

Evidence that protein B of the thiosulphate-oxidizing system of *Thiobacillus versutus* contains a binuclear manganese cluster

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Manganese was shown to be an essential trace metal for growth and thiosulphate oxidation by *Thiobacillus versutus* in chemostat culture. In the thiosulphate-oxidizing enzyme system of *T. versutus*, protein B was the only component found to contain manganese in significant amounts. When it was examined by electron spin resonance (ESR) spectroscopy, protein B gave a broad, complex spectrum, indicative of the presence of a dimeric manganese cluster, with manganese in the Mn(II) oxidation state.

Sulfur-oxidizing bacterium; ESR; Electron paramagnetic resonance spectroscopy; Manganese protein; (*Thiobacillus versutus*)

1. INTRODUCTION

Thiobacillus versutus uses a multienzyme system to oxidize thiosulphate to sulphate. Previous studies have shown it to consist of two colourless proteins and two *c*-type cytochromes located in the periplasmic space of the organism [1-7]. The mechanism of action of the components of the multienzyme system has not been established, other than to show that one, protein A, is a thiosulphate-binding protein [5].

2. MATERIALS AND METHODS

2.1. Isolation of protein B

T. versutus (DSM 582, originally provided by D.S. Hoare)

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was cultured, harvested and disrupted as previously described [1,2]. Protein B was purified by the procedures used earlier [3,4]. Preparations were from hydrophobic interaction chromatography [3] or from further purification of the 0.35 M NaCl(I) fraction from DEAE-Sepharose CL-6B chromatography by filtration through Sephadex G-100 or by high-performance liquid chromatography. Similar results were obtained in these preparations, which were all of 85-90% purity on the basis of polyacrylamide gel electrophoresis.

2.2. Chemostat cultures

To investigate the trace-element requirements a continuous culture, growth-limited by 38 mM thiosulphate, was established at a dilution rate of 0.06 h⁻¹. The thiosulphate consumption rate was estimated discontinuously by cyanolytic assay of thiosulphate [8], and continuously from the rate of alkali addition for automatic maintenance of the culture at pH 7.75 ± 0.05.

2.3. Metal analyses

Samples of the protein were digested in nitric acid. Metals were assayed both on a Varian AA-1275 series atomic absorption spectrophotometer with model 55 sample changer, and on a Jobin Yvon plasma emission spectrometer.

2.4. Electron spin resonance (ESR) spectroscopy

Most ESR spectra were recorded on a Varian E4 X-band (9 GHz) spectrometer with an E102 microwave bridge. The temperature was controlled with an Oxford Instruments ESR9

liquid helium flow cryostat. Samples were examined in quartz tubes of 3 mm internal diameter.

3. RESULTS

3.1. Demonstration that manganese is an essential trace metal for growth of *T. versutus* on thiosulphate

A chemostat culture, growth-limited by 38 mM thiosulphate, was established at a dilution rate of 0.06 h^{-1} . Its initial steady-state biomass concentration was $333 \pm 17 \text{ mg dry wt} \cdot \text{l}^{-1}$ (20 determinations). The medium supplied to the culture was changed to one lacking added calcium, cobalt, copper, manganese and zinc, but containing iron ($1.7 \text{ mg} \cdot \text{l}^{-1}$), molybdenum ($4.7 \text{ mg} \cdot \text{l}^{-1}$, as molybdate) and normal levels of other medium components (fig.1). After passage of three culture volumes of medium, the biomass concentration began to decline, falling to about $75 \text{ mg dry wt} \cdot \text{l}^{-1}$ during the subsequent 6.4 vol. replacements. Thiosulphate consumption also declined by about 70% over this period.

Manganese limitation had several striking effects on the behaviour of the chemostat culture: the alkali consumption rate (for automatic maintenance of the culture at $\text{pH } 7.75 \pm 0.05$) declined by 25% over a 54 h period (3.5 culture vols; 363–417 h in fig.1) during which there was no decline in the amount of thiosulphate consumed, but there was a reduction in the growth yield of the organism from the steady-state value of 8.4 to about $5.2 \text{ g} \cdot \text{mol}^{-1}$. This indicated that thiosulphate was not being completely oxidized to sulphate. However, no sulphur or polythionates were accumulated. Subsequently, unused thiosulphate began to accumulate in the culture, reaching about 70% of the input thiosulphate concentration. The quantity of alkali consumed indicated that, when thiosulphate consumption was still apparently complete, the production of sulphuric acid was only about 77% of that expected, and during the thiosulphate accumulation phase, acid production was halved, with a corresponding decrease in biomass production. The maximum decrease in growth yield (to 48% of the initial steady-state value) corresponded with the minimum alkali consumption value (47% of steady-state value) at 441 h.

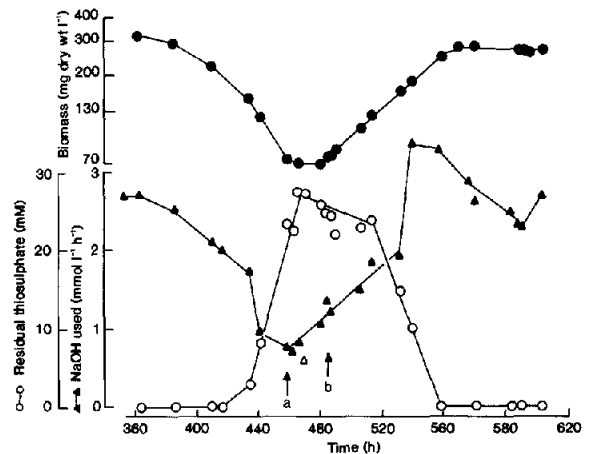


Fig.1. Effect of manganese limitation on a chemostat culture of *T. versutus*. Biomass (●), unoxidized thiosulphate (○) and consumption of NaOH (to maintain culture at $\text{pH } 7.8$) (▲) are shown. At the times indicated by the arrows (a) the culture vessel was supplemented with $5 \text{ mg Mn} \cdot \text{l}^{-1}$ (as manganese sulphate); and (b) the input medium supply was supplemented with $3.8 \text{ mg Mn} \cdot \text{l}^{-1}$.

The culture vessel was then supplemented with manganese sulphate ($5 \text{ mg Mn} \cdot \text{l}^{-1}$) and the input medium feed with $3.8 \text{ mg Mn} \cdot \text{l}^{-1}$. This resulted in progressive recovery of the culture to the original biomass concentration and thiosulphate consumption rate. The minimum manganese concentration required for maintenance of growth was not determined. The requirement for manganese was not relieved by the presence of calcium chloride ($6.5 \text{ mg Ca} \cdot \text{l}^{-1}$) in a manganese-limited culture (data not shown).

After the culture had recovered to a steady state ($303 \text{ mg} \cdot \text{l}^{-1}$, 36.6 mM thiosulphate being consumed, alkali consumption $2.18 \text{ mmol NaOH per mmol thiosulphate}$), it was pulse-labelled with $^{54}\text{MnSO}_4$ ($2.3 \text{ MBq} \cdot \text{l}^{-1}$) and the partition of ^{54}Mn between organisms and medium was assayed over the following 50 h. $7.0 \pm 1.8\%$ of the manganese was recovered in the organisms over this period, indicating a cell-associated concentration of $0.83 \text{ mg Mn} \cdot (\text{g dry wt})^{-1}$.

3.2. Demonstration of manganese in protein B by atomic absorption spectrophotometry

Protein B, purified by ammonium sulphate precipitation and chromatography on DEAE-Sephacel CL-6B followed by gel filtration on Sephadex G-100, gave only one major band on

polyacrylamide gel electrophoresis [3]. The manganese content of the protein was estimated to be 1.28 ± 0.07 atom Mn per mol protein. Variable amounts of iron were detected, which could be correlated with the presence of traces of cytochrome *c*-551. The levels of cobalt, copper, nickel and molybdenum were less than 0.05 atom per mol protein. Given a probable protein purity of about 85–90%, this indicated that the metal content of the isolated protein was approx. 0.71 Mn per monomer of protein B.

No significant amounts of Mn were detected in the other components of the thiosulphate-oxidizing system, protein A, cytochrome *c*-551 and cytochrome *c*-552.5.

3.3. ESR spectroscopy of protein B

The X-band spectrum of protein B as prepared

(fig.2), consisted of a pattern of broad lines separated by about 52 mT, with narrow hyperfine structure superimposed. The hyperfine structure was most prominent around 240 mT, with at least 21 discernible lines separated on average by 4.07 mT, and at 80 mT, with at least 11 lines separated by 4.25 mT (insets in fig.2). This hyperfine structure is immediately suggestive of a metal ion with a high nuclear spin, most probably manganese. In fig.2, signals around $g = 4.3$ and $g = 2$ may also be noted. These signals were of variable intensity, and are attributed to contamination by Fe^{3+} and Cu^{2+} , respectively. The broad features of the spectrum, together with the narrow hyperfine structure, were unaffected by addition of either thiosulphate or dithionite, though the signals assigned to Fe^{3+} and Cu^{2+} were reduced by dithionite.

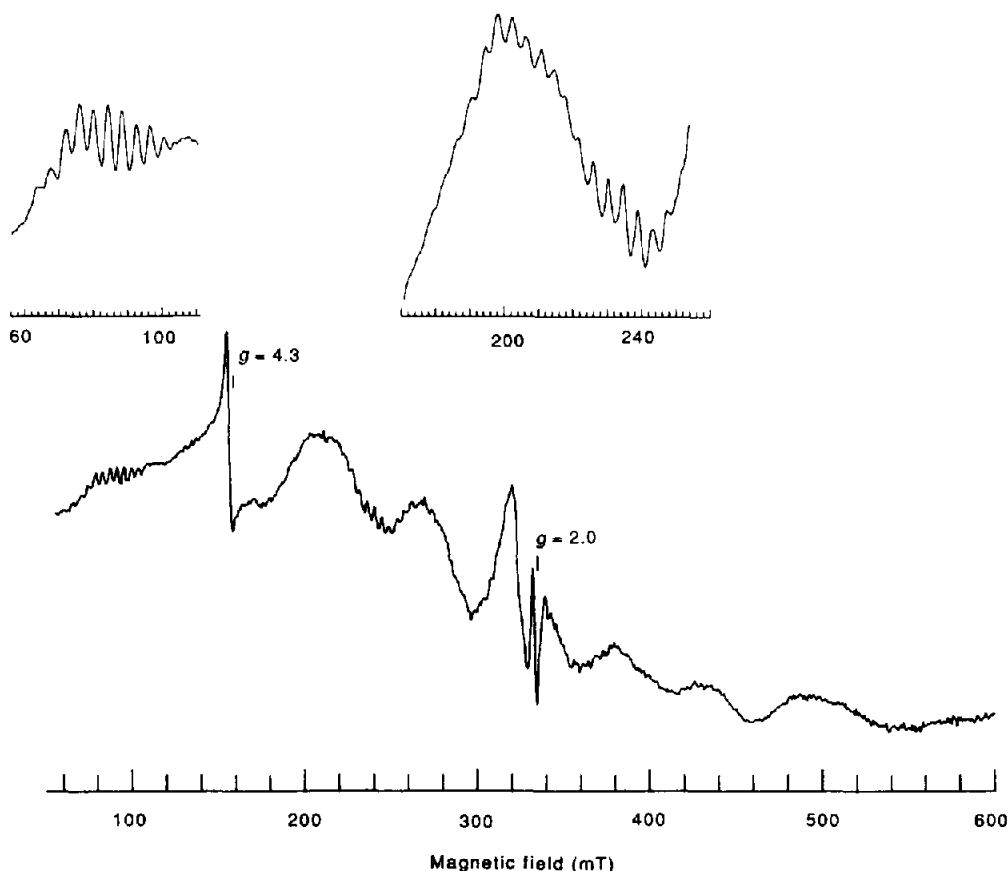


Fig.2. ESR spectrum of protein B, $15 \text{ mg} \cdot \text{ml}^{-1}$, recorded at a temperature of 60 K, with microwave power 20 mW, frequency 9.19 GHz, modulation amplitude 1.0 mT. The inset regions show details of the hyperfine structure, recorded at 22 K.

4. DISCUSSION

Manganese has been proved to be an essential trace element for growth of *T. versutus* on thiosulphate. A direct role for manganese in thiosulphate oxidation was shown by the failure of manganese-starved organisms to oxidize thiosulphate completely to sulphate even when all added thiosulphate was disappearing. A direct effect of metal deficiency on thiosulphate reduction is indicated by the parallel between the decline in biomass and accumulation of thiosulphate in the absence of Mn^{2+} . If some other manganese-requiring process had been growth-limiting, gratuitous oxidation of thiosulphate would have been expected. The nature of any sulphur-containing intermediates being accumulated has not yet been established, but was not polythionate ($(S_nO_6)^{2-}$, where $n = 4$ or more) or elemental sulphur.

Of the proteins involved in the reduction of thiosulphate, we have shown that only protein B contains manganese in significant amounts. The ESR spectra of protein B are not consistent with a mononuclear manganese site. An isolated Mn(II) ion would be expected to give a hyperfine pattern of six lines from the $I = 5/2$ nucleus. In manganese proteins the lines are typically separated by about 9 mT [9]. Instead, a 'multiline' hyperfine pattern was observed (fig.2).

Multiline patterns have been observed in proteins which contain two or more exchange-coupled manganese ions. The best-known manganese cluster of this type is in the oxygen-evolving complex of photosystem II in green plants and cyanobacterial photosynthesis [10,11]. This complex contains three or four manganese ions, though the signal probably derives from an exchange-coupled pair within the cluster. Spectra similar to those of photosystem II have been detected in dimeric manganese complexes containing either Mn(II) + Mn(III) or Mn(III) + Mn(IV) [10,12,13]. The spectra of the oxygen-evolving complex consist of a pattern of up to 36 lines, centred at $g = 2$. In some circumstances, a broad signal around $g = 4.3$ is also observed [14,15], arising from another conformation of the same complex: this does not show a discernible manganese hyperfine pattern [16].

The ESR spectrum of protein B differs from the photosystem II spectra. It consists of a series of very broad lines, spread over 600 mT. This indicates a spin system with $S > 1/2$, probably integer-spin. The spectrum is unaffected by the strong reducing agent dithionite, which is inconsistent with manganese in its higher oxidation states. A complex hyperfine pattern is superimposed on this spectrum, with splittings, about 4.5 mT, which are about half those observed in simple mononuclear manganese complexes. This indicates the interaction of at least two ^{55}Mn nuclei in the paramagnetic centre. More spectroscopic data would be needed to simulate this spectrum. The signal at $g = 4.3$ in the ESR spectrum of protein B is much narrower than that from photosystem II, and probably derives from an Fe^{3+} contaminant.

Spectra of this type have been observed in model complexes with a pair of Mn(II) ions [17,18] and in dimanganese concanavalin A [19]. In all these cases, the manganese oxidation state is Mn(II). The spectrum of protein B most strongly resembles the dimanganese complex studied by Mathur et al. [18], in which the exchange coupling J is less than the zero-field splitting parameter, D . A number of the features of the spectrum of protein B are reproduced, including the hyperfine structure around 100 mT and 300 mT, and the fact that the hyperfine pattern around $g = 2$ is more pronounced on the low-field side than the high-field side.

On the basis of the ESR spectra we conclude that protein B contains a spin-coupled binuclear Mn(II) cluster. This conclusion must be reconciled with the analytical data which indicated low values for manganese content, about 1.4 Mn per dimeric protein. It is probable that the protein lost some manganese during purification. It may be noted that manganese is readily lost from preparations of photosystem II [20], and low values were also first reported for the manganese content of Mn-superoxide dismutase [21]. The ESR spectrum of protein B contained no six-line pattern around $g = 2$, which might have been expected for mononuclear Mn sites, or for free Mn^{2+} in solution. From the present data it is not possible to establish whether the native protein contains one cluster, bound between two subunits, or one cluster in each subunit. The composition and functions of this novel protein are subjects for future study.

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