J|A|C|S
ARTICLES
Published on Web 03/20/2002

A General Method for Determining the Electron Self-Exchange Rates of Blue Copper Proteins by Longitudinal NMR Relaxation

Malene Ringkjøbing Jensen, D. Flemming Hansen, and Jens J. Led*

Contribution from the Department of Chemistry, University of Copenhagen, The H. C. Ørsted Institute, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

Received September 17, 2001. Revised Manuscript Received January 9, 2002

Abstract: A general NMR method is presented that allows a precise determination of the second-order rate constant, k_{ese}, for the electron self-exchange in blue copper proteins, from the longitudinal relaxation rates of the nuclei in the protein. The method relies on the use of partly oxidized (paramagnetic) samples of the protein. In contrast to previous NMR approaches for the determination of electron self-exchange rates, the applicability of the method extends beyond the slow-exchange limit, $k_{ese}c \ll R_{ip}$, $i = 1, 2$, where c is the protein concentration, and R_{ip} is the paramagnetic relaxation enhancement of the observed nuclei.

Introduction

The biological function of blue copper proteins is electron transfer. As an example, plastocyanins transfer electrons from cytochrome *f* to the chlorophyll reaction center P700 in photosystem I.[1] The determination of electron transfer rates therefore plays an important role in studies of the function of blue copper proteins. The rate and mechanism of the electron transfer process can be studied conveniently through the electron self-exchange (ESE) that characterizes blue copper proteins. In this context, plastocyanin from *Anabaena variabilis* (*A.v.* PCu) is of particular interest since its ESE rate is considerably larger than that of other plastocyanins.[2]

Studies of electron transfer processes have so far been hampered by the lack of precise methods for the determination of the rate constants. Previously it was shown that the electron self-exchange rate can be estimated from the paramagnetic line broadening in regular one-dimensional (1D) ^{1}H NMR spectra of mixtures of the oxidized (paramagnetic) and the reduced (diamagnetic) forms of blue copper proteins,[2,3] or from the paramagnetic enhancement of the longitudinal relaxation rates of the protons.[2] Recently the line broadening approach was improved by exploiting the super-WEFT technique to suppress the signals of slow relaxing nuclei.[4] This approach increases the spectral resolution by reducing the number of signals. Moreover, the fast relaxing signals observed in the spectra are also those that contain information about the electron-exchange rate.

The approaches just mentioned are based on the slow-exchange condition[5]

$$k_{ese}c \ll R_{ip} \qquad i = 1, 2 \tag{1}$$

Here k_{ese} is the second-order ESE rate constant, c is the concentration of the protein, and R_{1p} and R_{2p} are the paramagnetic enhancements of the longitudinal and transverse relaxation rates, respectively. When this condition is fulfilled, the observed relaxation rate, R_{io}, of the diamagnetic signal is given by[5]

$$R_{io} = R_{id} + k_{ese}cf_p \qquad i = 1, 2 \tag{2}$$

where R_{id} is the relaxation rate of the diamagnetic site in the absence of exchange, and f_p is the molar fraction of the paramagnetic species. Equation 2 also holds if the signals of the two sites overlap (see below). Thus, if eq 1 is fulfilled, the rate constant k_{ese} can be obtained from the dependence of R_{io} on the fraction f_p using eq 2.

However, normally it is unknown a priori whether eq 1 applies. Consequently, signals may be ruled out as potential candidates for the k_{ese} determination even if they are strongly affected, or erroneous exchange rates may be derived. A method for determining the exchange rates that does not require the fulfillment of eq 1 is therefore highly desirable. Here we present a general approach that allows the determination of the k_{ese} rate constants from the longitudinal relaxation rates, independent of the slow-exchange condition.

Theoretical Section

In partly oxidized samples of a blue copper protein, the time dependence of the longitudinal magnetizations, $M_d(t)$ and $M_p(t)$, in the diamagnetic and the paramagnetic site, respectively, is, in general, biexponential and is given by the McConnell

* To whom correspondence should be addressed. Telephone: (+45) 3532 0302. Fax: (+45) 3535 0609. E-mail: led@kiku.dk.

(1) Gross, E. L. *Photosynth. Res.* **1993**, *37*, 103−116.
(2) Dennison, C.; Kyritsis, P.; McFarlane, W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1993**, 1959−1963.
(3) Lommen, A.; Canters, G. W.; van Beeumen, J. *Eur. J. Biochem.* **1988**, *176*, 213−223.
(4) Ma, L.; Philipp, E.; Led, J. J. *J. Biomol. NMR* **2001**, *19*, 199−208.
(5) McLaughlin, A. C.; Leigh, J. S. *J. Magn. Reson.* **1973**, *9*, 296−304.

equations for a two-site exchange system.[6] Thus, $M_d(t)$ takes the form

$$M_d(t) = C_1 \exp(\lambda_1 t) + C_2 \exp(\lambda_2 t) + M_d^\infty \quad (3)$$

where λ_1, λ_2, C_1, and C_2 are constants that are given by[7]

$$\lambda_1 = -\frac{1}{2}\left\{(k_{1d} + k_{1p}) - \sqrt{(k_{1d} - k_{1p})^2 + 4(1 - f_p) f_p k_{ese}^2 c^2}\right\} \quad (4)$$

$$\lambda_2 = -\frac{1}{2}\left\{(k_{1d} + k_{1p}) + \sqrt{(k_{1d} - k_{1p})^2 + 4(1 - f_p) f_p k_{ese}^2 c^2}\right\} \quad (5)$$

$$C_1 = \{(\lambda_2 + k_{1d})(M_d^\infty - M_d^0) - (1 - f_p) k_{ese} c (M_p^\infty - M_p^0)\}/(\lambda_1 - \lambda_2) \quad (6)$$

$$C_2 = \{-(\lambda_1 + k_{1d})(M_d^\infty - M_d^0) + (1 - f_p) k_{ese} c (M_p^\infty - M_p^0)\}/(\lambda_1 - \lambda_2) \quad (7)$$

Here $k_{1d} = f_p k_{ese} c + R_{1d}$, and $k_{1p} = (1 - f_p) k_{ese} c + R_{1p} + R_{1d}$. Furthermore, M_d^∞ and M_p^∞ are the equilibrium magnetizations in the diamagnetic and the paramagnetic site, respectively. Finally, M_d^0 and M_p^0 are the corresponding magnetizations immediately after the perturbation in the two sites.

According to eqs 4 and 5, λ_1 and λ_2 are both negative. Also it can be shown that $|\lambda_1|$ is significantly smaller than $|\lambda_2|$, and that $|C_1|$ is significantly larger than $|C_2|$ for all f_p values used here. Consequently, the recovery of the longitudinal magnetization is dominated entirely by the first exponential in eq 3 and is, therefore, effectively single-exponential. This holds regardless of whether the reduced and oxidized species have separate signals or an average signal is observed. Under these conditions, the observed longitudinal relaxation rate, R_{1o}, is given by[8]

$$R_{1o} = -\lambda_1 = \frac{2R_{1d} + R_{1p} + k_{ese} c}{2} - \sqrt{\left(\frac{R_{1p} + k_{ese} c}{2}\right)^2 - k_{ese} c f_p R_{1p}} \quad (8)$$

For $f_p = 0$, eq 8 reduces to $R_{1o} = R_{1d}$, while R_{1o} depends approximately linearly on f_p in the case of paramagnetic samples with small f_p fractions and constant protein concentration. Therefore, the two parameters R_{1p} and k_{ese} in eq 8 are heavily correlated and cannot be determined independently from the variation of R_{1o} with f_p. However, the three parameters R_{1d}, R_{1p}, and k_{ese} can be determined independently from a *simultaneous* analysis of the dependence of R_{1o} on f_p *and* the protein concentration, c, using eq 8. This holds irrespective of the fulfillment of the slow-exchange condition. In contrast, the k_{ese} rate constant cannot be determined from R_{1o} in the *fast*-exchange limit, $R_{1p} \ll k_{ese} c$, since in this case R_{1o} is independent of k_{ese}.[5]

Materials and Methods

A sample of *A.v.* plastocyanin prepared and purified as described previously[9] was kindly supplied by Prof. Jens Ulstrup, of the Technical University of Denmark. 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 the reduced and the oxidized form of *A.v.* PCu. The protein concentrations of the samples used in the NMR experiments were 0.47, 0.95, and 1.89 mM, respectively, and the fraction of oxidized PCu, f_p, was in the range from 0 to 0.33. All samples contained 50 mM NaCl.

The NMR experiments were carried out at 298 K and ^{1}H frequencies of 500 and 800 MHz, using Varian Unity Inova 500 and Unity Inova 800 spectrometers. The R_{1o} relaxation rates of the four amide protons, $C89^{NH}$, $E90^{NH}$, $M97^{NH}$, and $N40^{NH}$, were used to evaluate the method. All four amide proton signals are well resolved in D_2O. Furthermore, they are slow exchanging under the applied experimental conditions, with signals still visible weeks after dissolution in D_2O. In comparison, the total time for each relaxation experiment was from 0.5 to 2 h depending on the protein concentration. Therefore, their signal intensities are unaffected by the proton-deuteron exchange during the relaxation experiments. The longitudinal ^{1}H relaxation rates were obtained at 500 MHz by conventional 1D ^{1}H inversion−recovery (IR) experiments consisting of 11 partly relaxed spectra. To eliminate systematic errors, the partly relaxed spectra were recorded in a random order. The number of data points in the spectra was 32 768, and the sweep width was 9000 Hz. No window function was used, while zero filling was applied once. The fraction of oxidized *A.v.* PCu in the samples was estimated from the relaxation rates of three nuclei that fulfill the fast-exchange condition ($R_{1p} \ll k_{ese} c$), as described previously.[8] For each sample the weighted average of the three oxidation degrees was used. This approach allows a highly precise determination of f_p, since the R_{1o} rates can be determined accurately and are obtained from the same experiments as the k_{ese} rates. The rates were normalized using a freshly prepared 5% oxidized sample. The oxidation degree of this sample was determined optically, using the strong absorbance at 597 nm of the oxidized form. The R_{1o} relaxation rates were extracted from the experimental data by using the linear prediction model method.[10] The reliability of the experimental R_{1o} rates is strongly indicated by the excellent and mutual agreement of the large amount of rates obtained at different f_p fractions and protein concentrations (see below and Figure 2).

Results and Discussion

Two regions of the one-dimensional (1D) ^{1}H NMR spectrum of a partly oxidized sample of *A.v.* PCu are shown in Figure 1. The indicated assignments are those obtained previously.[9]

A series of k_{ese} rate constants derived from the experimental R_{1o} data is given in Table 1. The rate constants in columns 2−4 were derived assuming that the slow-exchange condition is fulfilled. Thus, for each of the individual protein concentrations, 0.47, 0.95, and 1.89 mM, the rate constants were obtained by a least-squares fit of eq 2 to the R_{1o} data at different fractions of oxidized PCu, f_p. Immediately, the difference between the obtained rate constants reveals that the slow-exchange condition applies only in a few cases. Further, the trend observed for the data in columns 2−4 of Table 1 is supported qualitatively by the following two considerations.

(6) McConnell, H. M. *J. Chem. Phys.* **1958**, *28*, 430−431.
(7) Led, J. J.; Gesmar, H. *J. Magn. Reson.* **1982**, *49*, 444−463.
(8) Ma, L.; Jørgensen, A.-M. M.; Sørensen, G. O.; Ulstrup, J.; Led, J. J. *J. Am. Chem. Soc.* **2000**, *122*, 9473−9485.
(9) Badsberg, U.; Jørgensen, A. M. M.; Gesmar, H.; Led, J. J.; Hammerstad, J. M.; Jespersen, L.-L.; Ulstrup, J. *Biochemistry* **1996**, *35*, 7021−7031.
(10) Moss, R.; Gesmar, H.; Led, J. J. *J. Am. Chem. Soc.* **1994**, *116*, 747−749.

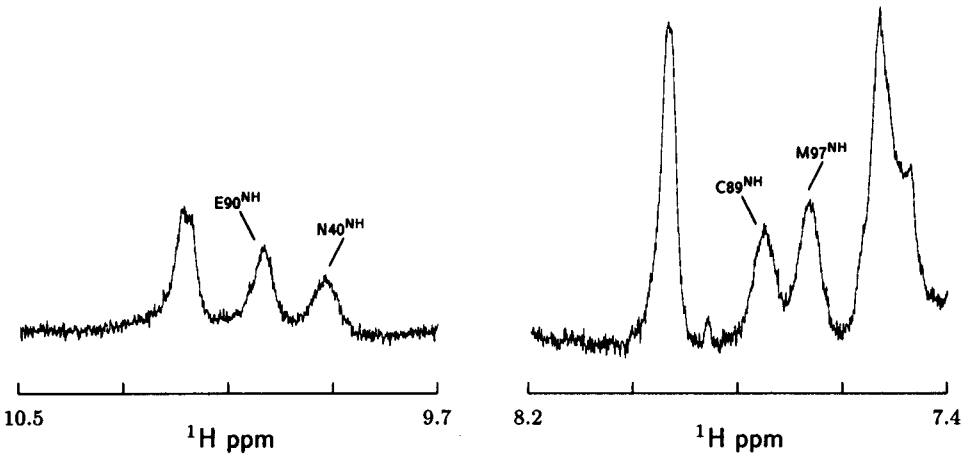

Figure 1. Two parts of the 500 MHz 1D ^{1}H NMR spectrum of a 1.89 mM solution of 14.2% oxidized *A.v.* PCu in 99.9% D_2O at pH 7.0 (meter reading) and 298 K. The indicated assignments were obtained previously.[9]

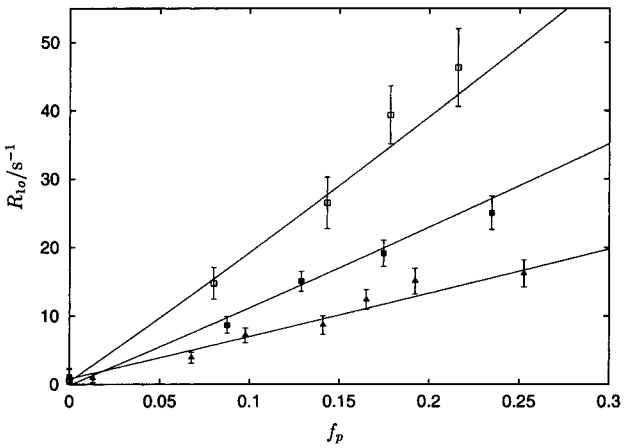

Figure 2. The longitudinal relaxation rates, R_{1o}, of the E90 amide proton at different PCu concentrations and at different fractions of oxidized PCu. The following concentrations were used: 0.47 mM (△), 0.95 mM (■), and 1.89 mM (□). The curves correspond to the least-squares fit of eq 8.

Table 1. Electron Self-Exchange Rate Constants k_{ese} (M^{-1} s^{-1}) of *A.v.* PCu[a]

	$k^b_{ese} \times 10^{-5}$			
nucleus	0.47 mM	0.95 mM	1.89 mM	$k^c_{ese} \times 10^{-5}$
$N40^{NH}$	1.42 ± 0.12	1.33 ± 0.08	1.52 ± 0.11	−
$C89^{NH}$	1.24 ± 0.07	0.96 ± 0.08	0.76 ± 0.14	1.45 ± 0.27
$E90^{NH}$	1.46 ± 0.10	1.09 ± 0.06	1.07 ± 0.07	1.48 ± 0.16
$M97^{NH}$	0.86 ± 0.07	0.49 ± 0.02	0.31 ± 0.01	1.79 ± 0.41
weighted average				1.50 ± 0.13

[a] At 298 K and pH 7.0. [b] Obtained from a least-squares fit of eq 2 for three different concentrations of the protein; that is, the slow-exchange condition is assumed to be fulfilled. [c] Obtained from a least-squares fit of eq 8, that is, without the slow-exchange restriction.

First, for nuclei close to the slow-exchange regime, that is, $k_{ese}c < R_{1p}$, a Taylor expansion of the square root term in eq 8,

$$R_{1o} = R_{1d} + k_{ese}cf_p\left[1 - \frac{k_{ese}c}{R_{1p}}(1 - f_p) + \left(\frac{k_{ese}c}{R_{1p}}\right)^2(1 - 2f_p)(1 - f_p) - \cdots\right] \quad (9)$$

shows that the k_{ese} rate constants derived from eq 2 are smaller than the actual value if the slow-exchange condition is not fulfilled. Experimentally, this tendency is observed for the protons $C89^{NH}$, $E90^{NH}$, and $M97^{NH}$ (Table 1). In particular, the small k_{ese} rate constants derived from the relaxation of $M97^{NH}$ indicate that this proton does not fulfill the condition at any of the applied protein concentrations. For the $N40^{NH}$ proton, on the other hand, the k_{ese} rate is approximately constant in the applied concentration range, indicating that the slow-exchange condition is fulfilled within this range.

Second, the protons spatially closest to the copper atom are those most likely to fulfill the slow-exchange condition, since they will have the largest R_{1p} rates if the point dipole approximation is valid for the paramagnetic Cu(II) site. According to the solution structure of *A.v.* plastocyanin,[8] the amide protons are located at the following distances from the copper atom: 3.70 ($N40^{NH}$), 5.89 ($C89^{NH}$), 5.41 ($E90^{NH}$), and 6.80 Å ($M97^{NH}$). Qualitatively, these distances support the suggestions that the $N40^{NH}$ proton fulfills the slow-exchange condition and that the $M97^{NH}$ proton is outside the slow-exchange regime at all the applied protein concentrations.

As described in the Theoretical Section, a determination of k_{ese} without a preknowledge of the slow-exchange condition requires a determination of R_{1o} as a function of both the protein concentration and f_p. The last column in Table 1 lists the k_{ese} values obtained from a least-squares fit of eq 8 to the R_{1o} rates of the three amide protons $C89^{NH}$, $E90^{NH}$, and $M97^{NH}$, respectively. For each one of the three individual protons, a simultaneous fit was performed to all the R_{1o} rates obtained at the applied f_p fractions and protein concentrations. Thus, for each proton the following five parameters were obtained: the k_{ese} rate, the R_{1p} rate, and one R_{1d} rate for each of the three protein concentrations. An example of the fits is shown in Figure 2. For the $N40^{NH}$ proton a least-squares fit of eq 8 could not be made since its R_{1o} rates are in the slow-exchange regime (eq 1) at all f_p fractions and protein concentrations applied here and are, therefore, independent of the paramagnetic relaxation enhancement R_{1p}, according to eq 2. As it appears from Table 1, the three k_{ese} rate constants derived from eq 8 are identical within the uncertainties obtained in the least-squares fit. Furthermore, these k_{ese} rate constants are larger than the constants obtained at the individual protein concentrations using eq 2. The most striking result is the k_{ese} value obtained for the $M97^{NH}$ proton. As shown by the data in columns 2−4 in Table 1, this proton is far from the slow-exchange regime. Neverthe-

less, the proton gives the same ESE rate constant as the $E90^{NH}$ and $C89^{NH}$ protons within the uncertainties. The larger uncertainty of the rate constant obtained from $M97^{NH}$ stems from the limited influence of the ESE rate on the relaxation of this nucleus.

The average k_{ese} rate constant of $(1.50 \pm 0.13) \times 10^5$ M^{-1} s^{-1} obtained here is smaller than the value found previously for *A.v.* PCu using the line broadening approach and the super-WEFT technique.[4] However, in that study the determination of the rate constant relied on an apparent double conformation around the metal site of *A.v.* PCu with separate signals from the nuclei of the metal bound residues in the two forms and with equal populations of the two conformations. Subsequently, we have found that the double conformation model was based on an incorrect assignment of some of the signals in the 1D super-WEFT spectrum and on difficulties in measuring the correct line width of partly overlapped signals in the spectrum. With only one conformation present, and by using only well-resolved signals that fulfill the slow-exchange condition, the previous line width study gives a k_{ese} rate constant of $(1.2 \pm 0.2) \times 10^5$ M^{-1} s^{-1} in agreement with the value found here.

In conclusion, the data in Table 1 show that the approach described here is generally applicable for precise determinations of k_{ese} rate constants, by including nuclei that do not fulfill the slow-exchange condition. Thus, the k_{ese} rate constants obtained from eq 8 are identical within the experimental uncertainties, independent of the fulfillment of this condition. In addition, the approach allows a determination of R_{1p} of the observed nuclei, that can provide valuable information about the geometric and electronic structure at the metal site.

Acknowledgment. Exploratory 800 MHz spectra were acquired at The Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. We are grateful to Lixin Ma and Søren M. Kristensen for helpful discussions, and to Lise-Lotte Jespersen, Else Philipp, Georg Ole Sørensen, and Jens Duus for technical assistance. This work was financially supported by the Danish Natural Science Research Council (J. No.'s 9400351, 9502759, and 9801801), Direktør Ib Henriksens Fond, Carlsbergfondet, and Novo Nordisk Fonden.

JA012186N