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Direct measurement of the self-exchange rate of stellacyanin by a novel e.p.r. method

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A method for reconstituting the blue copper protein stellacyanin with the stable copper isotopes ^{63}Cu and ^{65}Cu is reported. Small differences in the e.p.r. spectra of the two isotopic forms of stellacyanin have been used to monitor the electron self-exchange reaction of stellacyanin by rapid-freeze e.p.r. methods. The self-exchange rate constant (k_{11}) for stellacyanin has been determined as $1.2 \times 10^5 M^{-1} \cdot s^{-1}$ at 20°C. This value is in close agreement with values obtained from less-direct methods.

Electron-transfer reactions, when these occur via outer-sphere mechanisms, are often discussed in terms of the Marcus (1963) Theory. This theory attempts to express the electron-transfer rate constant for the reaction between two redox reagents in terms of the equilibrium constant for the reaction and the self-exchange rates for the individual components.

In its simplest form, and where the reaction is adiabatic and has a rather small equilibrium constant, the expression may be written as follows:

$$k_{12} = (k_{11} \cdot k_{22} \cdot K_{12})^{\frac{1}{2}}$$

where k_{12} is the second-order rate constant for the cross-reaction between components 1 and 2; K_{12} is the equilibrium constant for the reaction; k_{11} and k_{22} are the self-exchange rate constants for the components.

Even for well-behaved outer-sphere reactants the predicted and observed values of k_{12} may differ because the interaction energies between reagents in the reaction are different from those in self-exchange processes. For example, oppositely charged reagents will attract each other in the cross-reaction but will experience repulsive forces in self-exchange. This latter circumstance may be met by suitable choice of ionic strength in experiments and by calculating the values of rate constants at infinite ionic strength (Wherland & Gray, 1976). Deviations will also be expected if different electron-transfer mechanisms or different activation processes obtain in cross-reaction and self-exchange.

In spite of the constraints implicit in the assumptions, this theory has now been widely and successfully applied to reactions involving redox proteins. It is perhaps surprising that not only does this theoretical framework give insight into the mechanism of electron transfer between the buried metal centres of redox proteins and well-characterized small outer-sphere oxidants and reductants, but also into protein–protein electron-transfer mechanisms. The study of these mechanisms has been the subject of a large number of publications (see, for example, Wherland & Pecht, 1978; Lappin, 1981).

Surprisingly, although the theory requires a knowledge of the self-exchange rates for proteins, very few actual measurements exist. Only the self-exchange rates of cytochrome *c* (Gupta, 1973), cytochrome c_{551} (Keller *et al.*, 1976) and of azurin (Adman *et al.*, 1983) have been directly measured by n.m.r. techniques.

In the present paper we report a novel method of determining self-exchange rates by rapid-freeze e.p.r. methods. This method is applicable to a variety of copper proteins and takes advantage of small differences in the e.p.r. spectra of ^{63}Cu and ^{65}Cu. We have chosen to investigate first the protein stellacyanin. This protein contains a single copper atom, which may be removed and the apoprotein reconstituted with the desired copper

isotope. On mixing [^{65}Cu(I)]stellacyanin with [^{63}Cu(II)] stellacyanin, electron exchange occurs, resulting in the disappearance of part of the ^{63}Cu(II) and the appearance of a stoichiometric amount of ^{65}Cu(II). The approach to equilibrium may be monitored by rapidly freezing samples at known times after mixing and analysing the e.p.r. spectra of the resultant mixture of cupric ions.

We have performed such experiments and we find a value for the self-exchange rate constant of stellacyanin of between 1×10^5 and $1.3 \times 10^5 M^{-1} \cdot s^{-1}$. This result accords well with other estimates of this constant (Cummins & Gray, 1977) and gives confidence in the choice of stellacyanin as a 'bench-mark' protein in calculations relating kinetic parameters to structural features (Mauk *et al.*, 1980).

A preliminary report of this work was presented at the First International Conference on Bioinorganic Chemistry, held in Firenze, Italy, 13–17 June 1983.

Materials and methods

Protein and chemicals

An acetone-dried powder prepared from the natural lacquer of the Japanese lacquer tree (*Rhus vernicifera*) was purchased from Saito and Co., Tokyo, Japan. Stellacyanin was prepared from this acetone-dried powder by the method of Reinhammar (1970). The pure copper isotopes ^{63}Cu and ^{65}Cu were obtained as CuO from Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A., and were dissolved in 6M-HCl. The copper isotopes, as well as all other chemicals used, were of reagent grade. Solutions were prepared from deionized water. Phosphate buffer was treated with Dithizon in carbon tetrachloride to remove trace metal contaminants. All glassware for handling the apoprotein was washed in 2M-HNO_3 to remove metals. Optical-absorbance measurements were performed with a Zeiss M4Q III-PMQ II spectrophotometer and a Beckman ACTA M IV double-beam spectrophotometer. Dialyses were performed in ordinary dialysis bags and in Sartorius collodion bags. The concentrations of native, apo- and reconstituted stellacyanin were determined with measurements at 280nm, using $\varepsilon_{280}^{1\,cm} = 23200 M^{-1} \cdot cm^{-1}$, or by monitoring the blue colour with optical-absorbance measurements at 604nm, using $\varepsilon_{604}^{1\,cm} = 4080 M^{-1} \cdot cm^{-1}$ (Malmström *et al.*, 1970). The copper content was determined by the biquinoline method described previously (Broman *et al.*, 1962).

Exchange of the copper ion

Since the two copper isotopes show nearly identical e.p.r. spectra, it is necessary for a successful analysis of the reaction mixtures to have very good preparations of the two isotopic forms of stellacyanin uncontaminated by extraneous copper. A method for the exchange of copper in this protein has been published previously (Morpurgo *et al.*, 1974). This preparation, however, showed up to 90% restoration of the blue chromophore, but also the presence of Cu(II) with e.p.r. parameters different from those of the native protein. We therefore found it necessary to develop a new method for the metal exchange.

For the removal of copper ions, 300–750mg of stellacyanin in 5–10 ml of 0.2M-phosphate buffer, pH6, were dialysed for 20–22h against 750–1000ml of 0.1M-Tris/HCl buffer containing 0.1M-KCN, pH9.8, at 20°C (pH measured after CN^- addition). Extensive dialysis against 0.1M-phosphate buffer, pH7 (4 × 1 litre), removed the cyanide and the copper ions. The apo-stellacyanin concentration was determined and, if necessary, adjusted to between 0.2mM and 0.5mM and then the solution split into two equal volumes. The pure copper isotopes ^{63}Cu or ^{65}Cu were added (both as 40mM-CuO in 6M-HCl) to one apo-stellacyanin solution each in a ratio of 1.3–1.5 g-atoms of copper per mol of protein. The reconstitution reactions of [^{63}Cu]stellacyanin and [^{65}Cu]stellacyanin were monitored by measuring the increase in A_{604} in the double-beam spectrophotometer.

The reactions were allowed to proceed for 5–10h at room temperature and then for about 10h at 4°C before dialysis to remove unbound copper ions.

The rapid-freeze experiments

For the mixing of the two isotopic forms of stellacyanin and rapid quenching of the electron-transfer reaction, a rapid-freeze apparatus (Update Instruments, Madison, WI, U.S.A.) was used. The reactants were held in 1 ml Hamilton gas-tight syringes which were connected to a four-jet tangential Ballou (1971) mixing-cell. The ram of the rapid-freeze apparatus was interfaced to a Data General Nova 3 microcomputer which controlled ram speeds and push lengths. The ram velocities and the lengths of the reaction tubes corresponding to different reaction times were determined.

The concentrations of both isotopic forms of stellacyanin (in 0.1M-phosphate buffer, pH7), were determined and made equal. Solutions of [^{65}Cu]stellacyanin (2–3ml) were made anaerobic by alternately decreasing the pressure and flushing with O_2-free N_2. The protein was then anaerobically reduced with small additions (3–6μl) of 0.25M-ascorbate. The titration was stopped when a very small amount of stellacyanin remained oxidized (~1%). The pale blue colour did not vanish completely after leaving the solution under N_2 for

2h, showing that we had not introduced an excess of ascorbate.

The reduced [^{65}Cu]stellacyanin was mixed with oxidized [^{63}Cu]stellacyanin, allowed to react for various times and then sprayed through a nozzle with a diameter of 0.2mm into e.p.r. tubes which contained isopentane with a temperature of $-120°C \pm 5°C$. A Teflon-coated metallic plunger was used to pack 0.3–0.4ml of the frozen droplets in the e.p.r. tubes. To obtain different reaction times we varied the length of the reaction tubes and kept the spray velocity constant and high (12–15 m/s) in order to obtain a small droplet size. For reaction times longer than 150ms we used reaction tubes long enough to contain the whole sample. The computer was programmed to perform a double push, with a delay between the two pushes, this delay being the reaction time of the sample. In this way a series of mixtures were obtained with reaction times ranging from 8ms to 600ms. In addition, one sample was frozen after 7min to ensure a complete reaction. For comparative purposes, two samples, each containing only one of the isotopic forms of oxidized stellacyanin, were obtained in similar rapid-freeze experiments. The sample-containing e.p.r. tubes were stored in liquid N_2.

E.p.r. measurements of the stellacyanin mixtures

The e.p.r. spectra of the rapidly frozen samples were recorded at about 20K using a Varian E-9 e.p.r. spectrometer equipped with a low-temperature system, consisting of an ESR 9 continuous-flow cryostat and a Precision Temperature Controller manufactured by Oxford Instruments. This latter instrument was calibrated against carbon resistors placed in the spectrometer cavity. The spectrometer was interfaced to the Data General Nova 3 minicomputer, which was used to collect and store spectra in digitized form on disc. A Hewlett–Packard 7004 A x–y recorder and a Tektronix 564 oscilloscope were also interfaced to the computer for display of the spectra and their manipulation.

The following conditions in the e.p.r. analysis were constant throughout this work: temperature, 20.0 ± 0.2K; amplitude modulation, 1.00mT; microwave power, 0.2mW; and microwave frequency, 9.25 GHz. The spectra were recorded between 270mT and 345mT with 2.5mT/cm and a sweep rate and time constant of 9.4mT/min and 1.0s respectively. The computer sampled one data point every 0.1mT. To increase signal-to-noise ratio, two to four subsequent scans of each spectrum were collected and the average of these were stored on disc.

Analysis of the e.p.r. spectra

The second integral of every spectrum was calculated. The value of this integral is directly proportional to the amount of cupric ion giving the e.p.r. signal (Aasa & Vänngård, 1975). All spectra were normalized to the same second integral to correct them for slight differences in the amount of copper in the cavity resulting from differences in packing and in the inner diameters of the e.p.r. tubes. Secondly, the spectra were shifted along the field axis, correcting for small differences in the microwave frequency. A displacement of 0.2mT or more between the spectra had to be corrected before further analysis.

Results and discussion

Exchange of the copper ion

The method for the removal of copper from stellacyanin is rather slow, since about 30% of the copper is still bound to the protein after 5h of dialysis against cyanide. After 20–22h of dialysis, less than 3% of the copper remains bound.

On addition of the copper isotopes to the apoprotein the restoration of the blue chromophore was rapid, as seen from the development of blue colour. About 50% of the blue colour had returned after about 5min, and approx. 85% after another 20min of reaction time. The substituted isotopic forms of stellacyanin were subjected to determinations of total copper content, e.p.r.-detectable copper and optical-absorbance measurements. The results of two typical preparations are summarized in Table 1.

Fig. 1 shows the e.p.r. spectra of the two isotopic forms of the protein (A and B) and their difference (C). If spectra A and B are mixed in the ratio 7:3 (the ratio of the isotopes as they occur naturally), the resulting spectrum is identical with that of native stellacyanin.

These results show unambiguously that both isotopic forms of the protein contain 1.0 copper ion per molecule and that this metal has the same unique co-ordination as shown in the native protein. Moreover, all the metal is oxidized and there is no evidence for any non-specifically bound or extraneous copper in either preparation.

The kinetic experiments

The reaction under study may be written as follows:

$$^{63}\mathrm{Cu(II)} + {}^{65}\mathrm{Cu(I)} \underset{k}{\overset{k}{\rightleftharpoons}} {}^{63}\mathrm{Cu(I)} + {}^{65}\mathrm{Cu(II)}$$

The self-exchange rate constants are the same in both directions, and thus the equilibrium constant is unity. At the start of the electron-transfer reaction, only [^{63}Cu]stellacyanin was oxidized. As the reaction proceeded, the concentration of [^{65}Cu(II)]stellacyanin increased and finally each copper isotope was 50% oxidized. The rapidly

Table 1. *Properties of reconstituted stellacyanin*

Results were determined: (*a*) by the biquinoline method (Broman *et al.*, 1962); (*b*) from integration of e.p.r. spectra (Aasa & Vänngård, 1975); (*c*) at 280nm, using $\varepsilon = 23200\,M^{-1}\cdot cm^{-1}$ (Malmström *et al.*, 1970); and (*d*) at 604nm, $\varepsilon = 4080\,M^{-1}\cdot cm^{-1}$ (Malmström *et al.*, 1970).

	Concentration (mM)			
Copper isotope	(*a*) Total copper	(*b*) E.p.r.-detectable copper	(*c*) Protein	(*d*) Blue chromophore
^{63}Cu	0.58	0.58	0.58	0.55
^{65}Cu	0.54	0.53	0.56	0.52

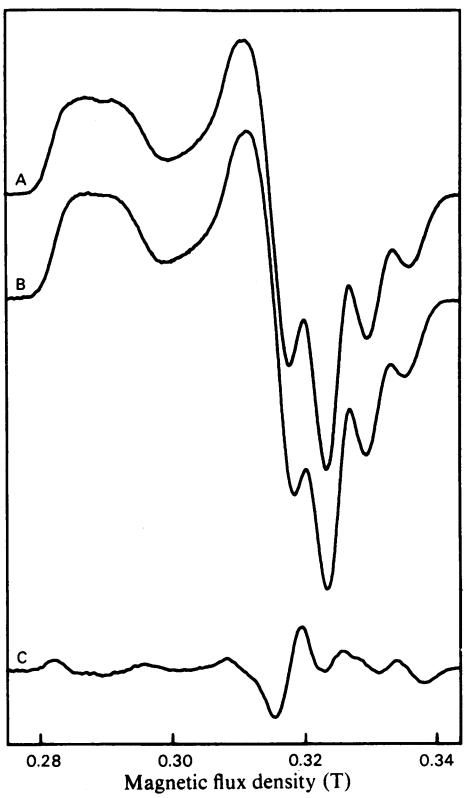

Fig. 1. *E.p.r. spectra of the two isotopically pure forms of stellacyanin*

A, [^{65}Cu]stellacyanin; B, [^{63}Cu]stellacyanin; C, the difference between these two spectra, A minus B. The spectra were recorded under the following conditions: microwave frequency, 9.25GHz; microwave power, 30dB; amplitude modulation, 1.00mT; the temperature was 20.0 ± 0.2K.

quenched samples thus give e.p.r. spectra corresponding to both isotopic forms of the protein in different ratios. The contribution from oxidized [^{65}Cu]stellacyanin increased with increasing reaction time. Provided the concentrations of both reactants are equal and because the equilibrium constant is unity, the electron-transfer reaction is pseudo-first-order, and the increase in the concentration of oxidized [^{65}Cu]stellacyanin is given by:

$$c = A(1 - e^{-k_{app.}\cdot t}) \quad (1)$$

where c is the concentration of [^{65}Cu(II)]-stellacyanin at time t and A is the concentration of [^{65}Cu(II)]stellacyanin at equilibrium (large t), i.e. half of the initial ^{63}Cu(II) concentration. The pseudo-first-order (apparent) rate constant is given by:

$$k_{app.} = k\cdot(\text{total [stellacyanin]})$$

Close agreement between the spectra of the rapidly quenched samples and spectra composed of A and B of Fig. 1 in different proportions could be achieved. However, the fit was never perfect. One possible reason for this observation is that stellacyanin, after rapid oxidation and freezing, does not have exactly the same spectrum as that of stellacyanin which has been in the cupric state for some time, i.e. there may be some small change in the co-ordination sphere of the copper atom after electron transfer. In rapid-freeze experiments one may then capture the form after electron transfer before relaxation around the metal site had occurred. This hypothesis finds support in the following observations.

(*a*) The e.p.r. spectra of rapidly frozen samples which had not undergone rapid valence change could be constructed from spectra A and B (Fig. 1).

(*b*) Samples taken at longer reaction times, i.e. $\geqslant$200ms, could more closely be simulated by mixtures of spectra A and B (Fig. 1).

In order, therefore, to determine the time course of the reaction, it was necessary to compare only spectra of samples which had undergone the rapid electron-transfer reaction. Each spectrum was thus subtracted from that of the sample frozen earliest in the reaction sequence (8–10ms). The shapes of the resulting difference spectra were very similar to the curve shown in Fig. 1, curve C. The amplitude of the largest difference (at about 0.32T; see Fig. 1, curve C) was measured and plotted against the cor-

responding reaction time for each of the samples, giving the experimental points shown in Fig. 2.

The continuous lines in Fig. 2 show exponential curves of the form given in eqn. (1), fitted to the experimental points by a least-squares non-linear regression procedure. It is evident that the experimental points are very well associated with eqn. (1), and this must be considered as evidence that the electron self-exchange reaction of stellacyanin is of pseudo-first-order under the conditions used.

The values of $k_{app.}$ for curves a and b of Fig. 2 are 73.1 s^{-1} and 53.3 s^{-1} respectively. Dividing each $k_{app.}$ value by the appropriate total concentration of stellacyanin, 0.551 mM and 0.532 mM, gives self-exchange rate constants for stellacyanin as $k = 1.33 \times 10^5 M^{-1} \cdot s^{-1}$ from curve a and $1.00 \times 10^5 M^{-1} \cdot s^{-1}$ from curve b. The average value for the self-exchange rate constant measured directly is therefore $1.17 \times 10^5 M^{-1} \cdot s^{-1}$. If curves a and b of Fig. 2 are extrapolated to zero reaction time, the total amplitude of each is 18.7 mm and 17.5 mm respectively. The ratio of these amplitudes is close to the ratio of the initial oxidized stellacyanin concentrations (0.551 mM and 0.532 mM). This is to be expected from eqn. (1) and gives confidence in both the correctness of the mechanism and the fitting procedure.

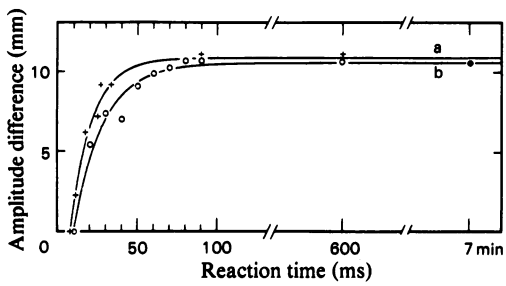

Fig. 2. *Rates of increase of the concentrations of oxidized [^{65}Cu]stellacyanin in the electron-transfer reaction between reduced [^{65}Cu]stellacyanin and oxidized [^{63}Cu]stellacyanin*

Difference amplitudes at 0.32T are shown between e.p.r. spectrum of sample quenched after the shortest reaction time and spectra of samples quenched at later times. The curves are simulations of eqn. (1) (see the text), giving the best least-square fits to the experimental points. All the points in a and b were given equal weights. a, Total stellacyanin concentration 0.551 mM. The shortest reaction time was 8 ms. The experimental points (+) are amplitudes of differences between e.p.r. spectra (8 ms minus t ms). The curve is drawn with $A = 18.71$ and $k_{app.} = 73.1\ s^{-1}$ ($\pm 0.46\ s^{-1}$). b, Total stellacyanin concentration 0.532 mM. The shortest reaction time was 10 ms. The experimental points (○) are amplitudes of differences between e.p.r. spectra (10 ms minus t ms). The curve is drawn with $A = 17.51$ and $k_{app.} = 53.3\ s^{-1}$ ($\pm 0.58\ s^{-1}$).

Although the salt concentration was not varied in our experiments, it is unlikely that ionic strength will markedly affect the value of the self-exchange constant, as it has no effect on reactions of stellacyanin with other proteins (Wilson *et al.*, 1979). The value we report may be compared (Table 2) with the values obtained by application of the Marcus (1963) Theory to the reactions between stellacyanin and other outer-sphere reagents and with other redox proteins thought to transfer electrons via an outer-sphere mechanism.

It is apparent that there is close agreement between the value of k_{11} directly determined and those values calculated from application of the Marcus (1963) Theory. This agreement strongly supports the view that stellacyanin possesses a kinetically accessible site close to the surface of the protein and that it transfers electrons via an outer-sphere mechanism both in self-exchange processes and with metal complexes. Even the values determined from the reactions of stellacyanin with other proteins are in reasonable agreement with the value reported here, being within an order of magnitude when calculated from the reaction of cytochrome c_{551} (*Pseudomonas aeruginosa*) and approx. 20-fold less when calculated from the reaction between stellacyanin and horse heart cytochrome *c* (Wilson *et al.*, 1979). This agreement again demonstrates the 'well-behaved' nature of stellacyanin acting as a simple accessible outer-sphere reagent.

Table 2. *A comparison of the estimated values of the self-exchange rate constant for stellacyanin*

Abbreviations used: $Ru(NH_3)_5py^{3+}$, pentamminepyridineruthenium(III); $Co(phen)_3^{3+}$, = tris-(1,10-phenanthroline)cobalt(III).

Reagent	k_{11} (stellacyanin) ($M^{-1} \cdot s^{-1}$)	Reference
$Ru(NH_3)_5py^{3+}$	1.6×10^5	Cummins & Gray (1977)
$Fe(EDTA)^{2-}$	2.3×10^5	Cummins & Gray (1977)
$Co(phen)_3^{3+}$	3.3×10^5	Cummins & Gray (1977)
Cytochrome c_{551}	2.9×10^4	Wilson *et al.* (1979)
Cytochrome *c* (horse)	6×10^3	Wilson *et al.* (1979)
Stellacyanin	1.17×10^5	The present work

Mauk *et al.* (1980) have attempted to relate the self-exchange constants of metalloproteins to the distance over which electron transfer occurs, i.e. to use kinetic data to obtain structural information. To do this they have used a modified form of Hopfield's (1974) equation:

$$R = -0.693 \ln(k/C) \quad (2)$$

where R is the intersite distance and k the electron-transfer rate constant. The value of C was obtained empirically by applying eqn. (2) to stellacyanin self-exchange. For this calculation the inter-site distance was assumed to be the closest approach between histidine ligands, i.e. twice the van der Waals' radius of an aromatic carbon atom. This distance and the self-exchange rate constant for stellacyanin were taken as 0.37nm (3.7Å) and $2.4 \times 10^5 M^{-1} \cdot s^{-1}$ respectively. This latter value, calculated from the redox reactions with metal complexes (average of values in Table 2), is in good agreement with the value of $1.17 \times 10^5 M^{-1} \cdot s^{-1}$ which we report, and this lends support to the inter-site distances calculated for other redox proteins by Mauk *et al.* (1980).

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