EPR STUDIES ON THE BLUE COPPER PROTEIN, RUSTICYANIN

A protein involved in Fe^{2+} oxidation at pH 2.0 in *Thiobacillus ferro-oxidans*

John C. COX*
Department of Biochemistry, University of Dundee DD1 4HN, Scotland

and

Roland AASA and Bo G. MALMSTRÖM
Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, Fack, S-402 20 Göteborg, Sweden

Received 10 July 1978

1. Introduction

Copper proteins may contain up to three different forms of copper, designated types 1, 2 and 3 [1]. Of the many copper proteins found in nature, one group which has been the focus of much recent attention is the blue copper proteins. Several blue copper proteins have been discovered, all characterised by an intense absorption around 600 nm and an unusual EPR spectrum, having a very small hyperfine splitting constant [2]. Blue copper proteins may contain one or more copper atoms per molecule (and one or more types of copper) but the intense blue colour and unusual EPR parameters are associated solely with the type 1 copper [3].

There are numerous blue proteins which contain only type 1 copper, e.g., azurin, stellacyanin, umecyanin and plastocyanin [2]. Although these proteins are basically similar with respect to their absorption and EPR properties, they differ significantly in terms of their function and minute properties such as g- and A-values from the EPR spectrum, absorption maxima, mid-point potential, response to pH variation [1]. Those blue proteins which exhibit some unusual characteristics are of special interest and it is useful to study them to see if an overall pattern emerges relating the variation in properties to the nature of the copper centre.

In this paper we examine the EPR properties of such a protein, namely rusticyanin, a blue copper protein recently purified from the acidophilic bacterium *Thiobacillus ferro-oxidans* [4], which contains one copper atom per molecule. Rusticyanin is thought to be the initial acceptor of electrons in the oxidation of Fe^{2+} by *Thiobacillus ferro-oxidans* [5]. It is stable at pH 2.0 and at this pH addition of Fe^{2+} causes a reversible loss of blue colour which may be a reduction of the Cu centre by Fe^{2+}. The mid-point potential at pH 2 of rusticyanin is 0.68 V [6] which is unusually high, being second only to the blue oxidase laccase from the fungus *Polyporus versicolor*. We report the effects of pH variation and certain anions on the EPR spectrum of rusticyanin and examine quantitatively the proposal that Fe^{2+} acts as a reductant for the copper centre.

Address correspondence to Roland Aasa

* Present address: Department of Microbiology, Division of Biological Sciences, University of Indiana, Bloomington, IN 47401, USA

2. Materials and methods

Rusticyanin was prepared as in [4]. EPR measurements were made at 77 K in a Varian E-3 spectrometer at 9.12 GHz and at about 90 K in a Varian V-4503 spectrometer operating at 34.8 GHz. Optical spectra were measured with a Beckman model 24 spectrophotometer at 20°C. EPR-detectable copper was measured by double integration of the 9 GHz EPR spectrum, comparing with a standard solution of Cu^{2+} in 2 M $NaClO_4$, pH 2. Total copper was determined in a Perkin Elmer model 303 atomic absorption spectrophotometer. Dialysis was carried out against 10 mM H_2SO_4, pH 2.0, using Visking dialysis tubing. Simulations of EPR spectra were performed with a Nova 3 minicomputer.

3. Results

Figures 1 and 2 show the EPR spectra of oxidized rusticyanin recorded at 9 GHz and 35 GHz, respectively. The figures also show computer simulated spectra using the g-values (2.019, 2.064, 2.229) and A-values (6.5, 2.0, 4.5) mT, which give a satisfactory

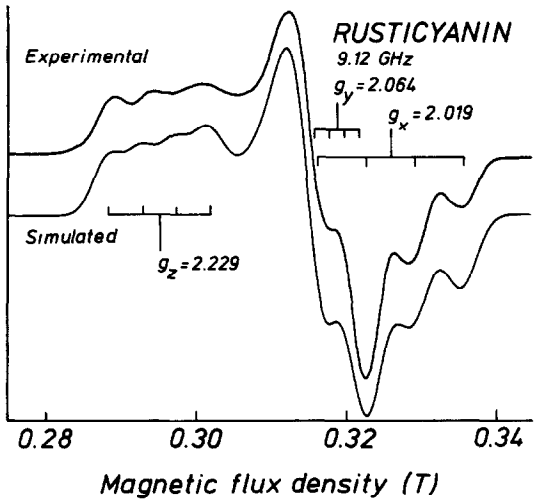

Fig.1. EPR spectrum of rusticyanin (pH 2, 0.9 mM Cu^{2+}) recorded at microwave frequency, 9.121 GHz, temp. 77 K and microwave power, 20 mW. Lower trace is a simulated spectrum using Gaussian line-shape with g-values (2.019, 2.064, 2.229), A-values (6.5, 2.0, 4.5) mT and line-widths (4.0, 5.0, 3.8) mT.

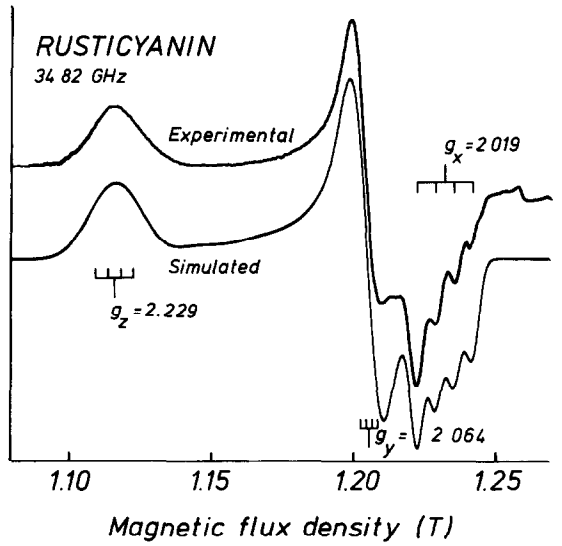

Fig.2. EPR spectrum of rusticyanin (same preparation as in fig.1) recorded at microwave frequency 34.82 GHz, temperature, 90 K and microwave power, ~10 mW. The lower trace is simulated with the same parameters as in fig.1 except linewidths (4.7, 8.0, 14) mT.

fit. The preparation contained a small amount of impurity copper, resembling free copper in water, which probably is the main reason for the small discrepancies between experimental and simulated spectra. The hyperfine coupling along g_y is not resolved and the given value can be taken as an upper limit. The fit along g_z can certainly be improved, using a larger A_z, if the line-width is allowed to be different for different hyperfine lines. The integrated intensity of the 9 GHz spectrum corresponds to 95% of the copper measured by atomic absorption spectrophotometry.

Addition of Fe^{2+} to a sample of rusticyanin causes a loss of the absorption band at 600 nm [4] which is restorable on addition of Fe^{3+} (J. C. C. and D. H. Boxer, unpublished observations). The data of fig.3 shows that this loss of blue colour is concomitant with a loss of the Cu^{2+} signal measured by EPR spectrometry. Addition of Fe^{2+} in a 1 : 1 ratio with Cu^{2+} resulted in the disappearance of the Cu^{2+} signal, indicating the absence of any paramagnetic copper, which suggests a reduction of Cu^{2+} to Cu^{+}. Figure 3 also shows the effect of Fe^{3+} on rusticyanin previously treated with 1.2 mM Fe^{2+}. Addition of Fe^{3+} resulted in the

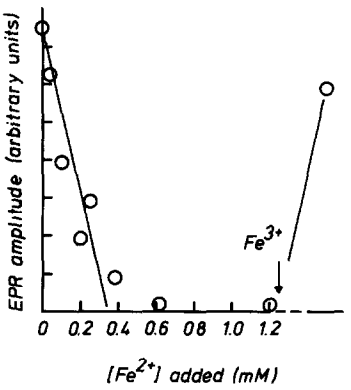

Fig.3. Titration of rusticyanin with $FeSO_4$. A 0.3 ml aliquot of rusticyanin (0.4 mM with respect to Cu^{2+}) at pH 2.0 was added to an EPR cuvette. Aliquots of 10 mM $FeSO_4$ in H_2SO_4, pH 2.0 were added and the mixture allowed to stand for 5 min. The sample was then frozen and the EPR spectra were recorded at 9 GHz and 77 K. The amplitude of the resulting Cu^{2+} signal was plotted against the concentration of Fe^{2+} added. After full reduction had occurred 20 mM Fe^{3+} was added, indicated by arrow, and rusticyanin was dialysed against 10 mM H_2SO_4, pH 2.0 for 4 h. The EPR spectrum was then recorded.

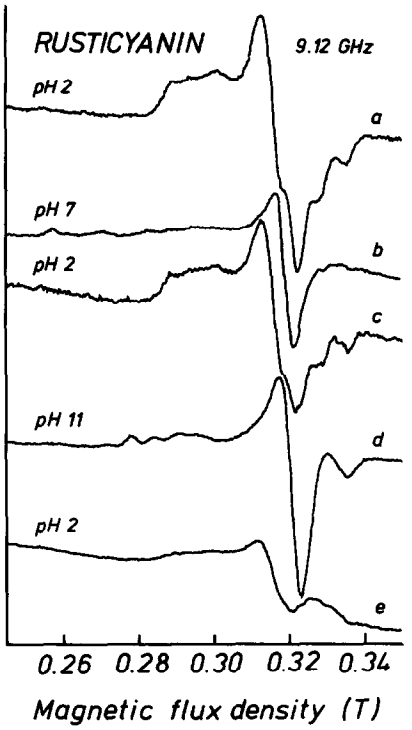

Fig.4. EPR spectra of rusticyanin (initial 2 mM Cu^{2+}) at varying pH. (a) oxidised protein in 10 mM H_2SO_4 buffer, pH 2. (b) 1 M potassium phosphate buffer, pH 7, allowed to stand for 15 min. (c) the protein used in (b) was dialysed for 24 h against 10 mM H_2SO_4, pH 2, re-oxidised with 20 mM $FeCl_3$ and dialysed against 10 mM H_2SO_4, pH 2. (d) a separate aliquot of protein was taken to pH 11 by addition of 1 M $NaHCO_3$: NaOH buffer. (e) the protein used in (d) was treated in the same way as (c). The spectrometer gain for each recording was adjusted to take account for the dilution. Microwave frequency, 9.12 GHz, temp. 77 K, microwave power, 20 mW.

re-appearance of the initial spectrum. About 80% of the original signal was regained.

Increasing the pH of a solution of a blue copper protein often causes a loss of the type 1 signal and a loss of blue colour [1]. Figure 4 shows the effect of pH variation on the EPR spectrum of rusticyanin. Rusticyanin retains most of the type 1 signal until pH 7.0 is reached whereupon the type 1 EPR signal rapidly disappears. The original EPR signal can be restored by returning the rusticyanin solution to pH 2.0 and re-oxidising it with Fe^{3+}. If the pH of the solution of rusticyanin is increased to 11.0 the protein precipitates and a completely new EPR signal appears. Dialysis against pH 2.0 (10 mM H_2SO_4) and re-oxidation with Fe^{3+} resulted in the re-appearance of ~30% of the original rusticyanin spectrum.

The effect of certain anions on the EPR spectrum of oxidised rusticyanin was investigated. There was no discernable change in the signal of type 1 copper in the presence of stoichiometric amounts of azide or thiocyanate. If these copper chelating anions were added to excess (500-fold) copper was removed from the protein and the type 1 EPR signal was replaced by a spectrum indicative of a more normal Cu (II) complex. Fluoride is a potent inhibitor of fungal laccase [8] to which it binds tightly to the type 2 copper, but F^- had no effect on the copper signal of rusticyanin even when present in 500-fold excess.

4. Discussion

The EPR spectra (fig.1,2) of rusticyanin show that the one atom of copper per molecule of protein can be classified as type 1 copper [1]. The spectrum

closely resembles that of stellacyanin with respect to the hyperfine splittings and departure from axial symmetry. The absorption spectra of the two proteins are also very similar [1,4,7]. However, the two proteins differ considerably in the value of their mid-point potentials, 0.184 V for stellacyanin [9] and 0.68 V for rusticyanin [6]. This big difference is apparently not manifested in the EPR spectra. Thus it is important to remember when drawing conclusions from EPR spectra that EPR parameters may sometimes be very insensitive indicators of certain chemical properties. Stellacyanin contains no methionine [10] whereas rusticyanin contains three methionine residues [4]. It has been shown that methionine is a ligand to the copper atom in the blue protein plastocyanin [11], and differences in the nature of the ligands to the copper atom may be responsible for the difference in properties of two otherwise similar proteins.

When present in equivalent concentrations to the Cu^{2+} in rusticyanin, copper chelating anions have no effect on the EPR spectrum of the protein. When present in excess these anions can, however, remove the copper from the protein enabling studies to be carried out on the apoprotein.

Increasing the pH of a solution of copper protein causes a loss of blue colour in several cases [1]. Rusticyanin also loses its blue colour and type 1 EPR signal if the pH is raised to 7.0 and the very low intensity of the spectrum at pH 7.0 (fig.4, trace b) makes it very probable that this is due to reduction of the copper. The process can be reversed by returning the solution of rusticyanin to pH 2.0, but it is also necessary to oxidise the protein (with Fe^{3+}) in order to regain the type 1 signal, further suggesting that the loss of colour at pH 7.0 is a reduction. The stability of rusticyanin is emphasised by the fact that even when the protein is precipitated (pH 11.0) about 30% of the native protein can be regained by returning to pH 2.0.

The data reported here show that at pH 2.0 Fe^{2+} reduces the copper centre. It is not auto-oxidisable, even at pH 2.0. This suggests that rusticyanin can accept electrons from Fe^{2+} though as yet it is uncertain whether cytochrome c_{551} acts as a physiological oxidant for the protein as suggested [5] in their scheme for the respiratory chain of *Thiobacillus ferro-oxidans*. But the reversible oxidation/reduction by iron and the fact that rusticyanin constitutes about 5% of the total protein of the cell strongly suggest a role in the oxidation of Fe^{2+} by *Thiobacillus ferro-oxidans*.

Acknowledgements

John C. Cox was the recipient of an EMBO short-term fellowship. We are grateful to Dr D. Boxer (University of Dundee) for helpful discussion and assistance in the preparation of rusticyanin. This work has been supported by grants from the Swedish Natural Science Research Council.

References

[1] Malkin, R. and Malmström, B. G. (1970) Adv. Enzymol. 33, 177–244.
[2] Fee, J. A. (1975) Struc. Bonding 23, 2–60.
[3] Malmström, B. G., Reinhammar, B. and Vänngård, T. (1968) Biochim. Biophys. Acta 156, 67–76.
[4] Cox, J. C. and Boxer, D. H. (1978) Biochem. J. in press.
[5] Ingledew, W. J., Cox, J. C. and Halling, P. J. (1977) FEMS Microbiol. Lett. 2, 193–197.
[6] Ingledew, W. J. (1976) Proc. Soc. Ital. Biochem. October 1976, p. 84.
[7] Malmström, B. G. Reinhammar, B. and Vänngård, T. (1970) Biochim. Biophys. Acta 205, 48–57.
[8] Malkin, R., Malmström, B. G. and Vänngård, T. (1968) FEBS Lett. 1, 50–54.
[9] Reinhammar, B. (1972) Biochim. Biophys. Acta 275, 245–259.
[10] Peisach, J., Levine, W. G. and Blumberg, W. E. (1967) J. Biol. Chem. 242, 2847–2858.
[11] Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M. and Venkatappa, M. P. (1978) Nature 272, 319–324.