Determination by High Field NMR Spectroscopy of the Longitudinal Electron Relaxation Rate in Cu(II) Plastocyanin from *Anabaena variabilis*

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The biological function of metalloproteins stems from the unusual geometric and electronic structures of their metal sites.[1] The relaxation of the unpaired electrons of the proteins depends on these unusual characteristics and is, therefore, a potential source of information about the structure and function of the proteins.[1,2] Also, precise knowledge of the electron relaxation is a necessary prerequisite of a determination of distances from the paramagnetic relaxations of the ligand nuclei.[3,4] An accurate determination of the electron relaxation in metalloproteins is, therefore, highly desirable. For Cu(II) complexes electron relaxation times in the range from 0.6 to 13 ns at low magnetic field strengths have been determined using nuclear magnetic relaxation dispersion.[2,5] Recently an approximate range from 0.2 to 0.8 ns was estimated for the copper electron relaxation time in spinach plastocyanin on the basis of the X-ray structure and proton relaxation rates obtained at 18.8 T.[6] Still, little is known about the electron relaxation and its field dependence at the high magnetic field strengths used nowadays in protein NMR studies.

Here we present a structure-independent NMR approach that allows a precise determination of the size and the field dependence of the longitudinal relaxation rate of the electron in blue copper proteins, *for example, Anabaena variabilis* plastocyanin (*A.v.* PCu(II)), at high magnetic field strengths. The approach is based on the field dependence of the longitudinal paramagnetic relaxation R_{1p} of ^{1}H and ^{13}C nuclei in the protein.

In Cu(II) proteins the R_{1p} relaxation of the ligand nuclei caused by interaction between the nuclear spin I and the unpaired electron spin S ($^1/_2$ for Cu(II)) is given by:[3,7]

$$R_{1p}\,\frac{2}{5}\left(\frac{\mu_0}{4\pi}\right)^2 S(S+1)g_e^2\,\mu_B^2\,\gamma_I^2\,\Delta^2\left[\frac{\tau_{c,1}}{1+\omega_I^2\,\tau_{c,1}^2}\right] \qquad (1)$$

Here $\tau_{c,1}^{-1} = R_{1e} + \tau_R^{-1} + \tau_j^{-1}$, where R_{1e} is the longitudinal relaxation rate of the unpaired electron, τ_R^{-1} is the reorientation rate of the nuclei, and τ_j^{-1} is the rate of exchange processes that modulate the $I-S$ interaction, that is, the electron self-exchange (ESE) rate in the case of *A.v.* PCu. Further, ω_I and γ_I are the nuclear Larmor frequency and gyromagnetic ratio, respectively, Δ is a parameter that depends on the metal−nucleus distance and the fraction of the unpaired electron spin delocalized to the ligand nuclei,[3,7] μ_B is the Bohr magneton and g_e the electron g-value.

If the ESE rate is sufficiently fast, as is the case with blue copper proteins, the R_{1p} rates can be obtained from experimental R_{1o} and R_{1d} rates, that is, the rates in a partly oxidized (paramagnetic) and a reduced (diamagnetic) sample, respectively, using generalized equations for relaxation in a two-site exchange system.[8] For such systems the relaxation is *bi*-exponential, in general, and *mono*-exponential in the fast- and slow-exchange limits,[9] i.e. for $\tau_j^{-1} \gg R_{1p}$ and $\tau_j^{-1} \ll R_{1p}$, respectively, $R_{1p} + R_{1d}$ being the total R_1 rate in the paramagnetic site. However, also in intermediate cases where $\tau_j^{-1} \gg R_{1p}$ the relaxation in the diamagnetic site is *mono*-exponential for all practical delay times (≥10 ms). Under these conditions R_{1p} is given by:[8]

$$R_{1p} = \frac{(R_{1o} - R_{1d} - k_{ese}\cdot c)\cdot(R_{1o} - R_{1d})}{R_{1o} - R_{1d} - k_{ese}\cdot c\cdot f_p} \qquad (2)$$

where k_{ese} is the ESE rate constant (3.2×10^5 M^{-1} s^{-1} for *A.v.* PCu at 298 K and pH 7.5),[10,11] c is the total protein concentration and f_p is the fraction of oxidized protein. For *A.v.* PCu(II) and other blue copper proteins $\tau_j^{-1} = k_{ese}\cdot c \gg R_{1d}$. Hence eq 2 applies to these proteins.

The R_{1p} rates for a series of protons in *A.v.* PCu(II) were determined at 9.4, 11.7, and 17.6 T.[12] The plots of proton R_{1p}^{-1} rates vs ω_I^2 in Figure 1 immediately reveal that $\tau_{c,1}$ is field dependent. Thus, according to eq 1 the nonlinearity of the plots is compatible only with a variation of $\tau_{c,1}$ in the observed field range. This is further supported by the R_{1p} rates of a series of α-carbons measured at 11.7 and 17.6 T. According to eq 1 the *increase* of these rates with increasing field strength is compatible only with a $\tau_{c,1}$ that increases with the field in combination with the condition $\omega_I^2\,\tau_{c,1}^2 < 1$. This conclusion is further illustrated by the plots of the normalized ^{1}H and ^{13}C R_{1p} rates vs $\tau_{c,1}$ that are shown in Figure 2.

Since R_{1e} is the only component of $\tau_{c,1}^{-1}$ that can be field dependent, the strong field dependences of R_{1p} for both ^{1}H and ^{13}C indicate that R_{1e} dominates $\tau_{c,1}^{-1}$ in the applied field range. In contrast, the exchange rate τ_j^{-1} can be ignored since the ESE rate ($k_{ese}\cdot c$) is of the order of 10^3 s^{-1} at the *A.v.* PCu concentration used here.[10] Consequently, the effective correlation time is given by $\tau_{c,1}^{-1} = R_{1e} + \tau_R^{-1}$. Previously[15] a value of 1.6×10^8 s^{-1} was obtained for τ_R^{-1} at 298 K ($\tau_R = 6.2$ ns) in good agreement with the size of *A.v.* PCu (M_r 10.5 kDa). It is assumed here that all ligand nuclei experience the same R_{1e} rate i.e. the electron

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(11) Variation of k_{ese} in the range from 2.0×10^5 to 4.0×10^5 M^{-1} s^{-1} has only minor influence on the ratios of the resulting R_{1p} rates at the two magnetic field strengths applied here. The k_{ese} rate estimated from the paramagnetic enhancement of the line widths observed in this study was well within this range and in good agreement with the published value.[10]
(12) *A.v.* PCu was purified and the NMR samples in 99.9% D_2O (2.9 mM, pH 7.1) were prepared as described previously.[13] The oxidized *A.v.* PCu(II) was prepared by adding an equimolar amount of $K_3[Fe(CN)_6]$ to *A.v.* PCu(I). The partially oxidized samples were obtained by mixing the appropriate amounts of *A.v.* PCu(I) and *A.v.* PCu(II). The NMR tubes were sealed off under nitrogen. 1D ^{1}H inversion−recovery relaxation experiments, and 2D ^{13}C heteronuclear R_1 relaxation experiments[14] with ^{13}C in natural abundance were recorded for reduced (R_{1d}) and 18% oxidized (R_{1o}) *A.v.* PCu at 298 K and ^{1}H-frequencies of 400, 500, and 750 MHz, and ^{13}C-frequencies of 125 and 188 MHz, using Varian Unity 400, Unity Inova 500, and Unity Inova 750 spectrometers.
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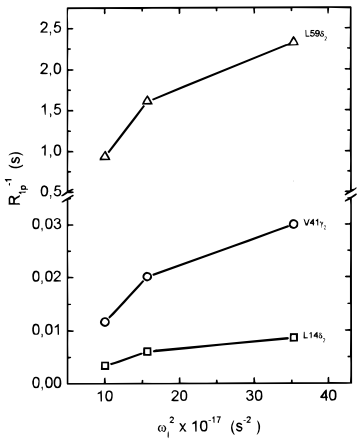

Figure 1. The (nonlinear) dependence on ω_I^2 of the paramagnetic ^{1}H R_{1p} rates of Leu(14) H$^\delta$ (□), Val(41) H$^\gamma$ (○), and Leu(59) H$^\delta$ (△) in *A.v.* plastocyanin. The rates correspond to the ^{1}H frequencies 400, 500, and 750 MHz, respectively.

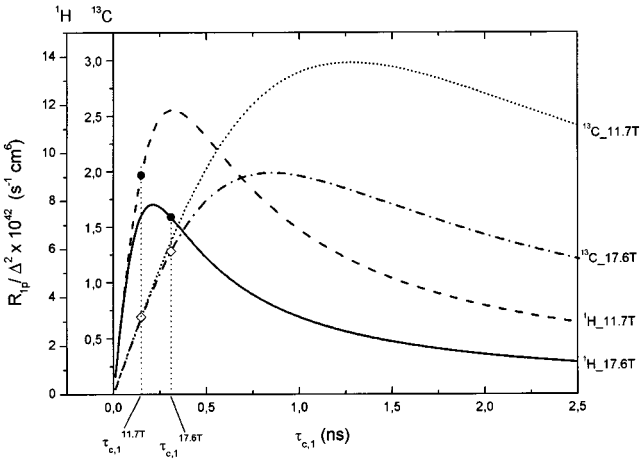

Figure 2. Normalized R_{1p} vs $\tau_{c,1}$ according to eq 1; solid line, ^{1}H at 17.6 T; dashed line, ^{1}H at 11.7 T; dotted-dashed line, ^{13}C at 17.6 T; dotted line, ^{13}C at 11.7 T. The specific R_{1p} rates that are indicated (●, ^{1}H and ◇, ^{13}C) correspond to the obtained R_{1e} rates.

relaxation is isotropic, and that possible differences in the τ_R^{-1} rates of the individual nuclei are negligible, or have no effect on $\tau_{c,1}^{-1}$. The former holds to a good approximation because of the moderate anisotropy of the g tensor of plastocyanins.[16] The latter was confirmed by a ^{15}N relaxation study[15] which showed that the backbone of the *A.v.* PCu molecule is rigid except for the C- and N-terminals.

According to eq 1 the ratio $R_{1p}^{11.7T}/R_{1p}^{17.6T}$ for a given nucleus depends only on the spectral density term, and thereby only on two unknowns, i.e. the R_{1e} rates at 11.7 and 17.6 T, respectively. Furthermore, within the experimental uncertainties the ratio will be the same for nuclei of a given kind, that is ^{1}H or ^{13}C, but different for the two sets of nuclei because of their different Larmor frequencies. Therefore, in principle only two different R_{1p} ratios, one from a ^{1}H nucleus and one from a ^{13}C nucleus, are needed to determine the electron relaxation rate. Here the R_{1e} rates at the two magnetic field strengths were determined from a least-squares fit of eq 1 to the experimental R_{1p} ratios of ten

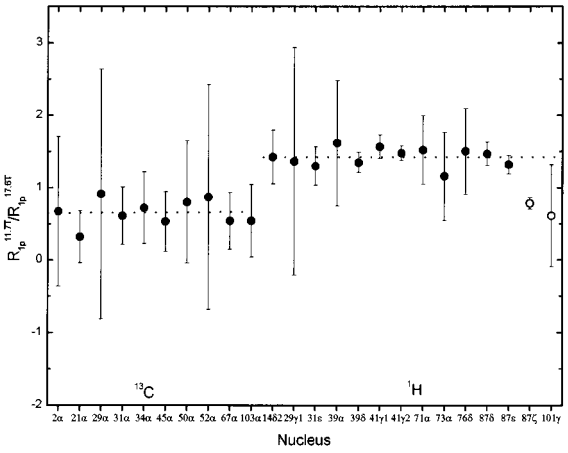

Figure 3. Simultaneous least-squares fit (− − −) of eq 1 to the experimental R_{1p} ratios of ten α-carbons and three α-protons (●). The error bars indicate the uncertainties of the R_{1p} ratios. The uncertainties of the experimental R_{1o} and R_{1p} rates and the f_p ratio were included in the calculations of the R_{1p} rates (eq 2) and the $R_{1p}^{11.7T}/R_{1p}^{17.6T}$ ratios. An additional eleven side chain protons were investigated. For nine of these the experimental $R_{1p}^{11.7T}/R_{1p}^{17.6T}$ ratios are in good agreement with the obtained R_{1e} rates (●). Therefore, $\tau_R^{-1} \ll R_{1e}$ also for these nuclei. For Phe87 H$^\delta$ and Ile101 H$^\gamma$ (○) the ratios are too small, indicating a higher mobility of these nuclei. For protons, $R_{1p}^{11.7T}/R_{1p}^{17.6T} \leq 1$ for $\tau_{c,1}^{11.7T} \leq 0.13$ ns according to eq 1 while $\tau_{c,1}^{11.7T} = 0.13$ ns gives $\tau_R = 0.5$ ns for $R_{1p}^{11.7T} = 5.8 \times 10^9$ s^{-1}. This suggests that the observed side chain protons have effective rotational correlation times $\tau_R \geq 0.5$ ns, except for Phe87 H$^\zeta$ and the Ile101 CH$_3^\gamma$ where $\tau_R \leq 0.5$ ns.

α-carbons and three α-protons as shown in Figure 3. Thus, only R_{1p} rates of nuclei of the rigid backbone of the molecule were used for the determination in order to exclude any possible influence on $\tau_{c,1}$ from the higher mobility of side chain nuclei. The obtained R_{1e} rates were $(5.8 \pm 0.5) \times 10^9$ s^{-1} and $(2.6 \pm 0.4) \times 10^9$ s^{-1} at 11.7 and 17.6 T, respectively. Hence, R_{1e} dominates $\tau_{c,1}^{-1}$.

In conclusion the proposed method applies to blue copper proteins with fast ESE rates and does not require any knowledge of the structure of the protein. It should be applicable also to other metalloproteins even when no exchange takes place, as long as the R_{1p} rates 1) depend on R_{1e} and 2) can be obtained for different kinds of nuclei at two field strengths where the rates are field-dependent. The latter condition is, in most cases, provided by the high field NMR spectrometers available nowadays. If no exchange takes place $R_{1p} = R_{1o} - R_{1d}$, where R_{1o} is the observed nucleus relaxation in the paramagnetic species.

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Supporting Information Available: Table of the R_{1d}, R_{1o}, and R_{1p} rates at 11.7 and 17.6 T used for the determination of the electron relaxation of Cu(II) in *A.v.* plastocyanin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org. This material has also been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under BMRB accession number 4738.

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