The Purification and some Properties of Rusticyanin, a Blue Copper Protein Involved in Iron(II) Oxidation from *Thiobacillus ferro-oxidans*

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The 'blue' copper-containing protein rusticyanin was purified to homogeneity from cells of the chemolithotrophic bacterium *Thiobacillus ferro-oxidans* by $(\text{NH}_4)_2\text{SO}_4$ fractionation and ion-exchange chromatography. The protein, which is stable at low pH, consists of a single polypeptide chain of mol.wt. 16500 and possesses $0.79 (\pm 0.28)$ g-atom of Cu/mol. The protein, which does not contain arginine residues, has optical absorbance maxima at 287, 450, 597 and 750 nm and is generally similar to azurin. The isolated protein is reduced directly by Fe^{2+} with a 1:1 stoichiometry to Cu. On reduction by Fe^{2+} the absorption peaks at 450, 597 and 750 nm are abolished, with the appearance of a new absorption band at 320 nm. The results obtained are consistent with rusticyanin being the initial acceptor of electrons from Fe^{2+} during respiratory iron oxidation.

Thiobacillus ferro-oxidans is a chemolithotrophic bacterium that uses Fe^{2+} as the sole energy source for growth and CO_2 fixation (Schlegel, 1975), and is unusual in that it grows optimally at pH 2.0.

The organism is an obligate aerobe and its respiratory chain contains *c*- and *a*₁-type cytochromes (Din *et al.*, 1967). Copley & Haddock (1975) showed that a copper protein, rusticyanin, which could be reduced by Fe^{2+} in a cell-free preparation, was present in *Th. ferro-oxidans*, and they suggested that rusticyanin may be part of the respiratory chain of the bacterium.

In the present paper we describe a procedure for the isolation of rusticyanin and report some of its chemical and physical properties. Comparisons are drawn with other copper proteins.

Materials and Methods

Growth and harvest of *Th. ferro-oxidans*

Th. ferro-oxidans (strain T.f.3) was kindly supplied by Dr. D. Herbert, Microbiological Research Establishment, Porton Down, Wilts., U.K., and grown in the chemostat mode as described by Copley & Haddock (1975). Chemostat effluent (45 litres) was harvested at 10°C in a continuous-flow rotor fitted to an MSE 18 centrifuge operated at 17000 rev./min with a flow rate of 15 litres/h, yielding a cell paste of 3–5 g wet wt. This was washed twice in 10 mM- H_2SO_4 , pH 2.0, to remove precipitated $\text{Fe}(\text{OH})_3$ and stored as a pellet at –15°C until required.

Purification of rusticyanin

All procedures were carried out at 4°C.

Cell breakage and extraction. Approx. 25 g wet wt.

of *Th. ferro-oxidans* cell paste was thawed and resuspended (1:4, w/v) in 10 mM- H_2SO_4 , pH 2.0. The cells were ruptured by passage three times through a French pressure cell at 20000 lb/in² (1.4×10^5 kPa). The broken-cell suspension was stirred for 15 min and then centrifuged at 10000 g for 10 min to remove debris. The blue-brown supernatant was removed and the pellet resuspended in 40 ml of H_2SO_4 , pH 2.0, stirred and centrifuged as previously. The supernatants were combined and centrifuged at 150000 g for 1 h. The resulting pellet consisted of the membrane fraction. The supernatant was deep-blue in colour and contained rusticyanin.

$(\text{NH}_4)_2\text{SO}_4$ fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant slowly over a period of 10 min, to give 70% saturation. The resulting solution was stirred for a further 10 min and then centrifuged at 18000 g for 15 min. The blue supernatant was then adjusted to 95% saturation by a further addition of solid $(\text{NH}_4)_2\text{SO}_4$, resulting in precipitation of rusticyanin, which was collected by centrifugation at 20000 g for 20 min. The pale-yellow supernatant was discarded and the dark-blue pellet resuspended in approx. 10 ml of 10 mM-sodium acetate buffer (pH 5.5) and dialysed against 2 litres of the same buffer for 18 h. The dialysis medium was changed twice during this time.

Ion-exchange chromatography. The dialysis residue was layered on a column (2 cm $\times$ 10 cm) of DEAE-cellulose (DE-52) equilibrated with 10 mM-sodium acetate, pH 5.5. The blue protein passed through in the non-adsorbed fraction.

The combined blue fractions from the DEAE-

cellulose column were applied directly to a CM-cellulose (CM-32) column (2cm×8cm) equilibrated with 10mM-sodium acetate, pH 5.5. Rusticyanin was adsorbed as a dark-blue band at the top of the column. The column was washed thoroughly with the equilibrating buffer and rusticyanin was subsequently eluted by the addition of 100mM-NaCl in 10mM-sodium acetate buffer (pH 5.5.). The fractions containing the blue protein were pooled and dialysed against H₂SO₄, pH 2.0. The pure protein could be stored either frozen at -15°C or dialysed against 10mM-HCl and freeze-dried.

Amino acid analysis

Preparations of rusticyanin were dialysed exhaustively against water and then freeze-dried. Samples, after performic acid oxidation (Moore, 1963), were hydrolysed *in vacuo* with 6M-HCl for 24h at 110°C and the hydrolysate was analysed with a Beckman Multichrom amino acid analyser (Cohen, 1973). Cysteine was also determined by the method of Ellman (1958) by using 5,5'-dithiobis-(2-nitrobenzoic acid) in a colorimetric assay. Tryptophan was measured by the method of Spies & Chambers (1949), by using procedure 'K'.

Ultracentrifugation

This was carried out with the assistance of Dr. P. Cohen with a Spinco model E analytical ultracentrifuge at 15°C equipped with a schlieren optical system, previously described by Cohen (1973). Low-speed (24000rev./min for 24h) sedimentation-equilibrium experiments were carried out at an initial protein concentration of 3.0mg/ml in 50mM-potassium phosphate buffer, pH 7.5, and M_z (Van Holde & Baldwin, 1958) was calculated from the schlieren pattern obtained. The partial specific volume of the protein was calculated from the amino acid composition and the appropriate temperature correction factor for 20°C was applied (Taylor *et al.*, 1975).

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969). Marker proteins used in the determination of the subunit molecular weight of rusticyanin were: bovine serum albumin (mol.wt. 67000), carbonic anhydrase (mol.wt. 29200), myoglobin (mol.wt. 16000) and cytochrome *c* (mol.wt. 11700).

Discontinuous-gel electrophoresis under non-dissociating conditions at pH 3.5 was carried out by the procedure of Reisfeld *et al.* (1962).

For both systems 10% polyacrylamide gels were used, electrophoresis being carried out at 5mA per tube for 1-2h. Gels were stained in 0.25% (w/v) Coomassie Blue and destained by immersion in a

mixture containing 7% (v/v) acetic acid and 5% (v/v) methanol. Gel densitometry was performed on a Joyce-Loebl Chromoscan 200 apparatus with the 5-040 filter.

Other techniques

Optical spectra were obtained with a Beckman model 24 spectrophotometer at room temperature in cells of 1cm light-path.

Protein was routinely measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K.) as standard. More precise determinations were obtained for purified preparations by using a synthetic-boundary method in the ultracentrifuge as described by Cohen (1973).

Copper content was measured in a Perkin-Elmer model 303 atomic-absorption spectrophotometer.

Reagents

All chemicals used except NaIrCl₆ were of the AnalaR grade and were purchased from BDH Chemicals, Poole, Dorset, U.K. NaIrCl₆ was obtained from K & K Laboratories, Plainview, NY, U.S.A. Dialysis was carried out in Visking dialysis tubing from the Scientific Instrument Co., London W.C.1, U.K. DEAE-cellulose (DE-52) and CM-cellulose (CM-32) were obtained from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, U.K., and prepared for use in accordance with the instructions of the manufacturer.

Results

Purification of rusticyanin

Throughout the isolation the purity of rusticyanin was monitored by the ratio of A_{597} to the protein concentration. Rusticyanin cannot be detected in stages preceding the pH 2 extract owing to the presence of other chromophores. Details of the purification method are given in the Materials and Methods section and a balance sheet for the purification is shown in Table 1. The stringent conditions (pH 2) used during the cell-breakage stage bring about a considerable purification of the protein. Reference to Table 1 shows that rusticyanin is obtained in 40% yield from the pH 2 supernatant and accounts for about 85% of the protein in the pH 2 supernatant. The subsequent overall purification factor of 1.21 is therefore low because it assumes the pH 2 supernatant as the starting material for the procedure and assigns to this stage a purification factor of 1.0 on the basis of A_{597} /mg per ml of rusticyanin (Table 1). These results are typical for the procedure. The remainder of the procedure is designed to remove the minor impurities. Of these, a substantial impurity is removed by the 70%-satd.-(NH₄)₂SO₄ fractionation. After centrifugation a pink

Table 1. Balance sheet for the purification of rusticyanin
Protein was determined by the method of Lowry *et al.* (1951). Abbreviation: n.t., not tested.

Fraction	Total protein (mg)	Total A_{597} (volume $\times A_{597}$ /ml)	A_{597} /mg of rusticyanin per ml	Purification factor	Yield (%) A_{597}	Total Cu (μ g)	Yield (%) Cu
pH2 supernatant	715.0	63.7	0.089	1.0	100	1584.4	100
70%-satd.-(NH ₄) ₂ SO ₄ supernatant	423.5	61.6	0.145	1.63	96.7	n.t.	n.t.
95%-satd.-(NH ₄) ₂ SO ₄ supernatant	365.0	34.4	0.094	1.06	54.0	867.0	54.7
DE-52 DEAE-cellulose	312.0	30.2	0.096	1.08	47.5	853.1	53.8
CM-32 CM-cellulose	247.5	26.0	0.108	1.21	40.8	738.8	46.6

Fig. 1. CM-cellulose ion-exchange chromatography. Details of the procedure are described in the Materials and Methods section. The combined eluate (312 mg of protein) from the DEAE-cellulose column was applied to the column (2 cm $\times$ 8 cm) and 3 ml fractions were collected. After removal of the non-adsorbed fraction, rusticyanin was recovered by elution with 100 mM-NaCl in 10 mM-sodium acetate, pH 5.5. A further protein fraction could be recovered by elution with 200 mM-NaCl in the same buffer. The fractions between the arrows containing rusticyanin were combined. Symbols: Δ , conductivity; $\square$, A_{280} ; $\circ$, A_{597} .

Fig. 2. Gel electrophoresis of rusticyanin. Curve (a) is a densitometer scan of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of rusticyanin. The method of Weber & Osborn (1969) was used. A portion (10 μ g) of the combined eluate from the CM-cellulose ion-exchange column was electrophoresed and stained for protein. Curve (b) is a densitometer scan of pH 3.5 electrophoresis of rusticyanin. The non-dissociating conditions of Reisfeld *et al.* (1962) were used. A portion (10 μ g) of the same sample as used in (a) was electrophoresed and stained for protein.

pellet remains and reduced-minus-oxidized difference spectra show it to contain a cytochrome of the *c* type that is stable at pH 2.0. Ingledew *et al.* (1977) have proposed a role for such a cytochrome in the respiratory chain of *Th. ferro-oxidans*. After treatment with 70% -satd. (NH₄)₂SO₄ the ratio of A_{597} to protein is higher than in subsequent stages. Coupled with the low overall purification factor of 1.21 this suggests that some copper is being lost at the second (NH₄)₂SO₄ fractionation stage. Such a problem has been encountered before during the purification of other copper proteins (Kato *et al.*, 1962; Milne & Wells, 1970).

Other impurities are removed by the ion-exchange steps (Fig. 1). However, some of the colourless fractions obtained probably represent breakdown products of rusticyanin.

As stated above, rusticyanin cannot be measured optically before the pH2-supernatant stage of the preparation. However, assuming that no loss of rusticyanin has occurred up to this stage, the rusticyanin content of whole cells can be estimated at the comparatively high concentration of about 4 nmol/mg of protein (this calculation uses the absorption coefficient at 597 nm described in a later section).

Criteria of purity and molecular weight

Rusticyanin showed a single protein-staining band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2a). A mol.wt. of 16600 was obtained for rusticyanin by using gels calibrated with proteins of known molecular weight. Use of the

Fig. 3. Sedimentation-equilibrium ultracentrifugation of rusticyanin

Rusticyanin (the combined fractions between the arrows of Fig. 1) at an initial protein concentration of 3.0mg/ml in 50mM-potassium phosphate, pH7.5, was centrifuged at 24000rev./min at 15°C. The plot of the ratio of refractive-index increment to radial position against concentration in arbitrary units was obtained from the schlieren pattern produced after centrifugation for 24h. The partial specific volume calculated by the method of Cohn & Edsall (1943) from the amino acid composition was 0.739 ml/g.

Table 2. Amino acid composition of rusticyanin
Hydrolysis and analysis of rusticyanin were carried out as described in the Materials and Methods section.

Amino acid	Content	
	(mol/100 mol)	(residues/16 500 mol.wt. of protein)
Asp	8.14	13
Thr*	12.28	19
Ser*	3.59	6
Glu	4.81	8
Pro	9.14	14
Gly	11.99	19
Ala	8.80	14
Val	9.66	15
Met	1.96	3
Ile	3.70	6
Leu	3.34	5
Tyr	3.70	6
Phe	5.94	9
His	3.41	5
Lys	9.09	14
Arg	0.015	0
Trp†	1.30	2
Cys‡	0.075	1
Total		159

* Serine and threonine were calculated by assuming a 10% and 5% loss respectively on hydrolysis.

† Determined colorimetrically by the method of Spies & Chambers (1949).

‡ Determined after performic acid oxidation and by titration with 5,5'-dithiobis-(2-nitrobenzoic acid).

onn-dissociating gel system of Reisfeld *et al.* (1962) also resulted in a single protein-staining band (Fig. 2b), demonstrating the homogeneity of the preparation. The preparation was also eluted as a single symmetrical peak after gel filtration on Sephadex G-75 at pH2 (results not shown).

Sedimentation-equilibrium experiments performed at pH7.5 showed that the protein sedimented homogeneously with no trace of other components, as indicated by the straight line obtained from the plot in Fig. 3. The molecular weight of the protein was calculated from this experiment to be 16500, corresponding closely to the value obtained by gel electrophoresis.

Therefore rusticyanin is monomeric in aqueous solution at pH7.5.

Amino acid composition

The analysis for rusticyanin is given in Table 2. The most striking feature of the composition was the absence of arginine and the low contents of cysteine, tryptophan and histidine in the protein, which bears strong comparison with the blue copper proteins azurin and plastocyanin (Milne & Wells, 1970; Ambler & Brown, 1967; Katoh *et al.*, 1962). Despite rusticyanin's unusual stability at low pH it does not contain an abnormal complement of acidic or basic residues.

Copper content

Copper analysis by atomic-absorption spectrophotometry showed that the protein contained $3.03 (\pm 0.74) \mu\text{g}$ of copper/mg of protein. The protein was measured by the synthetic-boundary method and the mean and s.d. refer to four independent preparations of rusticyanin. This gives a value of $0.79 (\pm 0.28) \text{g-atom of copper/16 500g of protein}$, indicating a copper content of 1 atom per molecule of rusticyanin.

Copper analysis throughout the isolation of rusticyanin (Table 1) shows that the overall yield of copper closely corresponded to the yield of rusticyanin measured by the A_{597} . The ratio of A_{597} to copper concentration is virtually constant throughout the preparations. This suggests that all the copper found in the pH2 supernatant is associated with rusticyanin.

Optical spectra

Fig. 4 shows the absolute spectrum of isolated rusticyanin. It consists of a large band between 280 and 290nm (λ_{max} , 287nm) due to the absorption of tyrosine and tryptophan residues, and two broad absorption bands in the visible region. The more prominent of these has an absorption maximum at 597nm and the other, smaller, band at 450nm. A small shoulder (at about 415nm) on the 450nm band is seen in Fig. 4; this may indicate a

Fig. 4. Optical absorption of rusticyanin

The spectra were obtained from a solution of purified rusticyanin (1.10 mg of protein/ml; 3.40 μ g of copper/ml) in 10 mM- H_2SO_4 , pH 2.0, in a 1 cm-light-path cell. Curve (a), baseline; curve (b), absolute spectrum of rusticyanin; curve (c), absolute spectrum of rusticyanin after reduction by 10 mM- FeSO_4 .

Table 3. Molar absorption coefficients of optical absorption bands of rusticyanin

The molar absorption coefficients were determined from spectrum (b) of Fig. 4. Protein (1.1 mg/ml) was determined by the synthetic-boundary method in the ultracentrifuge.

λ (nm)	Molar absorption coefficient (litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$)
287	4550
450	1060
597	1950
750	750

Fig. 5. Oxidized-minus-reduced difference spectrum of rusticyanin

Conditions were identical with those referred to in the legend of Fig. 4. Curve (a) shows the oxidized-oxidized rusticyanin baseline. Curve (b) shows the oxidized-reduced rusticyanin difference spectrum. Reduction was effected as described in the legend of Fig. 4.

Fig. 6. Titration of rusticyanin with Fe^{2+}

Purified rusticyanin (3.20 mg of protein/ml; 7.30 μ g of copper/ml) in 10 mM- H_2SO_4 , pH 2.0, was titrated with FeSO_4 . A_{597} was measured (1 cm light-path) initially and 10 min after each Fe^{2+} addition. The end point was estimated by extrapolation by assuming that at low $[\text{Fe}^{2+}]$ the change in A_{597} was directly proportional to the Fe^{2+} added.

small amount of cytochrome impurity, since in some preparations it was absent. Rusticyanin also exhibits a small absorption in the near-i.r. region (750 nm), which is partly masked by the 597 nm band. The molar absorption coefficients calculated from the absorption spectra are shown in Table 3. There is some variation in the absorption coefficients for rusticyanin when calculated on the basis of protein content. However, when the absorption coefficient at 597 nm is calculated on the basis of copper content there is less variation in the values obtained. A plot of A_{597} against copper concentration, with data obtained from six independent preparations of rusticyanin, was linear (correlation coefficient, 0.984) corresponding to a molar absorption coefficient of 2238 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. This value is higher than that usually obtained from calculations based on protein content, since it does not take into account any apoprotein that may be present. It assumes, however, that all the copper present is liganded to the protein.

Interaction of rusticyanin with Fe^{2+}

Cobley & Haddock (1975) showed that the A_{597} of rusticyanin in the pH 2 supernatant could be bleached by reduction with Fe^{2+} . In the present paper we demonstrate that rusticyanin interacts directly with Fe^{2+} , since addition of Fe^{2+} to the pure protein causes complete and immediate bleaching (less than 1 min) of the absorption bands at 450, 597 and 750 nm (Fig. 4). Although in this preparation a small amount of the absorbing species at 415 nm was present, identical results were obtained with preparations in which this absorption band was absent. This indicates that, whatever the nature of the

absorbing species at 415 nm, it has no effect on the interaction of rusticyanin with Fe^{2+} . In addition to this we report that on reduction of rusticyanin with iron an absorption band appeared at about 320 nm (Fig. 4b). This band is better resolved in the oxidized-minus-reduced difference spectrum (Fig. 5a), where it appears as a trough with an absorption minimum at 315 nm.

The stoichiometry of the Fe^{2+} -rusticyanin interaction was determined by direct titration (Fig. 6). The end point was determined by extrapolation, and it was shown that complete abolition of the A_{597} was effected by 0.91 g-ion of Fe^{2+} /g-atom of Cu. This demonstrates that all the copper in rusticyanin is reduced by Fe^{2+} . Between 70 and 80% of the A_{597} can be restored after reduction with Fe^{2+} , by the addition of three or four crystals of solid NaIrCl_6 , a powerful oxidant.

Storage and stability of rusticyanin

In a solution at 4°C the A_{597} of rusticyanin gradually decreases. This is a pH-dependent process, the time required for a 50% decrease of A_{597} being 1–2 weeks at pH 2.0, but only about 10 s at pH 8.0. This loss of absorption with time is distinct from the redox reaction described above, since it cannot be restored by the addition of NaIrCl_6 . The ion-exchange steps of the purification procedure were performed at pH 5.5. This is the highest pH to which the protein can be exposed for any length of time. The protein is best stored either frozen at -15°C, where it will be stable for up to 2 months, or freeze-dried, in which case rusticyanin is stable for at least 4–6 months. On freeze-drying, the absorption bands of the protein are bleached, the reduction possibly being effected by water (Malkin & Malmström, 1970). On rehydration of rusticyanin the blue colour can be restored by addition of a trace of NaIrCl_6 .

Discussion

Results obtained by ultracentrifugation and gel electrophoresis show that the rusticyanin molecule contains only one type of polypeptide chain. The values for the molecular weight obtained by these methods correspond closely. The copper analysis and iron-titration data clearly indicate the presence of one copper atom per molecule. Therefore rusticyanin has a mol. wt. of 16 500 and consists of one polypeptide chain to which is bound one atom of copper. The fact that the copper in rusticyanin can be observed by e.p.r. spectroscopy (Cobley & Haddock, 1975) and the optical properties of the molecule described above provisionally classify the copper environment in the molecule as type I (Malkin & Malmström, 1970).

The absence of arginine from rusticyanin, its loss of blue colour at relatively high pH and its copper content of one atom per polypeptide chain are features that indicate a strong similarity to other

small copper proteins such as azurin and plastocyanin (Katoh *et al.*, 1962; Ambler & Brown, 1967; Malkin & Malmström, 1970; Milne & Wells, 1970). These similarities extend to their optical absorption spectra in that they all exhibit absorption maxima in the regions of 450, 600 and 750–800 nm. However, the absorption band at 320 nm present in iron-reduced rusticyanin has not been reported for other copper proteins. The significance of this absorption is unknown.

The involvement of plastocyanin and azurin in electron transport is well known, and Cobley & Haddock (1975) proposed a role for rusticyanin as the initial acceptor of electrons from Fe^{2+} in the *Th. ferro-oxidans* respiratory chain. Our demonstration of a direct interaction of Fe^{2+} with rusticyanin and the large amount of the protein found in the organism supports this view.

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