THE RESPIRATORY CHAIN OF *THIOBACILLUS FERROOXIDANS*: THE REDUCTION OF CYTOCHROMES BY Fe^{2+} AND THE PRELIMINARY CHARACTERIZATION OF RUSTICYANIN A NOVEL 'BLUE' COPPER PROTEIN

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1. Introduction

Thiobacillus ferrooxidans is a chemolithotrophic bacterium which grows under acidic conditions and assimilates CO_2 at the expense of energy derived from Fe^{2+} oxidation with O_2 as terminal electron acceptor (for a recent review see [1]). Although Fe^{2+} is a particularly weak reducing agent (E_0 = +780 mV, for the Fe^{2+}/Fe^{3+} couple [2]), a variety of cytochromes in whole cells of *Th. ferrooxidans* are reduced by Fe^{2+} [3]. We have begun an investigation of the mechanism of Fe^{2+} oxidation by sub-cellular preparations from *Th. ferrooxidans* with the aim of characterizing the very high potential respiratory chain components involved in this process. In this paper we describe (a) the kinetics of Fe^{2+}-dependent cytochromes reduction and (b) some properties of rusticyanin, a novel, very high potential, and acid-stable 'blue' copper-protein which undergoes reduction during the oxidation of Fe^{2+} with O_2.

2. Materials and methods

2.1. *Bacterial strain and growth conditions*

Th. ferrooxidans (strain T.f.3) was obtained from Dr D. Herbert (Microbiological Research Establishment, Porton Down, Salisbury, Wilts., UK) and was grown in a medium containing: $(NH_4)_2SO_4$ (1 mM), KH_2PO_4 (0.2 mM), salts solution (0.2 ml/litre), H_2SO_4 (2 ml/litre of 98% w/w) and $FeSO_4 \cdot 7H_2O$ (180 mM). The components were added in the order listed to a sufficient quantity of distilled water; the pH of the final solution was 2.0 and was not further adjusted. The concentrated salts solution contained: HCl (250 ml/litre of 31% w/w), MgO (1.2 M), $CaCO_3$ (10 mM), ZnO (5 mM), $CoCl_2 \cdot 6H_2O$ (5 mM), H_3BO_3 (5 mM) and $Na_2MoO_4 \cdot 2H_2O$ (5 mM). The growth medium contained no added copper salt. The strain was maintained in the laboratory by inoculating each week 75 ml of fresh medium with 5 ml of the previous week's culture; incubation was in 250 ml pyrex conical flasks placed in a rotary shaker at 28°C.

Large scale cell growth was achieved in continuous culture in the medium described above. A 60-litre polythene aspirator served as the growth vessel (working volume, 40 litre). Both the medium (dilution rate, 0.013 h^{-1}) and air (flow rate, 20 litre/min) entered the growth vessel at the top through a nylon tube which extended almost to the bottom of the vessel. The effluent, composed of air and bacterial suspension, passes through a second nylon tube which extended from the surface of the culture to a collecting reservoir via the port at the base of the aspirator. The culture was maintained at 20°C (± 2°C) by circulating water from a constant temperature bath, and the collecting reservoir was lagged at the sides and placed on a cold plate.

2.2. *Preparation of electron transport particles*

50 litres of chemostat effluent was passed through an MSE continuous flow rotor (16 000 rev/min with a flow rate of 300 ml/min) to yield a cell paste of about 3 g wet weight. Electron transport particles were prepared in distilled water at pH 7.0 using a French press as described previously [4] except that

the French pressure cell was operated at 16 000 lbs/in^2, the broken cell suspension was spun twice at 10 000 *g* for 10 min to remove whole cells, and two stages, involving Millipore filtration and the addition of $MgSO_4$ (1 mM), were omitted.

2.3. *Preparation of the blue supernatant fraction*

Cells were broken as described above except that cells were passed through the French press in distilled water acidified with H_2SO_4 to pH 2.0, rather than in distilled water at pH 7.0. After two centrifugation steps at 10 000 *g* for 10 min each, the blue supernatant was obtained by centrifugation at 180 000 *g* for 1 h.

2.4. *Methods*

Rates of Fe^{2+} oxidation were assayed using a Clark oxygen electrode. Reduction of cytochromes was routinely monitored using a double beam spectrophotometer and kinetic measurements were made using an additional stopped-flow attachment. Protein determinations [5] and low temperature spectroscopy at visible wavelengths [6] were performed as described previously. Electron paramagnetic resonance (EPR) measurements were made with a Varian E-4 spectrometer in the laboratory of Dr I. Mason (Department of Biochemistry, University of Edinburgh) as described in the legends to the appropriate figures.

3. Results and discussion

3.1. *Fe^{2+}-reducible cytochromes in electron transport particles*

The Fe^{2+}-reducible cytochromes present in electron transport particles prepared from *Th. ferrooxidans* cells are shown in the low temperatures difference spectrum reproduced in fig.1. Although the absorption due to *c*-type cytochromes appears symmetric in the Soret region (400–428 nm), there is clear asymmetry in the α-band absorption region (545–550 nm) indicating the presence of at least two Fe^{2+}-reducible *c*-type cytochromes. In addition two Fe^{2+}-reducible *a*-type cytochromes are clearly discernible from their absorption in the Soret region (430–460 nm) but show only weak absorption in the α-band region (590–605nm) under these steady state conditions.

When Fe^{2+} was added to an aerobic suspension of electron transport particles, dual wavelength spectrophotometric measurements showed that cytochrome *c* underwent an immediate reduction (fig.2c), which in extent was 10% of that obtainable with $Na_2S_2O_4$. Then, as Fe^{3+} was produced, cytochrome *c* became slowly oxidized before anoxia was achieved, at which point only a slight reduction of cytochrome *c* occurred. By contrast, cytochrome *a* was more slowly reduced (fig.2d); the remaining two-thirds of cytochrome *a*, unreduced in the aerobic steady state, became reduced when the suspension became anoxic. Stopped-flow measurements showed that part of the cytochrome *c* was reduced within 3 msec, during the flow. A second phase of reduction with a half-time of about 25 msec occurred when flow stopped (fig.2b). Cytochrome *a* showed markedly biphasic kinetics; a small but fast phase when the flow stopped ($t_{1/2}$ about 50–100 msec) and then a slower but more extensive phase ($t_{1/2}$ about 1 sec, fig.2f). Thus time resolution confirms the presence of at least two *c*-type cytochromes and two *a*-type cytochromes in electron transport particles, presumably in a sequence from Fe^{2+} to oxygen as indicated by their half-time for reduction by Fe^{2+}.

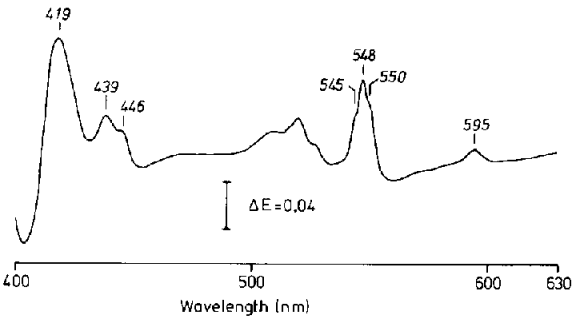

Fig.1. Wavelength scanning spectrophotometric measurements of Fe^{2+}-reducible cytochromes of electron transport particles derived from *Th. ferrooxidans*. Electron transport particles were suspended in air saturated β-alanine (100 mM) $-H_2SO_4$ (pH 3.0) at 2.6 mg protein/ml and placed in both compartments of the cell holder. To the test cuvette was added $FeSO_4$ (10 mM) and the cell holder was frozen in less than 30 sec. Under these conditions both cuvettes remained aerobic and the resultant steady state Fe^{2+}-reduced minus oxidized difference spectrum was recorded at 77° K.

3.2. *Optical properties of the supernatant fraction obtained following cell breakage at pH 2.0*

Functional electron transport particles capable of Fe^{2+} oxidation were prepared routinely from freshly

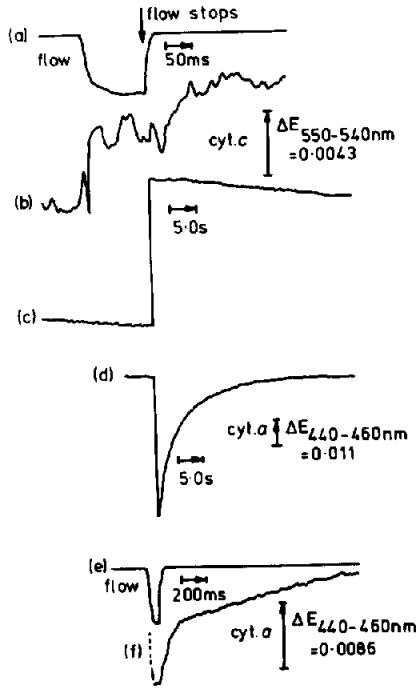

Fig.2. Stopped-flow dual-wavelength spectrophotometric measurements of the Fe^{2+}-reducible cytochromes of electron transport particles. Measurements were made with an apparatus having a 17:1 mixing ratio for the relative volumes of delivery from the major and minor syringes, and a dead-time of 3 ms. The optical path of the observation chamber was 10 mm long. Information was recorded with a transient recorder and, in parallel, a pen recorder. For each experiment the major syringe contained electron transport particles (3.5 mg protein/ml) suspended in air-saturated β-alanine (100 mM) $-H_2SO_4$ (pH 3.0) at 26°C and the minor syringe contained $FeSO_4$ (50 mM) in distilled water adjusted to pH 2.0 with H_2SO_4. The concentration of Fe^{2+} set up by mixing was 2.8 mM, and, under these conditions, Fe^{2+} was oxidized by the particles at a rate of 240 ng ions Fe^{2+}/min/mg protein. Cytochrome *c* was measured at 550–540 nm, cytochrome *a* at 440–460 nm and in each case reduction is shown as an upward deflection in the trace. Key to fig: (a) flow velocity trace for (b) rapid kinetics of cytochrome *c* reduction with a measuring time constant of 5 ms; (c) slower time base for cytochrome *c* measurement of the preceding trace; (d) cytochrome *a* reduction recorded on a slow time base; (e) flow velocity trace for (f) rapid kinetics of cytochrome *a* reduction with a measuring time constant of 20 ms. Spectrophotometric sensitivities and time scales are shown by labelled bars. The start and stop of flow are shown by the flow velocity traces (a) and (e), in which a downward deflection indicates increased flow velocity. The delivered volume was 0.75 ml. It should be noted that the mixing is initiated by displacing from the observation chamber the old mixture from the previous experiment, in which sufficient time had elapsed for cytochrome *c* to become largely re-oxidized because of Fe^{3+} accumulation from Fe^{2+} oxidation, whereas cytochrome *a* was fully reduced by the attainment of anoxia in the presence of Fe^{2+}, irrespective of the Fe^{3+} that had accumulated during oxygen utilization. However, the suspension freshly introduced from the major syringe in the new flow was air-saturated and with oxidized cytochromes.

grown cells, stored overnight at pH 7.0 and at 4°C, and subsequently broken at pH 7.0 [4]. If the cells were broken at pH 2.0, which is the pH of growth for the organism, it was found that the electron transport particles, prepared by differential centrifugation, were incapable of Fe^{2+} oxidation. It was noticed, however, that the supernatant fraction (180 000 *g* for 1 h) obtained following the preparation of particles at pH 2.0 was distinctly blue in colour, and that this blue colouration was absent from the supernatant obtained following cell breakage at pH 7.0. The absorption spectrum of the blue supernatant shows a broad band centered at 590 nm (fig.3a), which disappeared on

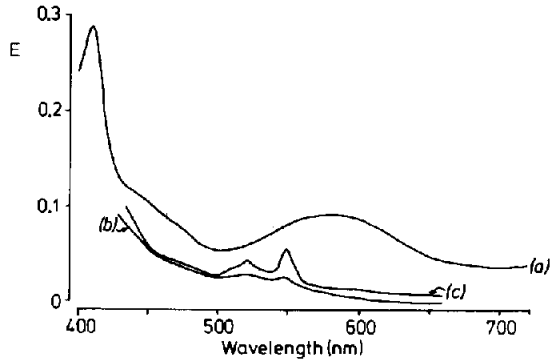

Fig.3. Absorption spectra of the supernatant fraction obtained following cell breakage at pH 2.0. Cells were suspended in H_2O–H_2SO_4 (pH 2.0) at a concentration of 1 g wet weight per 10 ml and broken and centrifuged to yield the blue supernatant fraction as described in the text. The oxidized spectrum of the supernatant was then recorded at room temperature (trace a). Spectrum (b) was recorded 2 min after the addition of $FeSO_4$ (5 mM) and spectrum (c) was recorded after the addition of a few grains of $Na_2S_2O_4$ to the Fe^{2+}-reduced sample.

reduction both with $Na_2S_2O_4$ (fig.3c) and, more significantly, with Fe^{2+} (fig.3b). No change in absorption at 590 nm was observed on adding Fe^{2+} to the supernatant fraction obtained following cell breakage at pH 7.0. By comparing Fig.3a and 3b, it can be seen that in addition to the band at 590 nm there is also a weaker absorption band at about 450 nm: these bands are characteristic of 'blue' copper-proteins [7]. The absorption bands at 410 nm (in fig.3a) and 550 nm (fig.3c) are due presumably to the oxidized and reduced forms of cytochrome *c* respectively. When the blue supernatant was stored at pH 2.0 and at 4°C, the absorption at 590 nm was stable for at least three weeks.

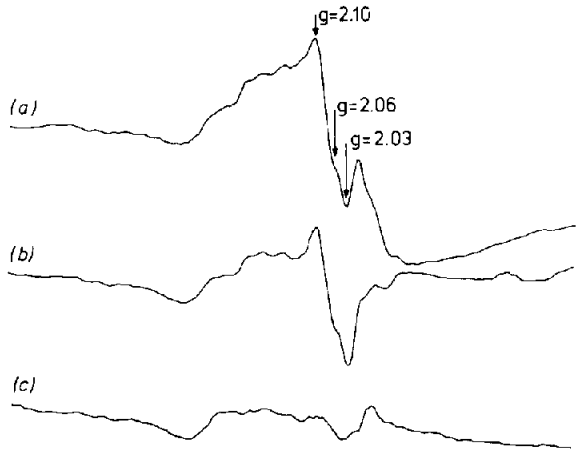

Fig.4. Electron paramagnetic resonance spectroscopy of whole cells and of the supernatant fraction obtained from cell breakage at pH 2.0. Whole cells were suspended in H_2O–H_2SO_4 (pH 2.0) at 18 mg protein/ml. After vigorous aeration, an aliquot of this suspension was frozen in an appropriate cuvette by slow immersion in liquid N_2 to give, subsequently, spectrum (a). An aliquot of the supernatant fraction described in the legend to fig.3 (2.1 mg protein/ml in H_2O–H_2SO_4 at pH 2.0) was similarly frozen and scanned (spectrum b). Spectrum c was obtained from the same preparation of the supernatant fraction, but pretreated with $FeSO_4$ (24 μM) for 2 min before freezing. Conditions for EPR spectroscopy: microwave frequency, 9.14 GHz; microwave power, 100 mW modulation amplitude and frequency 12.5 gauss and 100 kHz respectively; temperature, 101°K; scan, 500 gauss/min; and time constant, 1 s. Measurements were made between 2285 and 4285 gauss.

3.3. *EPR properties of cells and the blue supernatant fraction*

The presence of a 'blue' copper-protein in the pH 2.0 supernatant fraction was confirmed by EPR spectroscopy: the intense signal centered at about g = 2.05 is characteristic of some proteins containing Cu^{II} (fig.4b). A detailed examination of the spectrum, however, shows it to be more complex than that shown by most 'blue' copper-proteins, e.g. bacterial azurins [7], plastocyanin from higher plants [7], and umecyanin from horseradish [8], in that three g values are apparent rather than the two g values normally found. In this respect the copper-protein of the supernatant fraction resembles 'blue' copper-proteins like stellacyanin, isolated from the Japanese lacquer tree [9], or plantacyanin, from cucumber [10] or spinach [11].

A comparison of the EPR spectrum of *Th. ferrooxidans* cells (fig.4a) with that of the supernatant (fig.4b) show that breakage of the cells at pH 2.0 did not alter detectably the environment of the Cu^{2+} ion. Both in whole cells (data not shown) and in the supernatant fraction (fig.4c) the addition of $FeSO_4$ caused in disappearance of the signals attributable to Cu^{2+}.

Evidence that the EPR signal observed in fig.4a and b can be attributed to a single Cu^{2+} ion comes from a comparison of the intensity of the signal with the absorbance at 590 nm (fig.5b). An excellent correlation existed between E_{590} and the signal amplitude (between g = 2.10 and g = 2.03). Furthermore, as the chromophore was progressively bleached with $FeSO_4$, the signal amplitudes between the peak (g = 2.10) and the inflection (g = 2.06), and between the inflection (g = 2.06) and the trough (g = 2.03), were proportional to the E_{590}. It seems more likely that a single type of Cu^{2+} is responsible for the signals rather than two types of Cu^{2+} with similar E_0' (pH 2.0) values.

From fig.5a it can be seen that, at low concentrations of $FeSO_4$, the decrease in absorption at 590 nm was linearly related to the amount of $FeSO_4$ added. Assuming that in this part of the titration each Fe^{2+} ion added reduces one 'blue' Cu^{2+} ion, it can be calculated (using the extrapolations in fig.5a) that the supernatant fraction contains about 30 μeq/litre of 'blue' Cu^{2+} and that the millimolar extinction coefficient for the 'blue' Cu^{2+} is about 3.0 litre $mmol^{-1}$ cm^{-1}, a value in good agreement with that calculated for other 'blue' copper-proteins [7]. There was no autooxidation of the 'blue' copper-protein after reduction with $FeSO_4$.

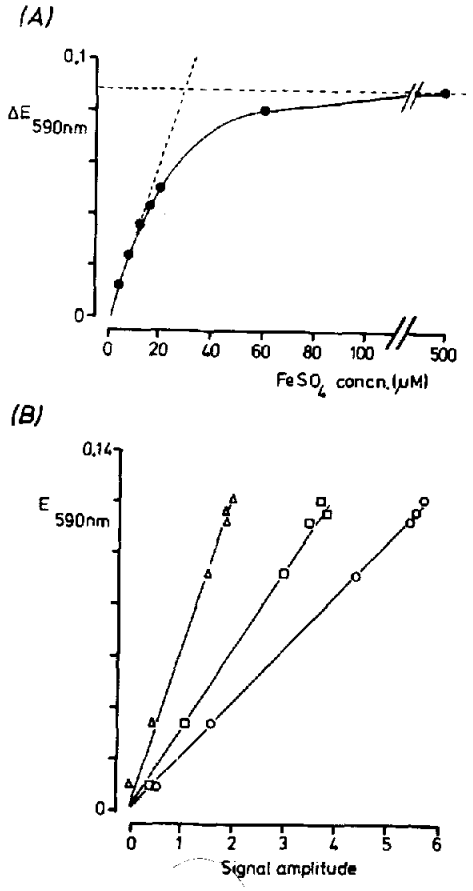

Fig.5. Titration of the supernatant fraction with $FeSO_4$. 4 ml of the supernatant fraction (2.1 mg protein/ml) described in the legend to fig.3 were placed in a cuvette of 1 cm light path, and the E_{590} recorded. An aliquot (0.3 ml) was then transferred to an EPR cuvette and frozen in liquid N_2. Additions of $FeSO_4$ were then made and when each ΔE_{590} was complete, a further aliquot (0.3 ml) was removed for EPR spectroscopy. In part A, the extent of bleaching of the broad absorption band at 590 nm is plotted as a function of the $FeSO_4$ concentration; the extrapolated lines are discussed in the text. In part B the amplitudes of the EPR signals of fig.4 are plotted as a function of E_{590}: (-△-) signal amplitude between g = 2.06 and g = 2.03; (-□-) signal amplitude between g = 2.10 and g = 2.06; and (-○-) signal amplitude between g = 2.10 and g = 2.03. The same arbitrary unit of signal amplitude has been used for each of the three plots. The conditions for EPR spectroscopy were as described in the legend to fig.4.

In conclusion, we have shown that Fe^{2+} oxidation by electron transport particles prepared from *Th. ferrooxidans* involves two *c*-type cytochromes, two *a*-type cytochromes and probably a 'blue' copper-protein, which can be readily removed from the membrane under certain conditions. It is proposed that this novel copper-protein be named rusticyanin to indicate the source of the protein without necessarily implying that the protein contains iron.

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