Ferrimycobactin Reductase Activity from *Mycobacterium smegmatis*

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Ferrimycobactin reductase activity from *Mycobacterium smegmatis* grown under iron-deficient conditions had a K_m for ferrimycobactin of less than $4\ \mu\text{M}$ and a K_m for NADH of $1.75\ \text{mM}$. Salicylate ($0.12\ \text{mM}$), which is synthesized by this bacterium, could substitute for EDTA as an acceptor of Fe^{2+} in the assay system. Reagents which react with thiol groups (HgCl_2 , *N*-ethylmaleimide) at $0.1\ \text{mM}$ inhibited activity by about 40%; other inhibitors (KCN, NaN_3 , carbonyl cyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol) were less effective (though these inhibited active iron transport, which uses exochelin and not mycobactin; Stephenson & Ratledge, 1979). The rate of ferrimycobactin reduction in extracts was about ten times faster than the rate observed *in vivo* following a shift-up of low-iron cells to high-iron status. Reduction of iron in ferri-ferrioxamine B, ferriexochelin MS and ferric ammonium citrate occurred at comparable rates to ferrimycobactin reduction.

Ferrimycobactin reductase activity was undiminished in *M. smegmatis* grown under iron-sufficient conditions. A similar activity was found in extracts of *Escherichia coli* and *Candida utilis* where it still required NADH. Yeast alcohol dehydrogenase reduced ferrimycobactin independently of NADH; this activity was attributed to the free thiol groups of this protein. Reductase activity therefore may be associated with a protein whose principal function need not be that of a siderophore reductase.

INTRODUCTION

The uptake of iron from various siderophores into microbial cells is becoming increasingly well-documented (Braun, 1978; Ernst *et al.*, 1978) though with little information about how iron is released from these chelates. Ernst & Winkelmann (1977) and Brown & Ratledge (1974) reported the occurrence of reductases in *Neurospora crassa* and *Mycobacterium smegmatis* which can reduce the iron in, respectively, coprogen and mycobactin, the natural siderophores of these species. Ferrous iron can then be transferred to other molecules within the cell as its affinity for the chelators is low. In *Escherichia coli* and *Fusarium roseum*, hydrolysis of the ferric chelate is needed before the iron can be released (Greenwood & Luke, 1978; Emery, 1976).

The finding by Dailey & Lascelles (1977) of an 'iron reductase' activity in *Spirillum itersonii* (using ferric citrate as the iron source) is relevant to an understanding of how iron may be released from its carrier. They suggested that this activity could be associated with one or more sites on the respiratory chain. Lascelles & Burke (1978) extended this work to *Staphylococcus aureus* and found that iron reductase activity in membrane preparations was associated with L-lactate dehydrogenase, glycerol-3-phosphate dehydrogenase and nitrate reductase activities using L-lactate and glycerol 3-phosphate as reductants. They concluded that reduced components preceding cytochrome *b* in the electron transport chain served as sources of reductant for ferric iron. The success of the iron reductase depended, however, on using ferric citrate; when ferri-ferrichrome and ferri-ferrioxamine

B (Desferal) were used with crude extracts from this organism, as well as from *Spirillum itersonii*, *Rhodopseudomonas spheroides*, *E. coli* and *Paracoccus (Micrococcus) denitrificans*, there was little or no reduction (Dailey & Lascelles, 1977). Thus the problem of iron release from the natural siderophore is still unresolved in these organisms as there is no evidence to suggest that uptake of iron is via formation of ferric citrate which is an easily reducible complex.

In this paper, we report our continuing studies on this problem in mycobacteria and suggest that ferrimycobactin reductase activity may be generally distributed rather than being the property of a specific enzyme found only in mycobacteria.

METHODS

Abbreviations. DNP, 2,4-Dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NEM, *N*-ethylmaleimide.

Organisms, growth and preparation of extracts. *Mycobacterium smegmatis* NCIB 8548 was grown in shake-flask culture (250 ml Erlenmeyer flasks) in iron-deficient ($0.05 \mu\text{g Fe ml}^{-1}$) or iron-sufficient ($2.0 \mu\text{g Fe ml}^{-1}$) medium (100 ml) as previously described (Ratledge & Hall, 1971). Three d-old cells, harvested by filtering through Whatman GF/C glass-fibre filters, were suspended in an equal volume of 0.1 M-Tris/HCl buffer (pH 7.0) at 2°C and disrupted by passage through a chilled French press at 35 MPa. The exuded material was incubated with DNAase (Sigma) for 30 min at 2°C. After centrifuging at 30000 *g* for 30 min at 2°C, the supernatant was made to 2% (w/v) with respect to streptomycin sulphate and re-centrifuged as before.

Escherichia coli K12 and *Candida utilis* were grown under iron-sufficient conditions and extracts were prepared from them in the same manner as for *M. smegmatis*.

Enzyme assay. Ferrimycobactin reductase activity was assayed under anaerobic conditions by Method 2 of Brown & Ratledge (1974) using 400 nmol ferrimycobactin, instead of 680 nmol, with NADH as electron donor. Extracts contained approx. 7 mg protein ml^{-1} .

Recovery of ferrimycobactin from growing cultures exposed to a shift-up of iron. To a 60 h iron-deficient culture of *M. smegmatis* [about 3 mg dry wt ml^{-1} , containing naturally approx. 1% (w/v) mycobactin] was added $2.0 \mu\text{g Fe ml}^{-1}$ (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (see McCready & Ratledge, 1978). The amount of ferrimycobactin which had been produced was determined by ultrasonically disrupting the harvested cells (using one flask per determination) suspended in 0.1 M-EDTA; ferrimycobactin, together with desferrimycobactin, was then extracted into ethanol and the absorbance was read immediately at 450 nm to give the concentration of the ferric chelate only. The extraction and all other manipulations were performed in iron-free glassware (see Ratledge & Hall, 1971) to ensure no acquisition of extraneous iron into the mycobactin. The proportion of the added iron which was bound to mycobactin could then be calculated. The total amount of mycobactin was determined by converting the remaining desferrimycobactin to ferrimycobactin in the manner of White & Snow (1968) and again reading the absorbance at 450 nm.

Materials. Ferrimycobactin S was prepared to an $A_{450}^{1\%}$ of 44.3 by the method described by Ratledge & Snow (1974) with final chromatography through Sephadex LH 20. Ferrioxamine B (Desferal; CIBA Laboratories) was converted to the ferric form by adding a stoichiometric amount of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (chelated iron is quickly oxidized into the ferric state). Ferriexochelin MS was fraction 3A purified as described by Macham *et al.* (1977) from *M. smegmatis* and was donated by Dr M. C. Stephenson. Alcohol dehydrogenase was purified to homogeneity from bakers' yeast by Dr F. M. Dickinson and had a specific activity of about 350 units (mg protein) $^{-1}$.

RESULTS

Various attempts were made to purify ferrimycobactin reductase activity using conventional procedures. Ammonium sulphate precipitation (precipitate from 20 to 40% saturation) gave a twofold improvement in specific activity but over 60% of the total activity was lost. Gel filtration through Sepharose 6B destroyed all activity. Though crude extracts treated with streptomycin sulphate were only about 15% more active than the original, they were markedly less turbid than the crude extracts and consequently gave more consistent assays. Such preparations were used throughout this work.

Table 1. *Effect of inhibitors on ferrimycobactin reductase activity in extracts of M. smegmatis grown under iron-deficient conditions*

Inhibitor	Concn (mM)	Inhibition of ferrimycobactin reductase* (%)
KCN	1	11
	10	74
NaN ₃ †	5	13
	30	31
CCCP†	0.01	0
	0.10	17
DNP	2.0	30
NEM	0.1	39
HgCl ₂	0.1	45

* Enzyme activity in absence of any inhibitor was 0.2 nmol ferrimycobactin reduced min⁻¹ (mg protein)⁻¹.

† Controls contained the appropriate concentrations of methanol, in which the inhibitors were dissolved.

General properties of the reductase activity

The activity of the reductase depended on the presence of NADH and EDTA and the maintenance of anaerobicity during the assay. The K_m for NADH was determined over the range 0.167 to 3.33 mM holding ferrimycobactin constant at 133 μ M. From a double reciprocal plot, the K_m was calculated to be 1.75 mM with a V_{max} of 3.33 nmol ferrimycobactin reduced min⁻¹ (mg protein)⁻¹. The K_m for ferrimycobactin was not measurable as the rate of reaction was still maximal at 8.3 μ M-ferrimycobactin, the lowest concentration of substrate which could be accurately determined spectrophotometrically. The K_m therefore cannot be greater than 4 μ M.

The equilibrium constant for the reaction:

was calculated using the Nernst equation to be 5.5×10^{-5} , assuming an E'_0 value (at pH 7) for ferrimycobactin of -0.265 V which was calculated by comparing reduction voltages in a differential cathode-ray polarograph (see Ratledge & Marshall, 1972) with other iron-containing compounds of known E'_0 values (K. A. McCready, unpublished work). This equilibrium constant is greatly in favour of the oxidized state, but for the reaction:

a value of 8.2 was obtained which is weakly in favour of the reduced state.

The strong electronegativity of the Fe(III)/Fe(II) mycobactin system precludes many compounds from acting as reductant if in low concentration. NAD⁺/NADH with its E'_0 of -0.320 V (Loach, 1970) can function in this capacity. However, although the acetoacetate/ β -hydroxybutyrate system has an even lower E'_0 of -0.346 V (Loach, 1970), β -hydroxybutyrate (at 3.3 or 33 mM) failed to reduce ferrimycobactin under the usual conditions of assay.

Of the various potential inhibitors of reductase activity (Table 1), NEM and HgCl₂, which react with thiol groups, were by far the most potent. The other inhibitors, KCN, CCCP, NaN₃ and DNP, were not as effective as NEM and HgCl₂, although all strongly inhibit iron transport from ferriexochelin into *M. smegmatis*, a process which is not mediated by mycobactin (Stephenson & Ratledge, 1979). Similar sensitivity of the ferrimycobactin reductase to *p*-chloromercuribenzoate and iodoacetate (each giving 75% inhibition at 1 mM), which are also inhibitors of thiol group activity, had been noted previously (Brown & Ratledge, 1974).

Salicylic acid (0.12 mM) could replace EDTA in the assay without loss of activity. This

Fig. 1. Loss of iron from ferrimycobactin following addition of $20 \mu\text{g Fe}^{2+} \text{ ml}^{-1}$ to a 60 h iron-deficient culture of *M. smegmatis*. At the time when iron was added, cells contained approx. 10 mg desferrimycobactin (g dry wt) $^{-1}$; within 2 min following the iron addition, the cells had turned a light red due to formation of ferrimycobactin. One complete culture flask (100 ml medium) was taken for each determination.

may be significant as salicylate is produced by mycobacteria though no firmly established role for it has yet been found (Ratledge *et al.*, 1974).

There was no evidence for hydrolysis of mycobactin by the extracts; recoveries of mycobactin by chloroform extraction after enzymic reduction were about 90% and were the same as when the substance was recovered following chemical reduction with dithionite in the presence of bovine serum albumin under anaerobic conditions.

Although the observed activity of ferrimycobactin reductase was low, it nevertheless exceeded a value calculated for the release of iron from ferrimycobactin *in vivo*. When a growing culture of *M. smegmatis* was shifted up from iron-deficient growth to iron sufficiency (see Methods) the additional iron was complexed immediately by the mycobactin, probably being completely taken up within 2 to 3 min and causing the cells to turn light red. (The rate of complexing of iron with mycobactin is evidently much faster than the rate of iron precipitation in the medium. It is not known, however, whether the added Fe^{2+} is oxidized before or after its chelation to mycobactin.) The iron was slowly released from the ferrimycobactin into the cells over the next 12 h at a linear rate of about $0.02 \text{ nmol Fe min}^{-1} (\text{mg cell dry wt})^{-1}$ (Fig. 1). This rate was approximately ten times slower than the rate measured in extracts but, of course, was taking place in the second half of the growth phase when the rate of iron acquisition may have been decelerating.

Evidence for the non-specificity of ferrimycobactin reductase activity

Activity of ferrimycobactin reductase was similar in extracts of *M. smegmatis* grown either iron-sufficiently or iron-deficiently (Table 2). This was somewhat surprising as the concentration of mycobactin in iron-sufficient cells is about 2000 times less than in iron-deficient cells (Ratledge & Marshall, 1972). Reductase activity was also found in crude extracts of organisms not synthesizing mycobactins. Extracts of *Escherichia coli* and *Candida utilis* (both grown under iron-sufficient conditions) reduced ferrimycobactin at rates comparable to those found in *M. smegmatis* (Table 2). The activity in each extract resembled that from *M. smegmatis* in being dependent on NADH and being inhibited by 50 to 60% after pre-incubation with $10 \mu\text{M}$ -iodoacetamide.

Since the reductase activity was sensitive to reagents that attack thiol groups (see above and Table 1) and was apparently non-specific in nature, and since thiol reagents [dithiothreitol (3.3 mM), mercaptoethanol (33.3 mM) and cysteine (33.3 mM)] reduced ferrimycobactin under anaerobic conditions in the absence of any enzyme and co-factor, alcohol dehydro-

Table 2. *Ferrimycobactin reductase activities in extracts of M. smegmatis and other micro-organisms and of yeast alcohol dehydrogenase*

About 10 mg protein of each microbial extract was added to the assay mixture (3 ml); for yeast alcohol dehydrogenase, 3.3 mg was added. Ferrimycobactin reductase activities are expressed as nmol ferrimycobactin reduced min⁻¹ (mg protein)⁻¹.

Source of activity	Condition of culture	Requirement of reductase for NADH	Activity of ferrimycobactin reductase
<i>M. smegmatis</i>	Iron-deficient	Yes	0.12
<i>M. smegmatis</i>	Iron-sufficient	Yes	0.13
<i>E. coli</i>	Iron-sufficient	Yes	0.21
<i>C. utilis</i>	Iron-sufficient	Yes	0.13
Bakers' yeast alcohol dehydrogenase	—	No	1.12

genase [which contains up to 36 thiol groups per molecule (Brändén *et al.*, 1970)] was also tested as a reductant. This protein reduced ferrimycobactin under anaerobic conditions in the absence of NADH (Table 2).

As reductase activity towards ferrimycobactin was seen in several unrelated micro-organisms, we investigated whether extracts of *M. smegmatis* could catalyse the reduction of iron in forms other than ferrimycobactin. Using a modified assay procedure, in which salicylate (0.12 mM) replaced EDTA to prevent spontaneous removal of iron, ferri-ferrioxamine B (Desferal), ferriexochelin and ferric ammonium citrate were reduced at the respective rates of 0.34, 0.14 and 0.16 nmol min⁻¹ (mg protein)⁻¹, with ferrimycobactin being reduced by the same extract at 0.38 nmol min⁻¹ (mg protein)⁻¹. NADH and anaerobic conditions were still essential for activity in each case.

DISCUSSION

The general properties of ferrimycobactin reductase activity for *M. smegmatis* are similar in many respects to those reported for the corresponding reductase from *N. crassa* (Ernst & Winkelmann, 1977). Both activities are sensitive to reagents which react with thiol groups, have similar pH optima (see Brown & Ratledge, 1974) and can reduce iron chelates other than their own 'natural' substrate. Both reduce ferri-ferrioxamine B at similar rates. The important differences between the two enzymes appear to be: (i) the mycobacterial enzyme has a much lower K_m (less than 4 μ M) for its substrate than the enzyme from *N. crassa* (K_m for coprogen, 100 μ M) though both have similar V_{max} values, and (ii) the mycobacterial enzyme is not decreased in activity in iron-sufficient cells whereas the fungal enzyme is only 30% as active after growth under iron sufficiency compared with growth under iron deficiency.

An important finding in the current work was that ferrimycobactin reductase activity is present in micro-organisms other than mycobacteria, which perhaps implies that the activity is associated with a ubiquitous protein or proteins whose principal function need not, therefore, be that of a siderophore reductase. Although reductase activity could also be manifested by yeast alcohol dehydrogenase, this is attributed to the free thiol groups of this protein reacting directly with ferrimycobactin since NADH was not required as a co-factor. With extracts from *M. smegmatis*, as well as *E. coli* and *C. utilis*, NADH was required and thus, unless we suppose the presence of an enzyme which uses NADH to generate thiol groups on another protein, we presume that direct reduction of the ferrimycobactin was occurring.

The nature of the reductase protein is unknown. It may be associated with the respiratory chain, paralleling the 'iron reductase' found in other micro-organisms (Dailey & Lascelles, 1977; Lascelles & Burke, 1978; see Introduction), but, because of the strong electro-

negativity of ferrimycobactin, this would have to be at a very early site in the sequence. 'Iron reductase' activity is clearly not synonymous with ferrimycobactin or coprogen reductases as this 'enzyme' cannot reduce several naturally occurring siderophores including ferri-ferrinoxamine B (see Introduction).

A non-specific protein having a somewhat high K_m for NADH as co-substrate may help to explain the slow rate at which iron is released from mycobactin both in extracts and *in vivo* (Fig. 1). Mycobactin does not participate in the active uptake of iron in *M. smegmatis* (Stephenson & Ratledge, 1979) – indeed, the results from studies with inhibitors show that the reductase plays no part in the uptake of iron via exochelin – but it might function as a store of iron if iron in any quantity suddenly became available to the organism (e.g. Fig. 1). The controlled release of this stored iron into the cytoplasm, as and when acceptor molecules are synthesized, is therefore necessary and, as these current experiments show, occurs at a slow rate. It remains an open question as to how the activity of the reductase, whatever its nature, is controlled to regulate the release of iron.

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