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Determination of the electron self-exchange rates of blue copper proteins by super-WEFT NMR spectroscopy

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Abstract

An NMR approach for determining the electron self-exchange (ESE) rate constants in blue copper proteins is presented. The approach uses the paramagnetic relaxation enhancement of resonances in 1D ^{1}H super-WEFT spectra of partly oxidized (paramagnetic) proteins. These spectra allow a more precise determination of the relevant paramagnetic linebroadenings than conventional 1D ^{1}H spectra and, thus, permit a more detailed investigation of the applicability of the linebroadenings for determining the electron exchange rates. The approach was used to estimate the ESE rate constant of plastocyanin from *Anabaena variabilis*. It was found that, although the rate constant can be determined accurately from a series of resonances, precise but erroneous constants are obtained from the resonances of the copper-bound residues, unless a narrow splitting of these resonances caused by the presence of two conformations is taken into account. As demonstrated here, this complication can be overcome by a correct analysis of the paramagnetic broadening of the *combined* double signals. Because of the high resolution and specific sensitivity of the approach it should be generally applicable to estimate electron transfer rates, **k**, if the paramagnetic relaxation enhancement R_{2p} of the resonances can be determined, and the conditions $\mathbf{k} \ll R_{2p}$ or $\Delta\omega_p \gg \mathbf{k} \gg R_{2p}$ are fulfilled, $\Delta\omega_p$ being the frequency separation between corresponding diamagnetic and paramagnetic sites.

Abbreviations: A.v., Anabaena variabilis; PCu, plastocyanin; 1FA4, PDB accession code for the solution structure of *A.v.* PCu (Ma et al., 2000); ET, electron transfer; ESE, electron self-exchange; WEFT, water-eliminating Fourier transformation; FID, free induction decay; FT, Fourier transformation; LP, linear prediction; IR, inversion recovery; HSQC, heteronuclear single quantum coherence.

Introduction

Electron exchange rates play a salient role in studies of the function and mechanism of electron transfer (ET) reactions of plastocyanin (PCu) and other blue copper proteins (Adman, 1991). The biological role of PCu is photosynthetic electron transfer from the cytochrome b_6/f complex to photosystem I. The rate and mechanism of the electron transfer can be investigated conveniently through studies of the electron self-exchange (ESE) process that characterizes PCu and other blue copper proteins. In this process an electron is being reversibly exchanged between the reduced and oxidized form of the protein, that is, PCu(I) + PCu(II) $\rightleftharpoons$ PCu(II) + PCu(I) in the case of PCu.

At the relatively high protein concentrations that are normally used in NMR studies, the ESE rates are sufficiently high to affect the NMR signals significantly. A convenient method for determining the rate constants of electron exchange is to use the enhancements of the longitudinal or transverse relaxation rates of the resonances of the nuclei near the

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copper ion. Previously it was demonstrated (Lommen et al., 1988; Dennison et al., 1993) that the ESE rates could be estimated from the linebroadening of selected ^{1}H resonances in conventional 1D spectra of the protein. However, this approach is hampered by the low spectral resolution inherently associated with 1D ^{1}H spectra of proteins. This drawback is further amplified by the severe linebroadening that often characterizes NMR spectra of paramagnetic metallo-proteins, and in particular those of paramagnetic copper proteins.

Here we present an alternative NMR approach that is based upon the super-WEFT experiment (Inubushi and Becker, 1983). This experiment allows the observation of resonances with fast R_1 relaxation rates while suppressing the slowly relaxing signals. Therefore, the super-WEFT spectra have fewer resonances and, thus, a better spectral resolution than conventional 1D spectra. In addition, the sensitivity of the super-WEFT spectra is relatively high because of the fast repetition rate used in the experiment. Most importantly, however, the fast relaxing resonances observed in the super-WEFT spectra are those that are most likely to fulfil the condition required for obtaining the electron transfer rates from the paramagnetic relaxation enhancement (McLaughlin and Leigh, 1973). Overall, the approach presented here is well suited for a determination of the paramagnetic linebroadenings of NMR resonances in metallo-proteins, and for a detailed investigation of the applicability of these linebroadenings for determining the electron exchange rates of blue copper proteins.

Theory

Relaxation under chemical exchange

The electron self-exchange affects the NMR spectra of partly oxidized blue copper proteins considerably. This holds in particular because of the paramagnetic nature of the oxidized form. For nuclei undergoing chemical exchange between two magnetically distinct environments, such as the nuclei in the reduced and oxidized forms of *Anabaena variabilis* plastocyanin (*A.v.* PCu), the relaxation decays of the observed resonances are in general double-exponential (McConnell, 1958; Led and Gesmar, 1982) and are given by $C_1 \cdot e^{-tR_i^-} + C_2 \cdot e^{-tR_i^+}$ (i = 1,2). The constants C_1 and C_2 are functions of the relaxation and exchange rate constants and the equilibrium magnetization and, in the case of transverse magnetization, also of $\Delta\omega_p$. The relaxation rates R_i^- and R_i^+ are (Leigh, 1971):

$$R_1^{\pm} = \frac{R_{1d} + R_{1p}^* + \mathbf{k}}{2} \pm \left[\left(\frac{R_{1p} + \mathbf{k}}{2}\right)^2 - \mathbf{k} f_p R_{1p}\right]^{1/2} \quad (1)$$

$$R_2^{\pm} = \frac{R_{2d} + R_{2p}^* + \mathbf{k}}{2} \pm \left[\frac{G + \sqrt{G^2 + H^2}}{2}\right]^{1/2} \quad (2)$$

where

$$G \equiv [R_{2d} - R_{2p}^* + \mathbf{k}(f_p - f_d)]^2/4 + \mathbf{k}^2 f_p f_d - \Delta\omega_p^2/4$$

$$H \equiv [R_{2d} - R_{2p}^* + \mathbf{k}(f_p - f_d)]\Delta\omega_p/2$$

Here the ESE rate **k** is given by $k_{ese} \cdot c$, where k_{ese} is the second order ESE rate constant and c is the total protein concentration. Further, f_d and f_p are the molar fractions of the diamagnetic and paramagnetic species, respectively, $\Delta\omega_p$ is the frequency separation of the resonances in the paramagnetic and diamagnetic sites, R_{ip}^* and R_{id} are the relaxation rates in the two sites if no exchange takes place, and R_{ip} (= $R_{ip}^* - R_{id}$) is the paramagnetic relaxation enhancement.

In diamagnetic–paramagnetic exchange systems, such as partly oxidized blue copper proteins, the R_{id} rates are normally very small compared with the exchange rate. In consequence of this, the R_i^+ component relaxes much faster than the R_i^- component according to Equations 1 and 2. Furthermore, for the resonances corresponding to the diamagnetic form $C_1 \gg C_2$ (Led and Gesmar, 1982; Ma, 2000), i.e. the amplitude of the slow relaxing R_i^- component is much larger than the amplitude of the fast relaxing R_i^+ component. Therefore, the relaxation decay in the diamagnetic site is controlled entirely by the first term, $C_1 \cdot e^{-tR_i^-}$, of the double-exponential decay, i.e. the decay is virtually single-exponential, while the relaxation rates R_{io} of the observed resonances in the diamagnetic site are given by R_i^- in Equations 1 and 2. Hence $R_{1o} = R_1^-$ and $R_{2o} = R_2^-$. Recently this was verified for the R_{1o} rates of nuclei in partly oxidized *A.v.* PCu (Ma et al., 2000). However, despite the single-exponential character the relaxation of the observed resonances depends on both the electron exchange rate, **k**, and the paramagnetic relaxation rate enhancement, R_{ip}, and, in the case of R_{2o}, also on the paramagnetic shift, $\Delta\omega_p$, as shown by Equations 1 and 2 and illustrated in Figure 1. Therefore, in the general case also R_{ip} and

$\Delta\omega_p$ must be determined in order to derive k_{ese} from the relaxation enhancement.

If the *slow exchange* condition ($\mathbf{k} \ll R_{ip}$) applies, the magnetization in the two sites relaxes with different rates, and Equations 1 and 2 corresponding to the diamagnetic site reduce to (McLaughlin and Leigh, 1973):

$$R_{io} = R_{id} + \mathbf{k} \cdot f_p \tag{3}$$

i.e. the relaxation rates are dominated by the electron exchange rate, $\mathbf{k} = k_{ese} \cdot c$, and are independent of R_{ip}. Hence $\mathbf{k}$ can be obtained from the relaxation enhancement. On the other hand, in the fast exchange limit ($\mathbf{k} \gg R_{ip}$) the relaxation rates are given by $R_{1o} = R_{1d} + R_{1p} \cdot f_p$ and $R_{2o} = R_{2d} + R_{2p} \cdot f_p + \Delta\omega_p^2 \cdot f_p \cdot f_d/\mathbf{k}$. Therefore, R_{1o} is independent of $\mathbf{k}$ while R_{2o} is still affected by $\mathbf{k}$ to an extent that depends on the size of $\Delta\omega_p$. If the *well-resolved* condition ($\Delta\omega_p \gg \mathbf{k}$) applies, the magnetization in the two sites relaxes with different rates and R_{2o} is again independent of R_{2p} and is given by Equation 3, even for $\mathbf{k} \gg R_{2p}$. Therefore, if $\Delta\omega_p \gg \mathbf{k}$ the exchange rate can be obtained from the paramagnetic linebroadening even in the fast exchange limit. In the intermediate region where R_{io} depends on both R_{ip} and $\mathbf{k}$, the full Equations 1 and 2 come into play. If this condition applies, a k_{ese} rate constant derived from R_{io} using Equation 3 will be too small, as can be inferred from Figure 1 where Equation 3 is presented as dotted lines.

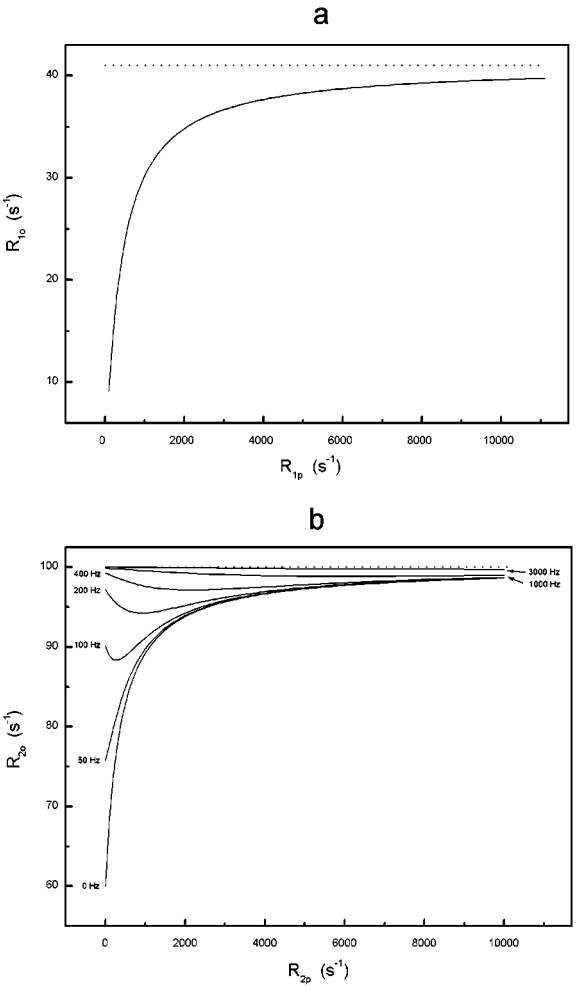

Figure 1. Plots of (a) R_{1o} and (b) R_{2o} versus R_{1p} and R_{2p} according to Equations 1 and 2, respectively, for $\mathbf{k} = 400\ s^{-1}$, $f_p = 10\%$, $R_{1d} = 1\ s^{-1}$ and $R_{2d} = 60\ s^{-1}$. The different R_{2o} curves correspond to different values of $\Delta\omega_p$, as indicated in (b). The two horizontal dotted lines correspond to $R_{1o} = R_{1d} + \mathbf{k} \cdot f_p$ (a) and $R_{2o} = R_{2d} + \mathbf{k} \cdot f_p$ (b).

Super-WEFT spectra

The super-WEFT technique (Inubushi and Becker, 1983) is a relaxation-selective NMR technique which is useful for the observation of paramagnetic NMR signals that relax too fast to be detected by the conventional NMR experiments. The 1D ^{1}H super-WEFT pulse sequence takes the same form as the inversion recovery (IR) pulse sequence, 180°-τ-90°-t_A, but uses a much faster repetition rate (5–15 s^{-1}). The recovery of the magnetization in the 1D super-WEFT experiment is given by (Inubushi and Becker, 1983):

$$M_t = M^\infty[1 - (2 - e^{-R_{1o}t_A})e^{-R_{1o}t}] \tag{4}$$

where M^∞ is the steady-state value of the magnetization, and M_t is the value of the magnetization at time t.

For the fast relaxing protons where $t_A \approx 2\text{–}3/R_{1o}$, the magnetization recovers efficiently after the acquisition time t_A. For the slow relaxing protons, where $t_A < 1/R_{1o}$, most of the magnetization is not recovered after the acquisition time t_A, and is therefore eliminated by the repetition of the pulse sequence. In practice, the sequence is repeated a number of times before data collection to produce a steady state with a null at τ for the slow relaxing magnetization. Thus, by using appropriate values of τ and t_A in the super-WEFT pulse sequence the fast relaxing resonances can be observed while the slow relaxing resonances are suppressed.

Concerning the applicability of Equations 1 and 2 under the conditions of the WEFT experiment, the question arises whether a partial saturation of the dia-

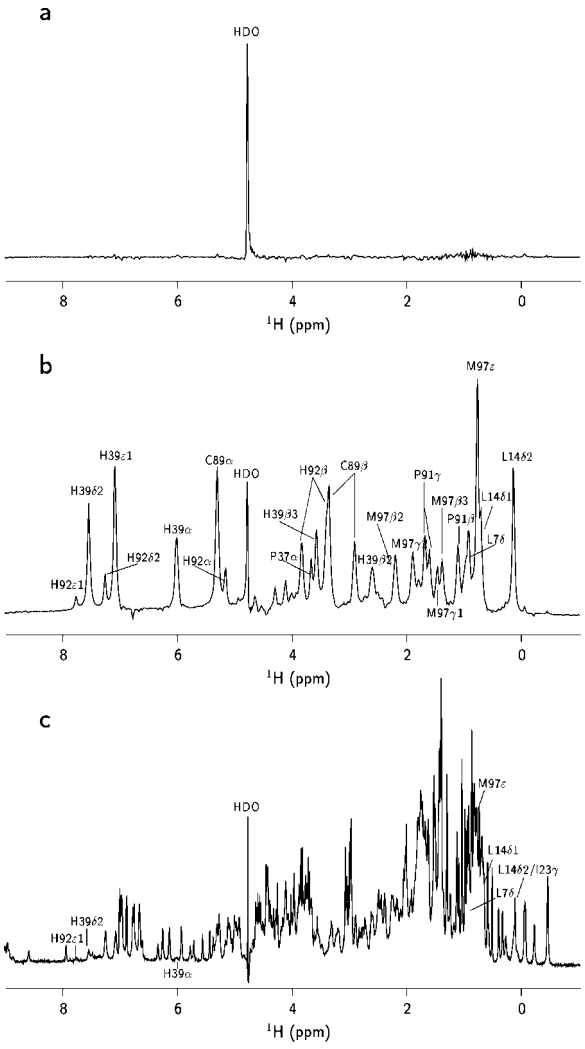

Figure 2. One–dimensional 750 MHz ^{1}H super-WEFT spectra of (a) reduced and (b) 9% oxidized *A.v.* PCu in D_2O (2.86 mM, pH 7.1, 298 K); both spectra were obtained using the same pulse sequence (see text); (c) the spectrum with the longest delay time in a series of 750 MHz ^{1}H inversion recovery spectra of the 9% oxidized *A.v.* PCu sample.

magnetic resonances in the WEFT spectrum can affect the ratio of the magnetizations corresponding to f_p and f_d and, thereby, invalidate Equation 1 and 2. As discussed below the fast ESE rate assures that the ratio of the magnetization in the oxidized and the reduced form still corresponds to f_p and f_d under the conditions applied here, and that R_{2o} and the linewidths of the observed resonances are given by the R_2^- rate of Equation 2.

In the super-WEFT spectra of partly oxidized *A.v.* PCu the relaxation of the diamagnetic resonances is affected by the fast relaxation and the chemical shift in the paramagnetic site, because of the ESE between the reduced (diamagnetic) and the oxidized (paramagnetic) form of the protein. In fact, the ESE rate is sufficiently fast (300–1000 s^{-1} at the applied *A.v.* PCu concentration) to allow many exchanges between the slow relaxing diamagnetic form and the fast relaxing paramagnetic form during the super-WEFT pulse sequence ($\approx$ 100 ms). Consequently, the paramagnetic site acts as an effective relaxation pathway for the spins in the diamagnetic site because of the fast ESE rate. This holds in particular for the nuclei of the metal-bound residues because of a very fast paramagnetic relaxation in the oxidized form ($R_{1p} \approx$ 400–1500 s^{-1}; Ma et al., 2000). Therefore, the super-WEFT resonances of these residues are not saturated, despite the fact that separate signals are observed for the reduced and the oxidized form because of large Fermi contact shifts (Bertini et al., 1999).

The nuclei of the non-metal-bound residues experience only a small pseudo-contact shift in the paramagnetic form. Therefore the chemical shifts of these nuclei change only slightly upon oxidation (Ma et al., 2000), and averaged signals are observed for the reduced and oxidized form because of the ESE. Although the spins of these nuclei might be partly saturated due to a relatively slow R_{1p} rate, the fast ESE rate will assure that the saturation is proportionately distributed between the diamagnetic and the paramagnetic form, leaving the f_p and f_d fractions unchanged.

Therefore, for sufficiently high ESE and R_{1p} rates the super-WEFT experiment gives the same f_p and f_d fractions and, thus, the same k_{ese} rate constants as conventional NMR spectra. This conclusion was confirmed experimentally (*vide infra*).

Experimental procedure

The ESE rate of the blue copper protein *A.v.* PCu was determined using the approach presented here. The protein was prepared and purified as described previously (Badsberg et al., 1996). Aprotinin was added during the purification to minimize enzymatic degradation of *A.v.* PCu. The protein was dissolved in 99.9% D_2O at pH 6.9–7.1 (meter reading). The partly oxidized samples were prepared by mixing the appropriate amounts of *A.v.* PCu(I) and *A.v.* PCu(II).

The NMR spectra were recorded at 298 K and ^{1}H frequencies of 500 MHz, 750 MHz or 800 MHz using

Varian Unity Inova 500, 750 and 800 spectrometers. A series of 1D ^{1}H super-WEFT spectra of *A.v.* PCu were recorded using different protein concentrations (from 2.86 mM to 0.13 mM), each with four or five different fractions of the oxidized form (from 4.9 to 20% *A.v.* PCu(II)). The ^{1}H super-WEFT spectra were obtained by using the 1D super-WEFT pulse sequence 180-τ-90- t_A (Inubushi and Becker, 1983). The interpulse delay (τ) was 40 ms and the acquisition time (t_A) was 51 ms, corresponding to a repetition rate of $\approx$ 11 s^{-1}. The numbers of data points were 1024 or 2048, corresponding to sweep widths of 10 000 and 20 000 Hz, respectively. The experiments consisted of 1024 to 2048 scans while 512 dummy scans were applied before acquisition to reach the steady state of the slow relaxing resonances. The total time for each experiment was $\approx$ 2–5 min. Zero-filling was applied to obtain a digital resolution of 1.2 Hz point^{-1} in the super-WEFT Fourier transformation (FT) spectra. Longitudinal ^{1}H relaxation rates were obtained from 1D IR experiments recorded on reduced *A.v.* PCu (R_{1d}), and the oxidized *A.v.* PCu samples used for the super-WEFT experiments (R_{1o}). For each concentration of *A.v.* PCu(II) the R_1 experiment was performed immediately after the super-WEFT experiment, and the fraction of *A.v.* PCu(II) in the partly oxidized samples was estimated from the enhancement of the paramagnetic relaxation (R_{1p}) as described previously (Ma et al., 2000) using the 5% oxidized sample for normalization.

The spectral parameters, including the linewidth, frequency, intensity and phase of the individual resonances in the super-WEFT spectra were obtained directly from the FIDs by backward linear prediction (LP) analysis (Led and Gesmar, 1988). A total of 400 LP coefficients were used in the analysis. The IR FT-spectra were analyzed and the R_{1d} and R_{1o} rates were obtained by using a simultaneous least squares fitting procedure (Kristensen et al., 1996; Ma et al., 2000). No window function or zero-filling were applied to obtain the 1D IR FT-spectra.

Results and discussion

The virtues of the super-WEFT spectra are illustrated in Figure 2. The spectrum of a 9% oxidized *A.v.* PCu (Figure 2b) includes all the fast relaxing protons from the four residues bound directly to Cu^{2+}, that is, H39, C89, H92 and M97, and from L7, L14, P37 and P91, which are the four non-bonded residues that are spatially closest to the Cu^{2+} ion according to the solution structure of *A.v.* PCu (PDB code 1FA4; Ma et al., 2000). In contrast, the resonances from the slower relaxing protons located further away from the Cu^{2+} ion are absent in the spectrum, while only the water signal is left in the super-WEFT spectrum of the reduced sample (Figure 2a). The advantage of the super-WEFT spectrum over a conventional 1D spectrum is immediately apparent from a comparison of the super-WEFT spectrum (Figure 2b) and the 1D spectrum of the same oxidized sample (Figure 2c). In the latter spectrum the large amount of overlapping signals allows only a few resonances to be analyzed quantitatively, and unlike the super-WEFT spectrum it does not select the signals with the largest paramagnetic relaxation enhancement.

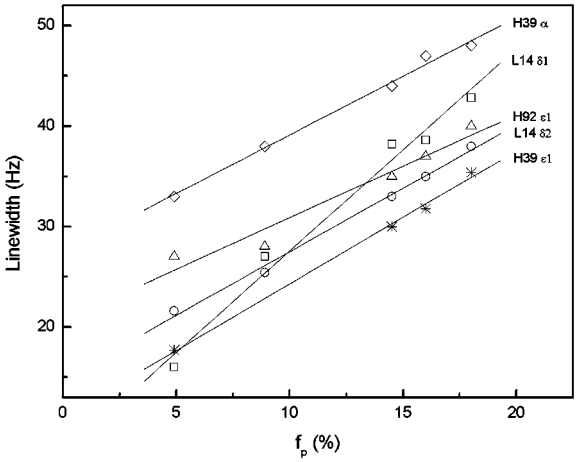

Figure 3. Examples of plots of linewidths versus fraction of oxidation (f_p) for a series of resonances in 750 MHz super-WEFT spectra of *A.v.* PCu (2.86 mM, pH 7.1, 298 K).

Figure 3 shows examples of least squares fits of Equation 3 to the linewidth ($R_{2o}/\pi = \Delta\nu_{\frac{1}{2}o}$) of resonances in the super-WEFT spectra as a function of f_p. In the least squares fit the individual linewidths were weighted with the relative uncertainties obtained in the LP analysis of the experimental FIDs.

Observation of two conformations

From Figure 3 it is immediately apparent that the discrepancies between the **k** rates, given as the slopes of the plots, are considerably larger than the experimental uncertainties. Clearly this suggests that Equation 3 does not apply to at least some of the observed resonances. However, also signal multiplicity gives rise to the discrepancies of the plots. Thus the observed resonances of the ligand residues are double signals, except the resonances of M97. As shown in Figure 4,

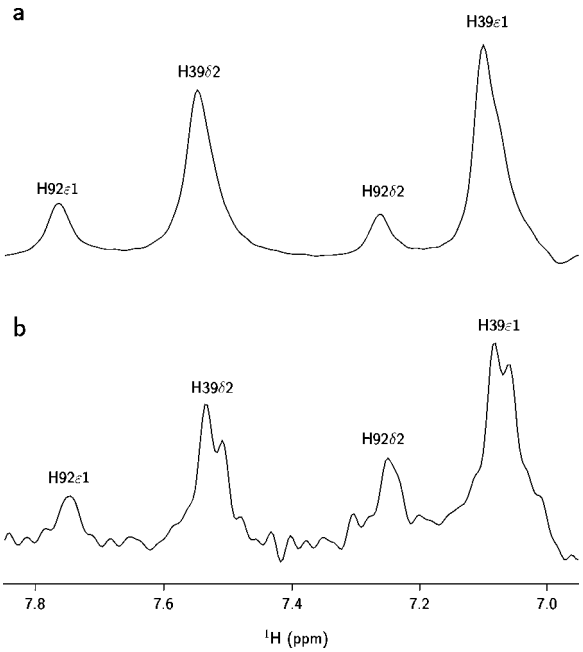

Figure 4. One–dimensional 800 MHz super-WEFT spectra of *A.v.* PCu in D_2O at pH 6.9, (a) 1.89 mM 20% oxidized *A.v.* PCu and (b) 0.47 mM 31.7% oxidized *A.v.* PCu. The double signals that are visible at the lower concentration are also present at the higher concentration, as shown by a linear prediction analysis (see text), but are concealed by a larger linebroadening caused by the higher **k** rate at this concentration.

the double signals are clearly visible at low concentration where the exchange rate and the linewidth are smaller, according to Equation 3. At higher concentrations (e.g. Figure 4a), the presence of double signals is revealed by the LP analysis of the FIDs. Thus for instance, for H39 the frequency difference of the two signals is 12 Hz for H39 $H^{\delta 2}$, 20 Hz for H39 $H^{\epsilon 1}$ and 26 Hz for H39 H^{α} in the super-WEFT spectra at 750 MHz and a PCu concentration of 2.86 mM. The magnitude of these differences, and the fact that all the signal splittings (in Hz) decrease proportionally to the magnetic field strength, show that the double signals correspond to different chemical environments. This, in turn, indicates the presence of two different local conformations in the protein. It is worth noticing that the two conformations are present also in the reduced protein, where double signals with a splitting of 12 Hz were observed for H39 $H^{\delta 2}$ in the 1D spectrum of the reduced *A.v.* PCu at 750 MHz and 2.86 mM.

Furthermore, an increase of the signal splitting with decreasing *A.v.* PCu concentration shows that slow exchange takes place between the two conformations. Thus for example, in the super-WEFT spectra of 32% oxidized *A.v.* PCu(II) at 800 MHz the H39 $H^{\delta 2}$ signal has splittings of 20, 25 and 26 Hz at *A.v.* PCu concentrations of 0.47, 0.30 and 0.14 mM, respectively. At the same time, the observation of two individual signals shows that the well-resolved condition ($\mathbf{k}_{conf} \ll \Delta\omega$) applies to the resonances of the ligand residues H39, H92 and C89. In contrast, the exchange-narrowing condition ($\mathbf{k}_{conf} \gg \Delta\omega$) applies to the resonances L7, L14 and P37, if any conformational differences exist for these residues.

It should be emphasized that the two conformations do not correspond to the protonated and the de-protonated form, respectively, of any of the histidines, since the pK values of the NH protons of the imidazole rings of these residues are about 5.0 (Kojiro and Markley, 1983), i.e. about two pK units smaller than the pH value applied here. Also it should be noted that splittings caused by scalar couplings are absent in the super-WEFT spectrum since the inter-nuclear scalar interactions are effectively decoupled by the fast ESE rate ($\mathbf{k} \approx 300$–$1000\ s^{-1}$) and the fast relaxation in the paramagnetic site ($R_{1p} \approx 400$–$1500\ s^{-1}$; Ma et al., 2000). However, the ^{13}C spectrum suggests the presence of both a *cis* and a *trans* conformation around the P37-P38 peptide bond, even though the ^{1}H spectra showed no indication of isomerization (Badsberg et al., 1996). Thus two signals separated by 0.5 ppm were observed here for $^{13}C^{\gamma}$ of P38 (data not shown), indicating a *cis-trans* isomerization around the P37-P38 peptide bond (Dorman and Bovey, 1973). The double signals observed for the protons of the ligand residues could result from this isomerization close to H39.

The slow exchange condition

Fulfillment of the slow exchange condition for the ESE process can be examined qualitatively from the variation of the linewidth of the resonances with temperature or concentration (Groeneveld and Canters, 1988; Dennison et al., 1993). Thus the linewidth increases with increasing temperature or concentration only if Equation 3 applies. In contrast, if both the fast exchange condition and the exchange narrowing conditions apply, i.e. $R_{2p} \ll \mathbf{k} \gg \Delta\omega$, the linewidth decreases with increasing temperature since $R_{2o} = R_{2d} + f_p \cdot R_{2p} + \Delta\omega_p^2 \cdot f_p \cdot f_d/\mathbf{k}$ under these conditions, and R_{2d} and R_{2p} decrease with increasing temperature. In the super-WEFT spectrum in Figure 2b the linewidth of all the resonances increases with temperature. A similar observation was made for increasing *A.v.* PCu concentrations. There-

Table 1. Electron self-exchange rate constant[a] of *A.v.* PCu

	$k_{ese} \cdot 10^{-5}/M^{-1}s^{-1}$			
	500 MHz			750 MHz
Nuclei	2.86 mM[b]	1.89 mM[c]	0.95 mM[d]	2.86 mM[e]
L7 H^{δ}	[1.2 ± 0.4[f]]	–	–	2.1 ± 0.5[f]
L14 $H^{\delta1}$	–	–	–	2.2 ± 0.2[f]
L14 $H^{\delta2}$	[0.8 ± 0.1[f]]	[1.6 ± 0.1[f]]	–	[1.4 ± 0.2[f]]
P37 H^{α}	[0.7 ± 0.4[f]]	[2.0 ± 0.8[f]]	–	[1.5 ± 0.2[f]]
M97 H^{ϵ}	[1.9 ± 0.7[f]]	2.4 ± 0.7[f]	–	2.2 ± 0.2[f]
H39 H^{α}	2.3 ± 0.3	2.4 ± 0.1	–	2.3 ± 0.6[g]
H39 $H^{\delta2}$	2.5 ± 0.1	2.1 ± 0.3	3.2 ± 0.6	2.3 ± 0.8
H39 $H^{\epsilon1}$	2.6 ± 0.3	2.0 ± 0.1	–	2.8 ± 0.3[g]
C89 H^{α}	2.9 ± 0.8	–	–	3.0 ± 0.8
C89 $H^{\beta3}$	–	–	–	2.4 ± 0.5
H92 $H^{\delta2}$	2.1 ± 0.5	–	–	3.3 ± 0.8
H92 $H^{\epsilon1}$	2.4 ± 1.4	2.5 ± 0.4	2.4 ± 1.1	2.4 ± 0.3
Average[h]	2.5 ± 0.1	2.2 ± 0.1		2.3 ± 0.1

[a]Obtained at pH from 6.9 to 7.1 (meter reading, in 99.9% D_2O) and 298 K by a least squares fit of Equation 3 to the linewidths of resonances in the super-WEFT spectra of *A.v.* PCu samples as a function of the fractions of *A.v.* PCu(II). Combined double signals were used (see text) unless indicated otherwise.
[b]Obtained from samples with 6.9%, 8.9%, 15.5% and 18.0% *A.v.* PCu(II).
[c,d]Obtained from samples with 5%, 10%, 15% and 20% *A.v.* PCu(II).
[e]Obtained from samples with 4.9%, 8.9%, 15.0%, 15.5% and 18.0% *A.v.* PCu(II).
[f]Derived from a single signal using $\mathbf{k} = k_{ese} \cdot c$ (see text).
[g]Derived from the sum of the **k** rates obtained from the two individual resonances of double signals (see text).
[h]Weighted average value not including the bracketed k_{ese} values, [ ].

fore, the R_{2o} rates are clearly affected by the exchange rate.

Still it remains unclear whether Equation 3 applies rigorously to the R_{2o} rates obtained from the super-WEFT spectra. To clarify this (1) the **k** rate was decreased by decreasing the total protein concentration, and (2) the R_{2p} was increased by increasing the magnetic field strength. The former variation holds as $\mathbf{k} = c \cdot k_{ese}$, while the latter applies since the electron relaxation time T_{1e} that controls the correlation time for the R_{2p} rates of *A.v.* PCu varies from 0.17 ± 0.01 ns at 11.7 T to 0.38 ± 0.06 ns at 17.6 T (Ma and Led, 2000). In both cases the derived k_{ese} rate constant remains unchanged only if Equation 3 applies.

Values of the k_{ese} rate constant obtained for a series of signals in the super-WEFT spectra are given in Table 1. The smallest k_{ese} rate constant is obtained from the linewidths of L14 $H^{\delta2}$ and P37 H^{α} at the highest PCu concentration (2.86 mM) and the lowest field strength (11.7 T, 500 MHz). When the concentration is decreased to 1.89 mM, or the field strength is increased to 17.6 T (750 MHz), larger values of the k_{ese} rate constant are obtained. Therefore, none of the two extreme conditions under which Equation 3 applies are fulfilled for L14 $H^{\delta2}$ and P37 H^{α} at low field and high concentration. This conclusion can also be reached from a calculation of the relaxation rate of L14 $H^{\delta2}$. Thus, assuming that R_{2p} of this nucleus is purely dipolar, R_{2p} rates of 451 s^{-1} and 825 s^{-1} are obtained at 11.7 and 17.6 T, respectively, for a Cu^{2+}–L14 $H^{\delta2}$ distance of 5.6 Å as found in the solution structure of *A.v.* PCu (PDB code 1FA4; Ma et al., 2000), using correlation times for the dipolar interaction of 0.17 and 0.38 ns at 11.7 T (500 MHz) and 17.6 T (750 MHz), respectively (Ma and Led, 2000). Clearly, for these R_{2p} rates and a **k** rate of 300–1000 s^{-1} the slow exchange condition is not fulfilled. The same conclusion can be made for P37 H^{α}.

In contrast, similar calculations for L14 $H^{\delta1}$ and L7 H^{δ} result in dipolar R_{2p} rates of 5500 and 7000 s^{-1}, respectively, at 17.6 T, using a Cu^{2+}–L14 $H^{\delta1}$ distance of 4.0 Å and a Cu^{2+}–L7 H^{δ} distance of 3.9 Å (PDB code 1FA4; Ma et al., 2000). Hence, for these resonances the slow exchange condition applies at 17.6 T and the PCu concentration of 2.86 mM used here, where the **k** rates are of the order of 1000 s^{-1}. Therefore, the k_{ese} rate constant of *A.v.* PCu must be close to the values obtained from the L14 $H^{\delta1}$ and L7 H^{δ} resonances (Table 1), i.e. $2.1 \cdot 10^5$ $M^{-1}s^{-1}$. This value is slightly smaller than the rate constant of 3.2×10^5 M^{-1} s^{-1} found previously at pH = 7.5, 298 K and an *A.v.* PCu concentration of 2 mM (Dennison et al., 1993), using the paramagnetic linebroadening of the copper-bound histidines in a conventional 1D spectrum. However, Dennison et al. also found values in the range from 1.6×10^5 M^{-1} s^{-1} to 2.1×10^5 M^{-1} s^{-1} using an *A.v.* PCu concentration of 1 mM.

The effect of signal multiplicity

The slow-exchange condition is fulfilled for the R_{2p} rates of the protons of the copper-bound residues since these protons are spatially close to the copper ion (PDB code 1FA4; Ma et al., 2000) and therefore have a considerable dipolar R_{2p} relaxation. In addition, they have a significant scalar R_{2p} relaxation. However, a determination of the k_{ese} rate constant from the resonances of these protons is hampered by the observed double resonances (*vide infra*).

Table 2. Simulated paramagnetic linebroadening of a combined double signal

f_p (%)	Simulated double signals			Combined signal[d]		
	I[a]	ν (Hz)[b]	$\Delta\nu_{\frac{1}{2}}$ (Hz)[c]	I	ν (Hz)	$\Delta\nu_{\frac{1}{2}}$ (Hz)[e]
0	1.0 & 1.0	0 & 8	13 & 13	2.08±0.02	3.9±0.1	16.2±0.2
5	1.0 & 1.0	0 & 8	18 & 18	2.06±0.02	4.3±0.2	20.9±0.3
10	1.0 & 1.0	0 & 8	23 & 23	2.06±0.02	3.8±0.2	25.9±0.4
15	1.0 & 1.0	0 & 8	28 & 28	2.02±0.02	3.9±0.2	30.8±0.4
20	1.0 & 1.0	0 & 8	33 & 33	2.05±0.02	3.5±0.3	35.8±0.5

[a]Normalized intensities of the two individual signals.
[b]Frequencies of the two individual signals.
[c]Linewidths of the individual signals calculated as $\Delta\nu_{\frac{1}{2},o} = \Delta\nu_{\frac{1}{2},d} + \frac{c}{2} \cdot k_{ese} \cdot f_p/\pi$, for $k_{ese} = 3.14 \times 10^5\ s^{-1}M^{-1}$ and c = 2 mM, $\Delta\nu_{\frac{1}{2},d} = 13$ Hz.
[d]Parameters obtained by an LP analysis of the combined double signal.
[e]Fitting of Equation 3 to the f_p-variation of the linewidth gives $\mathbf{k} = 307 \pm 3\ s^{-1}$ corresponding to $k_{ese} = 2 \cdot \mathbf{k}/c = (3.07 \pm 0.03) \times 10^5\ s^{-1}\ M^{-1}$ (see text).

The intensities of the individual signals of the double resonances indicate that the two conformations are present in approximately equal amounts. Also the **k** rates derived from the two individual signals of the H39 H^{α} resonance at 750 MHz and 2.86 mM are identical within the experimental uncertainty (Table 1), suggesting that the two conformations have similar activities. Furthermore, these rates are half the size of the **k** rates obtained from the L14 $H^{\delta 1}$ resonance, in agreement with Equation 3 and the concentration of the two forms. Accordingly, a k_{ese} value derived from the *sum* of the **k** rates obtained from the two individual H39 H^{α} signals corresponds to the rate constant obtained from the L14 $H^{\delta 1}$ and L7 H^{δ} resonances (Table 1). A similar result was obtained from the two signals of the H39 $H^{\epsilon 1}$ resonance, although a slightly larger rate constant was obtained from this double resonance.

The remaining H39 resonances and the resonances of the copper-bound residues C89 and H92 are also double signals. However, the smaller frequency difference and the larger linewidth of these double signals prevent a reliable estimation of the k_{ese} rate constant from the individual resonances. Nevertheless, reliable rate constants can be derived from the linebroadening of the *combined* signal. This is demonstrated by the series of simulated signals with increasing linebroadening that are shown in Figure 5 and Table 2. As illustrated in Figure 5 two closely spaced Lorentzian signals with similar intensity and similar linewidth combine to a near-Lorentzian signal with a linewidth that is only slightly larger than the linewidth of one of the two individual signals. More importantly, within the experimental uncertainty the f_p-dependence of the paramagnetic linebroadening of the combined signal is identical to the f_p-dependence of one of the two individual signals in the double resonance. This is shown in Figure 5 and Table 2 by the variation of the linewidths derived by an LP analysis of the combined signals. Thus, for a series of simulated double signals (Table 2) with a linebroadening corresponding to **k** = 314 s^{-1} for each of the two individual signals, the variation of the LP-derived linewidths of the combined signal corresponds to $\mathbf{k} = 307 \pm 3\ s^{-1}$. Hence, in the case of overlapping double signals the **k** rate derived from the combined signal is 50% of the total exchange rate, i.e. $k_{ese} = 2 \cdot \mathbf{k}/c$. It should be emphasized that this simple relation applies only if the two signals have equal intensity and equal linewidth, as found here.

In agreement with this conclusion, the linebroadenings of the combined signals of the double resonances of H39 $H^{\delta 2}$ and H39 $H^{\epsilon 1}$ and the resonances of H92 and C89 all correspond to **k** rates that are half the size of the rates obtained from the L14 $H^{\delta 1}$ and L7 H^{δ} resonances, within the experimental uncertainties. Accordingly, the k_{ese} values in Table 1, which are derived from these resonances using the relation $k_{ese} = 2 \cdot \mathbf{k}/c$, are in good agreement with those obtained from L14 $H^{\delta 1}$ and L7 H^{δ} using the relation $k_{ese} = \mathbf{k}/c$.

As for the exchange between the two conformations, an estimation of the exchange rate, $\mathbf{k}_{conf}$, based on the frequency difference measured for the double signals at a series of *A.v.* PCu concentrations (*vide supra*), shows that this rate is at least two orders of magnitude smaller than the ESE rate and can therefore not contribute to the linebroadening of the signal. Moreover, the agreement between the values

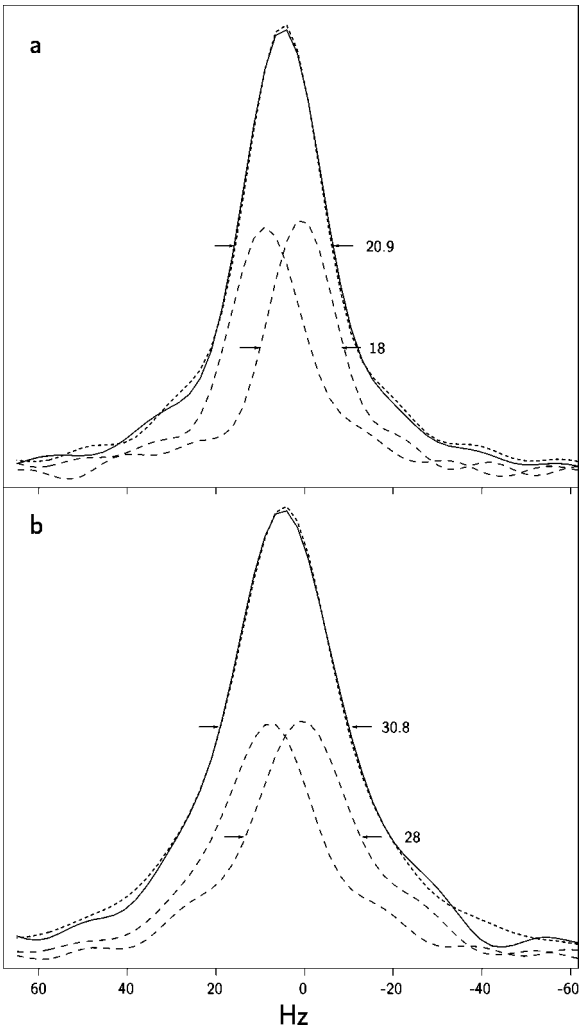

Figure 5. Two overlapping simulated signals. Lower curves (dashed lines): (a) two overlapping Lorentzian signals with equal intensity, a frequency difference of 8 Hz, $\Delta\nu_{\frac{1}{2}} = 18$ Hz, and S/N = 8/1; (b) as (a) except for $\Delta\nu_{\frac{1}{2}} = 28$ Hz. Upper curves (solid lines): combined signals (sum) of the two overlapping Lorentzians. Upper curves (dotted lines): signals generated from the parameters obtained by an LP analysis of the combined signals. The linewidths of the LP signals are (a) 20.9 Hz and (b) 30.8 Hz, as indicated in the figure.

of the ESE rate constant, k_{ese}, obtained from the double signals and those obtained from L14 $H^{\delta 1}$ and L7 H^{δ} indicates that the linewidths of the double signals are unaffected by the exchange between the two conformations.

Finally, to further establish the applicability of the approach presented here the k_{ese} rate constant was determined from the linewidths of three signals that are sufficiently isolated in the regular 1D ^{1}H spectrum of *A.v.* PCu to be investigated. Thus k_{ese} rate constants of 2.3 $\pm$0.4 $\times$ 10^5, 3.0 $\pm$0.6 $\times$ 10^5, and 2.5 $\pm$0.5 $\times$ 10^5 M^{-1} s^{-1} were obtained from the linewidths of the H39 H^{α}, H39 $H^{\delta 2}$ and V41 $H^{\gamma 2}$ signals, respectively, in the 1D R_1 spectra of 1.89 mM partly oxidized *A.v.* PCu samples (pH = 7.0; f_p = 5%, 10%, 15%, and 20%) recorded at 298 K and 11.7 T. Also here the rate constants derived from the H39 signals were calculated as $k_{ese} = 2 \cdot \mathbf{k}/c$, while the rate constant derived from the V41 signal was calculated as $k_{ese} = \mathbf{k}/c$. These results not only agree with the k_{ese} rate constant obtained from the WEFT spectra; they also confirm the applicability of the WEFT approach presented here.

Conclusions

The approach presented here allows an accurate estimation of the electron self-exchange rate if the slow exchange condition or the well-resolved condition applies. The super-WEFT spectra have relatively few and isolated resonances which, furthermore, are the fastest relaxing resonances in the protein. Therefore, not only is the resolution of the super-WEFT spectra high, allowing a precise determination of the linewidths; the resonances of the spectra are also those most likely to fulfil the slow exchange condition. In this study the merits of the approach allowed the detailed analysis of the linewidth that led to the disclosure of the double resonances from the copper-bound residues in *A.v.* PCu, corresponding to two interchanging conformations at the metal site. The presence of the double resonances, however, emphasizes the precaution that must be taken when deriving k_{ese} rate constants from the paramagnetic linebroadenings of resonances in crowded NMR spectra of metallo-proteins.

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