of the 0.7 MHz line indicates the presence of a small perturbation in the ^{14}N quadrupolar field. Thus, for example, in urocanic acid, where a –CHCOO group is substituted for –H at the C_4 position in the imidazole ring, the 3 zero field quadrupolar frequencies for the protonated ^{14}N become 0.565, 0.851 and 1.416 MHz [30]. It will be noted here that the lower two frequencies (ν_0,ν_-) are shifted by more than the high frequency (ν_+) in relation to the values obtained in unsubstituted imidazole. Similar observations were made [33] by measuring the zero field quadrupolar frequencies for the protonated nitrogen in a number of diamagnetic metal complexes involving imidazole.

In interpreting the results in [33], variations in the ^{14}N NQR parameters for the amino nitrogen of imidazole have been suggested to be primarily due to variations in hydrogen bonding [33,34]. It seems not unlikely therefore that the splitting of the ≃0.7 MHz line which we observe in type 2 copper centers originates in a similar manner. Within the protein structure, the proton on the remote ^{14}N of metal-ligated imidazole may not be completely insulated from its environment and, as suggested for the proximal imidazole in hemoglobin and myoglobin [35], may be hydrogen-bonded to the protein structure. In model compounds and simple amino acid and peptide complexes, this type of hydrogen bonding may not be present, or may be insufficient to shift the nuclear quadrupolar frequencies ν_0 and ν_1 far enough apart to give 2 resolved peaks in the echo envelope spectrum.

We have demonstrated that both types 1 and 2 Cu(II) in ascorbate oxidase and laccase are ligated to an imidazole. Type 1 copper differs from type 2 copper in that the 0.7 MHz line that appears in the superhyperfine spectrum of type 1 copper is split in the spectrum of type 2 copper. Consideration of published data on imidazole derivatives and on imidazole containing complexes suggests that this splitting is indicative of weak hydrogen bonding between the remote nitrogen of the imidazole and the protein structure.

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Appendix: The elimination of unwanted 2-pulse echoes

A problem occurs in making a 3-pulse echo envelope recording because of the fact that pairs of pulses (e.g., I, III, or II, III in fig.1) are able to generate echoes by the 2-pulse mechanism. For certain times *T* these unwanted echoes overlap the stimulated echo and thus cause a bump or 'glitch' to appear in the envelope.

The echo overlap problem was eliminated in this work by applying a magnetic field pulse to the sample between transmitter pulses II and III. This 'spoiler' pulse scrambles the phase relationships underlying the the 2-pulse echo generating mechanism, but has no effect on the stimulated echo. The required spoiler pulse was timed to occur immediately before pulse III. It had a duration ≃70 ns and a maximum amplitude ≃4 G. The pulse was applied to the coil in an ENDOR cavity of the type described elsewhere [36]. The best result (i.e., the most effective elimination of 2-pulse echoes) was obtained by winding an ENDOR coil with reversal of the winding sense halfway along the coil. This produced an extremely inhomogeneous magnetic field pulse, thus maximizing the disturbance of phase relationships in the system of processing spins.

An equivalent result can be obtained by reversing the microwave phase between pulses II and III in alternate electron spin echo cycles [37]. The 3-pulse echo is unaffected by this phase reversal, but 2-pulse echoes, involving pulses I and III or II and III, undergo a change of sign. In a measurement obtained by averaging many spin echo amplitudes the 2-pulse echo contributions would therefore average to zero and would not affect the 3-pulse envelope. This method requires an additional microwave component to produce a rapid phase reversal but has the obvious advantage of not requiring a microwave cavity containing a special coil.