TRANSPORT OF IRON BY MYCOBACTIN IN MYCOBACTERIUM SMEGMATIS

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SUMMARY

The uptake of $^{55}FeCl_3$ and ^{55}Fe-mycobactin has been followed. $FeCl_3$ was the more effective source of iron for the cell although Fe-mycobactin was adsorbed on to the cell very quickly. The mycobactin within the cell is the prime receptor for Fe^{3+} and it probably functions as an ionophore for the transport of iron across the lipid envelope of the cell. Release of iron from mycobactin occurs by a NADH-dependent reductase forming Fe^{2+} and conditions for observing the activity of this unique enzyme are given.

Mycobactin, an intracellular lipid-soluble, iron-binding compound, with a stability constant for Fe^{3+} of over 10^{30}, is only produced by mycobacteria (1). Iron represses its formation (1-4): in iron-sufficient cultures (2.0 μg Fe/ml) growing exponentially, mycobactin is repressed to about 3 μg/g cell dry weight (2) whereas in iron-deficient cultures (0.05-0.1 μg Fe/ml), its derepressed concentration is 80 mg/g cell dry weight (2, 3). Although the function of mycobactin is uncertain, Snow has suggested that it may function in some capacity in iron metabolism (1). This preliminary communication presents evidence that mycobactin participates in the uptake of iron into the cell.

METHODS

M. smegmatis was grown under stationary conditions as previously described (5). For uptake of iron, $^{55}FeCl_3$ or ^{55}Fe-mycobactin was injected into the medium (100 ml) which had

supported iron-deficient growth (0.1 μg iron added/ml) for 3 days. Entire cultures were harvested in triplicate at intervals, filtered with suction through Whatman no. 1 paper and the cells rapidly washed with water, 1% citric acid and water to remove loosely bound Fe^{3+}. Cells were sucked as dry as possible and mycobactin was extracted with ethanol for 24 hr at 15°C. The activity of ^{55}Fe in the ethanol was determined (all ^{55}Fe in the ethanol was also chloroform-soluble and was chelated with mycobactin) and the mycobactin, after conversion wholly to the ferriform, was extracted into chloroform. The chloroform was washed with water, dried over $MgSO_4$, evaporated and the residue dissolved in methanol which was then assayed for mycobactin (6). Cell residues were dried, weighed and their radioactivities counted by scintillation techniques; (radioactivity of mycobactin in methanol was measured similarly and corrected for quenching).

Cell extracts were prepared with a Hughes press from 4 or 5 day-old iron-deficient cultures; 3 hr before harvesting 1 μg Fe^{3+}/ ml was added to each culture to activate iron transport processes. For the formation of ferrimycobactin *in situ*, the uncentrifuged extract was gently homogenized in 0.1 M tris-HCl buffer, pH 7.5, + 1 mM $MgCl_2$ + 1 mM mercaptoethanol + 3 mM reduced glutathione; the final protein concentration, as determined by the method of Lowry *et al.* (7), was 6.5 mg/ml. The mixture was incubated with 0.5 mM Fe(salicylate)$_3$ with occasional homogenization at 37°C. The rate of formation of ferrimycobactin was followed at 460 nm in a Unicam SP 1800 spectrophotometer using a band width of 3 nm against cell extract without Fe(salicylate)$_3$ as reference.

For the reduction of ferrimycobactin, 2.5 ml of the above mixture after 40 minutes' incubation was added to each of two cuvettes suitable for anaerobic incubation. The reference

cuvette was flushed with N_2 for 1 minute and, using the utmost precautions to exclude air, deoxygenated NADH solution, 20 mg in 0.2 ml buffer, was injected into it; tris-HCl buffer, pH 7.5, 0.2 ml, having already been added to the sample cuvette. Spectra were scanned from 410 to 510 nm at 2 nm/sec and the cuvettes held at 37°C.

RESULTS

Uptake of iron. The uptake of $^{55}FeCl_3$ or ^{55}Fe-mycobactin by established cultures was monitored after 0, 1, 4 and 24 hr (Fig 1).

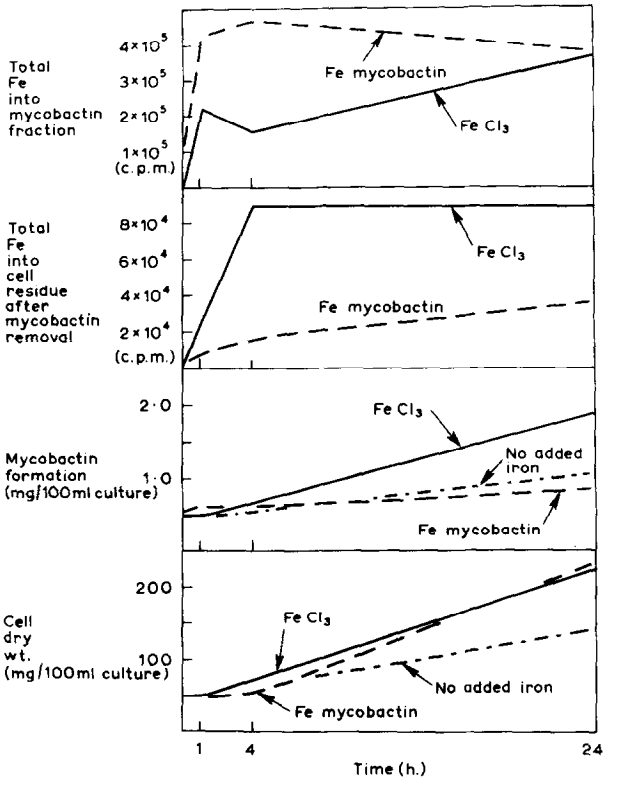

Figure 1. Uptake of iron into M. smegmatis: Iron-55, 5μg containing 5 x 10^5 cpm, as $FeCl_3$ or Fe-mycobactin, was added to 3-day-old iron-deficient cultures. Cultures were sampled in triplicate after 0, 1, 4 and 24 hr and average values for the various determinations are given. Progress of cultures to which ^{55}Fe was not added is indicated in the lower two graphs.

With $^{55}FeCl_3$, 0.05 μg ^{55}Fe/ml added to the culture, the mycobactin within the cells became labelled very rapidly (0 to 1 hr) but from 1 to 4 hr, its specific activity fell with there being a corresponding increase in the activity of the cell residue (Fig 1) thus indicating that iron was only transiently attached to the mycobactin. Similar results for the uptake of iron were obtained when 1 μg ^{55}Fe/ml (as $^{55}FeCl_3$) was added to cultures, although, unlike with the lower concentration of $FeCl_3$, not all the added ^{55}Fe was taken up by cells: only about 35% was utilized over 24 hr. Upon addition of ^{55}Fe-mycobactin to the cultures, rapid association of ^{55}Fe with the cells occurred (Fig 1), probably because of the lipophilic natures of both Fe-mycobactin and the mycobacterial cell envelope (8). Entry of ^{55}Fe into the cell residue was, however, more rapid using $^{55}FeCl_3$ than ^{55}Fe-mycobactin although there was little difference in the resulting increases in cell dry weight over 24 hr.

The addition of $^{55}FeCl_3$ at 0.05 or 1.0 μg/ml produced no precipitate of $Fe(OH)_3$ presumably because of chelation with the constituents of the medium and with salicylic acid which is excreted at about 1 μg/ml under these growth conditions (3).

Removal of iron from mycobactin. As mycobactin was labelled with ^{55}Fe before the remainder of the cell, the cell must possess some mechanism for the subsequent removal of iron from the mycobactin. This mechanism was most likely to be by reduction to Fe^{2+} which would have little affinity for mycobactin and could be used as substrate for such enzymes as ferrochelatase for the synthesis of heme (9).

The reduction of ferrimycobactin in cell extracts was accomplished using NADH as co-factor (Fig 2). The formation of

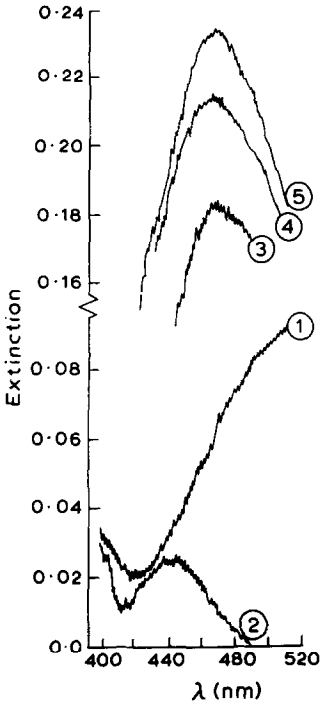

Figure 2. Reduction of ferrimycobactin: Sample and reference cuvette both contained cell extracts with ferrimycobactin generated in situ (Spectrum 1); reference cuvette was then flushed with N_2 (Spectrum 2), NADH added to it and spectra read after 1, 2 and 3 min (Spectra 3, 4 and 5). Formation of ferromycobactin in the reference cuvette results in the appearance of the spectrum of ferrimycobactin in the sample cuvette.

ferrimycobactin in situ, by incubating Fe^{3+} with uncentrifuged extracts of cells already containing desferrimycobactin produced during cell growth, was checked by extracting the compound formed into chloroform and verifying that its spectrum and properties agreed with those of mycobactin obtained more conventionally. Under these conditions, the formation of ferrimycobactin, which could easily be seen by its redness, took about 20 to 30 minutes to become maximal and, furthermore, could be halved by incubation at 0°C. $Fe(salicylate)_3$ at an equimolar concentration as $FeCl_3$ produced the same rate of ferrimycobactin formation though $Fe(EDTA)_3$ was inactive.

Reduction of mycobactin could only be successfully observed when high concentrations of NADH were added under anaerobic conditions. Addition of NADH to cell extracts in which ferrimycobactin had not been formed did not show changes in extinction corresponding to the reduction of ferrimycobactin. Ferrimycobactin was not reduced in the absence of cell extract when held under anaerobic conditions, with or without the inclusion of glutathione or ascorbate along with NADH. Ferrimycobactin was completely reduced, however, by $Na_2S_2O_4$: the ferro form of mycobactin is colourless and quickly re-oxidizes in air.

DISCUSSION

The content of mycobactin in the cell is about 1% of the cell dry weight and because of its low solubility in water (1) and content in the cytoplasm - about 4 μg/ml - (2), it must be almost entirely associated with the lipoidal regions of the mycobacterial cell, i.e. the thick cell wall (8) and the membrane. This is supported by its rapid extraction with ethanol or 1% Tween 80 (2). Both desferri- and ferrimycobactin have high and equal interfacial tensions (2) and the participation of mycobactin as an iron transport agent across the boundary layers of the cells becomes evident from the data given in Fig 1. Mycobactin appears to be the prime receptor for Fe^{3+} and the loss of iron from mycobactin into the rest of the cell occurs as a subsequent event by reduction to Fe^{2+} which then has little, if any, affinity for mycobactin. Other reduced co-factors besides NADH might function with the reductase responsible for this event. A scheme summarizing these findings is given below (Fig 3).

Movement of ferrimycobactin from the outer surface to the inner one and the movement of desferrimycobactin in the reverse

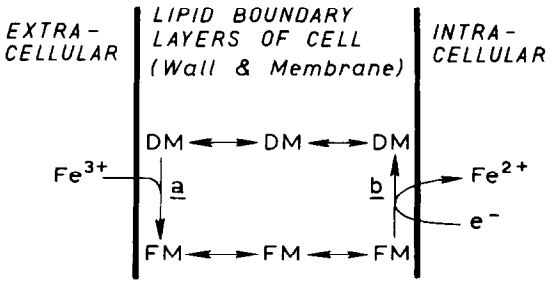

Figure 3. Proposal for transport of iron into the mycobacterial cell: DM = desferrimycobactin; FM = ferrimycobactin. Reactions a and b occur at the lipid/water interfaces and are considered irreversible.

direction are, of course, equal and opposite events and occur because of the continual changes in concentrations of both compounds at each surface. Thus the transport of iron into the mycobacterial cell is by a process of facilitated diffusion.

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