IRON TRANSPORT IN *MYCOBACTERIUM SMEGMATIS*: FERRIMYCOBACTIN REDUCTASE (NAD(P)H:FERRIMYCOBACTIN OXIDOREDUCTASE), THE ENZYME RELEASING IRON FROM ITS CARRIER

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1. Introduction

Mycobactin is a lipid-soluble iron-binding compound, which is located in the mycobacterial cell envelope where it acts as a transporter of ferric ions across the thick lipoial boundary layers [1]. The release of iron from its carrier is mediated by a ferri-reductase but assay of this activity was severely hampered by having to use very turbid cell extracts and also by the facility with which the ensuing ferrous ion reoxidized and then recombined with mycobactin [1]. Very little could therefore be learnt about the enzyme. In this paper we describe an improved method for determining its activity, which may have a general applicability to other microbial iron transport systems, and are now able to describe some of its properties.

2. Methods

2.1. *Growth of organism and preparation of cell extracts*

M. smegmatis NCIB 8548 was grown on iron-deficient medium as previously described [1,2]. Cells were washed with distilled water after harvesting and mixed with a little 0.1 M Tris–HCl, pH 7.0, buffer before disrupting by a single passage through a Hughes' press or French pressure chamber at 3.5×10^4 knm^{-2}. Disrupted material was diluted as required using 0.1 M Tris–HCl buffer and gently homogenized in an all-glass homogenizer.

2.2. *Assay of ferrimycobactin reductase*

Two assay methods were developed both relying upon changes in extinction at 450 nm, i.e. the absorption maximum of ferrimycobactin.

Method 1. This method was developed for assay of the enzyme activity in extremely dense ($E_{450} > 15$) uncentrifuged cell extracts. Ferrimycobactin was generated *in situ* by adding Fe(salicylate)$_3$, at about 0.25 mM final concentration, to the extract which being from iron-deficient cells contained mycobactin, and then holding at 37°C for about 10 min. The assay mixture in Thunberg tubes contained, in a final vol of 3 ml, cell extract, 2.8 ml; EDTA 10 μmol, and NAD(P)H, 10 μmol, in the sidearm. The Thunberg tubes were closed, repeatedly evacuated and flushed with N_2 (O_2 free; see below) before sealing under a slight positive pressure of N_2. Tubes were equilibrated for 10 min before starting the reaction by tipping in the NAD(P)H. After incubation, the extinctions of the solutions were read at 450 nm using a Cary 14 UV recording spectrophotometer fitted with a scattered transmission accessory and high intensity light source. As reference, an incubation mixture which had lacked NAD(P)H was used.

Method 2. After establishing the validity of Method 1 and the suitability of EDTA as a chelator of Fe(II) (see Results), routine assays of ferrimycobactin reductase were conduced using cell-extracts, centrifuged at 30 000 *g* for 30 min at 2°C, and incubated in anaerobic glass cuvettes each fitted with a sidearm. The assay

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mixture in a final vol of 3 ml, contained cell-extract up to 2.7 ml; EDTA 10 μmol; ferrimycobactin S 680 nmol in 1% Triton N101 (0.2 ml) and, placed in the sidearm, NAD(P)H up to 10 μmol. N_2 (O_2 free) was blown across the surface of the contents of the cuvette for 5 min. The cuvette was then equilibrated at 37°C for 10 min and the reaction started by tipping the NAD(P)H. The change in E_{450} was followed against a reference cuvette lacking NAD(P)H.

2.3. *Preparation of O_2-free nitrogen*

Commercial O_2-free N_2 was bubbled through a de-oxygenator containing 2% (w/v) $Na_2S_2O_4$ in 0.2 M Na_2HPO_4 and then through two Dreschel bottles containing 2 M NaOH to remove any traces of $Na_2S_2O_4$.

3. Results

3.1. *Requirement for an Fe(II) chelator for assay of ferrimycobactin reductase*

The original assay of ferrimycobactin reductase [1,3] involved the direct spectrophotometric measurement of changes in the concentration of ferrimycobactin but considerable difficulty was experienced in achieving reproducible assays due to the very dense solutions which had to be used and also to the rapidity with which the Fe(II) was re-oxidized to Fe(III) which then recombined spontaneously with the free mycobactin. Some chelator of iron was needed which would prevent either this re-oxidation or the subsequent recombination of Fe(III) with mycobactin.

To find a suitable chelator, ferrimycobactin (0.2 mM) was reduced non-enzymatically using $Na_2S_2O_4$ under anaerobic conditions in the presence of a variety of chelating agents, each at 0.66 mM. The procedure followed was similar to that given in fig.1. Of the chelators tried, EDTA appeared the most suitable as the resulting mixture was almost colourless. *o*-Phenanthroline and 2,2′-dipyridyl did form complexes with the iron which was released but had high extinctions around 450 nm and therefore could not be distinguished from ferrimycobactin. Nitrilotriacetic acid was ineffective in this system. In the absence of $Na_2S_2O_4$, but still under N_2, EDTA did not remove iron from ferrimycobactin during 6 hr incubation. The efficacy of EDTA is due to the formation of a colourless Fe(II) chelate which, when the reaction is complete and air re-introduced into the tubes, is rapidly re-oxidized to a pale yellow Fe(III) chelate [4] with ϵ = 6.0–6.5 at 450–470 nm (C. Ratledge, unpublished) whereas that of ferrimycobactin at this wavelength is 3780 [5]. Thus, as iron is not readily transferred from EDTA into mycobactin, the concentration of ferrimycobactin can be determined even in the presence of excess ferri EDTA (fig.1).

When EDTA was incorporated into an assay for ferrimycobactin reductase (Method 1), it produced

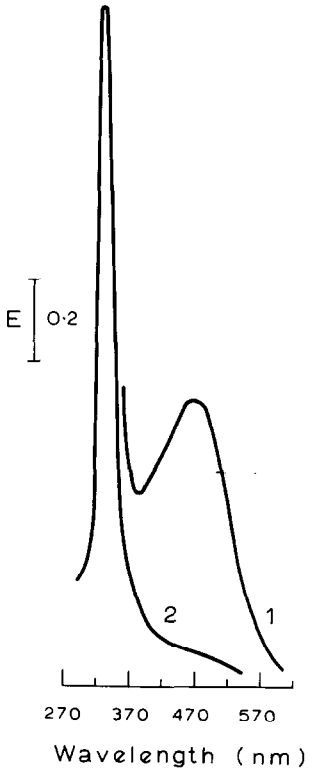

Fig.1. Spectra of ferrimycobactin S in the presence of EDTA before (line 1) and after (line 2) reduction with $Na_2S_2O_4$ under N_2 and subsequent re-oxidation. EDTA was at 3.3 mM and ferrimycobactin at 0.2 mM and was dissolved in 0.09 M Tris buffer, pH 7.5, containing 1% Tween 80. For reduction the components were held in a Thunberg tube and flushed with N_2. $Na_2S_2O_4$ (approx. 1 mg) was tipped in from the side-arm and after 5 min the tube opened, air blown in for 15 min and the spectra then determined.

Table 1
Reduction of ferrimycobactin formed in situ in an uncentrifuged extract of iron-deficiently grown *M. smegmatis*

Additions to extract + ferrimycobactin in assay system	Ferrimycobactin reduced (nmol)
None	55
NADH	74
NADPH	76
EDTA	176
NADH + EDTA	682
NADPH + EDTA	680

Cell extract, 2.8 ml, 15.0 mg protein/ml, containing ferrimycobactin was incubated at 30°C for 4.5 hr under anaerobic conditions as given in Methods (assay Method 1). NAD(P)H and EDTA were each at 10 μmol.

a 9-fold increase in activity (table 1). In the absence of cell extract ferrimycobactin was not reduced even in the presence of a high concentration of NADH (20 mg). A boiled cell extract had no reactivity in the complete assay system.

Adding EDTA, in the absence of NAD(P)H, tripled the amount of ferrimycobactin reduced (table 1) probably due to endogenous reductants within the cell extracts. The high control value of 55 nmol ferrimycobactin reduced in the period of assay is probably due to the same reason. There was no increase in the specific activity of the enzyme when cell extracts were prepared in buffer containing either 1 mM dithiothreitol or 1 mM mercaptoethanol.

3.2. *Some properties of ferrimycobactin reductase*

Preliminary work [1] indicated that ferrimycobactin reductase was a particulate enzyme, but upon using the improved assay for the enzyme, between 70 and 80% of the total amount of enzyme present remained in solution after centrifuging the disrupted cells (table 2). Even centrifuging at 150 000 *g* failed to sediment it. Thus, the direct attachment of the enzyme to a membrane is doubtful. This result meant that subsequent assays of the enzyme could be carried out on centrifuged cell extracts.

The stringent requirement for anaerobicity during assay of the enzyme was shown by gassing the assay mixture with N_2 for various times (fig.2). Traces of oxygen are tolerated by the enzyme as there was no difference in the rate of reaction when it was gassed for different times; but all oxygen had to be consumed (by endogenous respiratory processes) before activity of ferrimycobactin reductase was manifested.

Ferrimycobactin reductase was rapidly inactivated when held at temperatures over 37°C. At 45°C, 50% inactivation occurred in 10 min and at 80°C, 90% inactivation occurred in the same time; the enzyme was completely inactivated by holding at 100°C for 10 min. At 37°C for 10 min, however, the enzyme was just as stable as a preparation held at 0°C for the same time. The stability of the enzyme was such that it could be stored at 4°C for up to 4 days without loss of activity.

The enzyme had a sharp optimum activity at pH 7.0 (fig.3). It had only 40% of this activity at pH 7.4 and 75% of this activity at pH 6.5.

Uncentrifuged cell extracts had shown little

Table 2
Distribution of ferrimycobactin reductase in cell fractions

Cell fraction	Specific activity (nmol ferrimycobactin) reduced/30 min/mg protein)	Total activity in fraction (nmol ferrimycobactin reduced/30 min)
Whole cell extract	26	9880 (100%)
Pellet after initial centrifuging*	16	1760 (18%)
Supernatant	27	7020 (71%)
Supernatant after re-centrifuging**	26	6052 (61%)

Enzyme activity determined as given in Methods (assay Method 1).
* 30,000 *g* at 2°C for 30 min.
** 150,000 *g* at 2°C for 30 min.

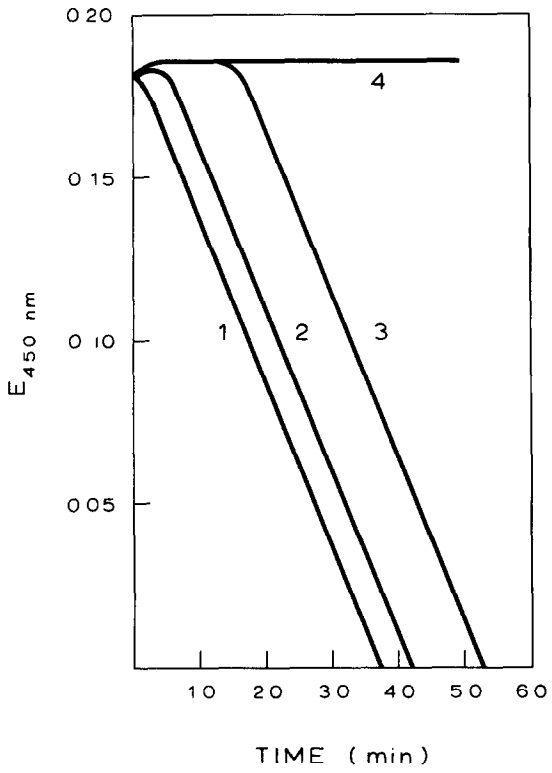

Fig.2. Effect of time of gassing with N_2 on activity of ferrimycobactin reductase. Enzyme assayed as given in Methods (assay Method 2) with gassing for 5 min (line 1), 2 min (line 2), 1 min (line 3) and without gassing, aerobic, (line 4).

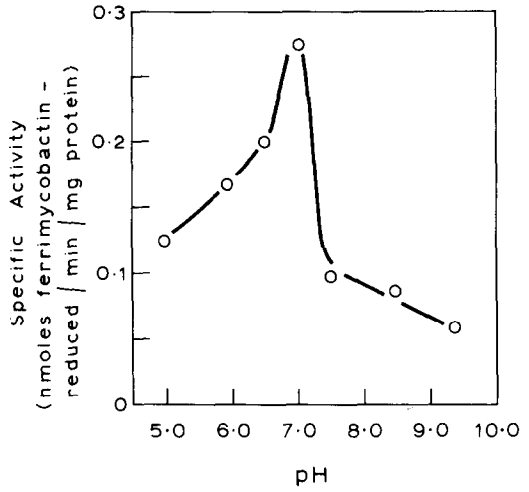

Fig.3. pH profile of ferrimycobactin reductase. Enzyme assayed as given in Methods (assay Method 2). Enzyme prepared in 10 mM Tris–HCl buffer pH 7.5 and 1.0 ml added to 1.6 ml 100 mM Bis-Tris–HCl of various pH values.

preference between NADH and NAD(P)H as electrons donors for the enzyme (see table 1) and using centrifuged extracts, in the assay Method 2, NAD(P)H was, in fact, 80% as effective as NADH (each at 3.3 mM) as cofactor for the reduction of ferrimycobactin. However, utilization of NADPH may not have been direct as the crude extracts may well have contained an active $NAD(P)^+$ transhydrogenase serving to generate NADH *in situ*. $FADH_2$ (FAD + sodium succinate each at 3.3 mM) though had 25% of the effectiveness of NADH as reductant. The specificity of the enzyme would therefore appear to be confined to NADH and NADPH.

3.3. *Effect of inhibitors on enzyme activity*

Strong inhibitors of the enzyme's activity (followed by Method 2) were the sulphydryl reagents, *p*-chloromercuribenzoate and iodoacetate, each producing about 75% inhibition when present at 1 mM. Sodium amytal, being an inhibitor of NADH oxidation [6], was tested also at 1 mM and inhibited activity by 80%. Triton, used routinely in the assay at 0.066% (w/v) as a solubilizer for mycobactin, inhibited activity by 30% only when added at 10 times this concentration. *p*-Aminosalicyclic acid (PAS) was tested against the enzyme as other work [7] has shown this compound to be a probable inhibitor of iron assimilation but even at 1 mM it was ineffective. Salicylate, which appears to have some role in iron transport [8] and for which PAS is probably antimetabolite, also had no effect when at 1 mM on the activity of the reductase.

4. Discussion

Although a wide variety of iron ionophores are now recognized in microbial systems [9–15] there has not been much work done on the mechanism by which iron is released from them. O'Brien et al. [14] described the properties of an esterase from *E. coli*

which hydrolysed enterochelin whilst still co-ordinated with $Fe^{(III)}$. This allowed the iron to become available to the cell presumably by subsequent reduction and insertion into porphyrins via ferrochelatase [16,17]. Bryce and Brot [18], however, failed to find this esterase which is somewhat disconcerting for the presentation of a unified view of iron transport in this bacterium.

In *Ustilago sphaerogenes* a NAD(P)H ferri–ferrichrome reductase, presumably similar to the enzyme reported here from *M. smegmatis*, was found by H. Komai and reported by Neilands [10]. But apart from this report, there have been no other reports of the existence of similar enzymes in micro-organisms but it would not be surprising to find that reduction of the iron was a widely used method for removing iron from its carrier. Reduction is not only energetically economic but, as most carriers have little affinity for $Fe^{(II)}$, the iron can then be easily removed. Provided no re-oxidation of $Fe^{(II)}$ occurs, it is then in a suitable state for immediate insertion into porphyrins and other acceptor molecules. The carrier, being unchanged by the action of the reductase, is free to return to the outside of the cell envelope to recommence the transport process. The systematic name for this enzyme from *M. smegmatis* is NAD(P)H: ferrimycobactin oxidoreductase.

Although some $Fe^{(II)}$ released from mycobactin will be incorporated into porphyrins or porphyrin–enzyme complexes, to give haem or haem–enzymes, most will be incorporated into non-haem iron proteins which probably constitute at least 90% of the total intracellular iron [19,20]. Mechanisms for insertion of iron into both these materials have yet to be elucidated in the mycobacteria but they may not necessarily be associated with the membranes of the cell. Kurup and Brodie [20] found that 65% of the total iron within *M. phlei* was recovered in the soluble cell-free extract rather than in sedimented particles and thus our finding that 70% of the total amount of ferrimycobactin reductase could be recovered in the soluble fraction may be in accord with their results.

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References

[1] Ratledge, C. and Marshall, B. J. (1972) Biochim. Biophys. Acta 279, 58–74.
[2] Ratledge, C. and Hall, M. J. (1971) J. Bacteriol. 108, 314–319.
[3] Ratledge, C. (1971) Biochem. Biophys. Res. Commun. 45, 856–862.
[4] Harris, D. C. and Aisen, P. (1973) Biochim. Biophys. Acta 329, 156–158.
[5] White, A. J. and Snow, G. A. (1969) Biochem. J. 111, 785–792.
[6] Appleby, C. A. (1969) in: Data for Biochemical Research, 2nd ed. (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M., eds.) pp. 380–387, Oxford Univ. Press, London.
[7] Brown, K. A. and Ratledge, C. (1975) Biochim. Biophys. Acta, in press.
[8] Ratledge, C., Macham, L. P., Brown, K. A. and Marshall, B. J. (1974) Biochim. Biophys. Acta 372, 39–51.
[9] Lankford, C. E. (1973) C.R.C. Crit. Rev. Microbiol. 2, 273–331.
[10] Neilands, J. B. (ed.) (1974): Microbial Iron Metabolism, Academic Press, New York.
[11] Emery, T. F. (1971) Adv. Enzymol. 35, 135–185.
[12] Haydon, A. H., Davis, W. B., Arceneaux, J. E. L. and Byers, B. R. (1973) J. Bacteriol. 115, 912–918.
[13] Luckey, M., Pollack, J. R., Wayne, R., Ames, B. N. and Neilands, J. B. (1972) J. Bacteriol. 11, 731–738.
[14] O'Brien, I. G., Cox, G. B. and Gibson, F. (1971) Biochim. Biophys. Acta 237, 537–549.
[15] Winkelmann, G. (1974) Arch. Microbiol. 98, 39–50.
[16] Porra, R. J. and Jones, O. T. G. (1963) Biochem. J. 87, 181–185.
[17] Porra, R. J., Langman, L., Young, I. G. and Gibson, F. (1972) Arch. Biochem. Biophys. 153, 74–78.
[18] Bryce, G. F. and Brot, N. (1972) Biochemistry 11, 1708–1715.
[19] Righelato, R. C. (1969) J. Gen. Microbiol. 58, 411–419.
[20] Kurup, C. K. R. and Brodie, A. F. (1967) J. Biol. Chem. 242, 2909–2916.