Exochelins of *Mycobacterium tuberculosis* Remove Iron from Human Iron-binding Proteins and Donate Iron to Mycobactins in the *M. tuberculosis* Cell Wall

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Summary

To multiply and cause disease in the host, *Mycobacterium tuberculosis* must acquire iron from the extracellular environment at sites of replication. To do so, the bacterium releases high-affinity iron-binding siderophores called exochelins. In previous studies, we have described the purification and characterization of the exochelin family of molecules. These molecules share a common core structure with another type of high-affinity iron-binding molecule located in the cell wall of *M. tuberculosis*: the mycobactins. The water-soluble exochelins differ from each other and from the water-insoluble mycobactins in polarity, which is dependent primarily upon the length and modifications of an alkyl side chain. In this study, we have investigated the capacity of purified exochelins to remove iron from host high-affinity iron-binding molecules, and to transfer iron to mycobactins. Purified desferri-exochelins rapidly removed iron from human transferrin, whether it was 95 or 40% iron saturated, its approximate percent saturation in human serum, and from human lactoferrin. Desferri-exochelins also removed iron, but at a slower rate, from the iron storage protein ferritin. Purified ferri-exochelins, but not iron transferrin, transferred iron to desferri-mycobactins in the cell wall of live bacteria. To explore the possibility that the transfer of iron from exochelins to mycobactins was influenced by their polarity, we investigated the influence of polarity on the iron affinity of exochelins. Exochelins of different polarity exchanged iron equally with each other. This study supports the concept that exochelins acquire iron for *M. tuberculosis* by removing this element from host iron-binding proteins and transferring it to desferri-mycobactins in the cell wall of the bacterium. The finding that ferri-exochelins but not iron transferrin transfer iron to mycobactins in the cell wall underscores the importance of exochelins in iron acquisition. This study also shows that the variable alkyl side chain on the core structure of exochelins and mycobactins, the principal determinant of their polarity, has little or no influence on their iron affinity.

Mycobacterium tuberculosis, the primary etiologic agent of tuberculosis, is a facultative intracellular pathogen. In the host, the organism appears to multiply both intracellularly in mononuclear phagocytes, especially lung macrophages, and extracellularly in lung cavities. Multiplication to very high levels in lung cavities is especially important to disease transmission, as it is from such sites that the organism is coughed up and disseminated to new hosts.

As with essentially all bacterial pathogens, *M. tuberculosis* requires the element iron to multiply in the host and cause disease. However, free iron is limited in at least one of the bacterium's two major sites of replication in the host. At the extracellular site of *M. tuberculosis* replication, iron is limited owing to the high affinity with which it is bound in the extracellular space by host iron-binding proteins, chiefly transferrin and lactoferrin. At the intracellular site of *M. tuberculosis* replication, the bacterium's phagosome in mononuclear phagocytes, the availability of iron is not known. However, in the nonactivated monocyte, studies in this laboratory of the intracellular bacterial pathogen *Legionella pneumophila* suggest that iron in the monocyte's intermediate labile iron pool may be readily available. Furthermore, the *M. tuberculosis* phagosome in human monocytes interacts with the transferrin receptor endocytic pathway (1), and exogenously administered iron transferrin is transported to the *M. tuberculosis* phagosome (2). In the IFN-γ–activated monocyte, however, iron may be limited as a result of marked downregulation of transferrin receptor expression, iron uptake, and intracellular ferritin concentration, and, consequently, shrinkage of the intermediate labile iron pool (3, 4). Hence, as a direct result of iron deprivation, *L. pneumophila*, which lacks a high-affinity iron

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uptake system, cannot multiply in the IFN-γ–activated human monocyte (3). In contrast, *M. tuberculosis*, which has a high-affinity iron uptake system, can multiply in the IFN-γ–activated human monocyte (5).

To compete for iron in environments where it is limited, such as the extracellular space and potentially within its phagosome in the IFN-γ–activated macrophage, *M. tuberculosis* uses high-affinity siderophores known as exochelins. In a previous paper, we described the composition and structure of the *M. tuberculosis* exochelin family of molecules (6). The exochelins are low molecular weight molecules, with the masses of the major species ranging from 744 to 800 daltons in the iron-loaded state. The molecules form two 14-dalton-increment series, one saturated and the other unsaturated, with the increments primarily reflecting different numbers of CH_2 groups on a side chain. The series further subdivide into serine- or threonine-containing species. The molecules share a common core structure with another high-affinity iron-binding molecule in the cell wall of *M. tuberculosis*: the mycobactins. The exochelins differ from the mycobactins in that they have a shorter alkyl side chain, and the side chain terminates in a methyl ester or, sometimes, carboxylic acid moiety. These differences render the exochelins smaller and more polar than the highly lipophilic mycobactins and hence soluble in the aqueous milieu in which the exochelins scavenge iron.

To scavenge iron for the bacterium at the extracellular site of *M. tuberculosis* replication, and perhaps too within the mildly acidified transferrin-containing *M. tuberculosis* phagosome in human monocytes (7), exochelins must be capable of removing iron from host iron-binding molecules. To determine if exochelins are capable of so doing, in the first part of this study we have explored the capacity of purified desferri-exochelins to remove iron from human transferrin and lactoferrin. At the same time, we have investigated the capacity of purified desferri-exochelins to remove iron from the intracellular iron storage protein ferritin, which also circulates at low levels in the blood.

The mechanism by which exochelins transfer iron to mycobacteria is not known. One plausible mechanism, originally hypothesized by Macham and colleagues (8), is that exochelins transfer iron to mycobactins in the bacterial cell wall, and then the mycobactins in turn transfer the iron to the interior of the organism. To explore this hypothesis, in the second part of this study we have investigated the capacity of purified ferri-exochelins to transfer iron to desferri-mycobactins in the cell wall of live *M. tuberculosis* organisms. Since exochelins differ from mycobactins and from each other primarily with respect to their polarity, we have additionally explored the influence of polarity on the iron affinity of exochelins.

Materials and Methods

Bacterial Cultures. *M. tuberculosis* Erdman strain (ATCC 35801; American Type Culture Collection, Rockville, MD) was cultured in modified iron-deficient Sauton's broth medium (1 μM iron, no Tween) in 1.9 liter tissue culture flasks (Falcon; Fisher Scientific Co., Pittsburgh, PA), 300 ml per flask, at 37°C in 5% CO_2/95% air for 6 wk (6).

Reagents. Human iron transferrin (95% iron saturated) and lactoferrin (90% iron saturated) (Sigma Chemical Co., St. Louis, MO) and horse ferritin (Calbiochem Corp., La Jolla, CA) were dissolved in PBS, pH 7.2, or in water. Apotransferrin and apolactoferrin (Sigma Chemical Co.) were 5% iron saturated. 40% iron-saturated transferrin was prepared by mixing 95% iron-saturated transferrin and apotransferrin in a 2:3 ratio in PBS and incubating the solution overnight at 4°C.

Purification of Exochelins and Mycobactins. Exochelins and mycobactins were purified as previously described (6). Briefly, the culture supernatant fluid was saturated with iron, and ferri-exochelins were extracted into chloroform. The chloroform extract was dried and exochelins were purified by reverse-phase HPLC on a C-18 column from which they eluted in 60–90% buffer B (50% acetonitrile, 0.1% TFA). Individual exochelins were subsequently purified on an alkyl phenyl column. Mycobactins were extracted from the bacterial cells in 95% ethanol for 24 h and then saturated with iron. The ethanol extract was diluted with water, and ferri-mycobactins were extracted into chloroform. The rest of the purification procedure was the same as for exochelins, except that mycobactins eluted in 95% acetonitrile, 0.1% TFA. Ferri-exochelins and ferri-mycobactins were identified in the HPLC eluate by their high 450-nm absorbance, and their identity was subsequently confirmed by mass spectrometry (MS)[1] (6). After purification, exochelins and mycobactins were again extracted in chloroform, dried, and stored at 4°C. Ferri-exochelins were converted to their desferri forms by incubation in 50 mM EDTA, pH 4, until the 450-nm absorbance of the solution declined to 10% of its initial value. The desferri-exochelins and remaining ferri-exochelins were then extracted into chloroform. By this method, ∼60–90% of the iron was removed from the exochelins without altering their structure, as confirmed by MS.

Assay for Capacity of Desferri-Exochelins to Remove Iron from Iron-binding Proteins. In each experiment, a single species of purified exochelin in its desferri form was dissolved in PBS, pH 7.2, and incubated with iron transferrin, iron lactoferrin, or ferritin at an iron/exochelin ratio of 4:1 or 1:1. As a control, the desferri-exochelin was also incubated with apotransferrin, apolactoferrin, or apoferritin. The conversion of desferri-exochelin to ferri-exochelin was assayed after 1-min, 1-h, and 3-h incubations with transferrin or lactoferrin, and after 1-, 2-, and 4-d incubations with ferritin. Ferri- and desferri-exochelins present in the solution at each time point were extracted into chloroform, and the chloroform was evaporated. The amount of ferri-exochelin in the preparation was quantitated by reverse-phase HPLC by measuring the 450 nm absorbance of the exochelin. The total amount of exochelin (ferri plus desferri) in the preparation was quantitated in the same way after saturating the preparation with iron. The percentage of saturation was equal to the ratio of these two measurements ×100.

Assay for Capacity of Exochelins to Donate Iron to Desferri-Mycobactins in Live Bacteria. *M. tuberculosis* Erdman was cultured for 6 wk in modified Sauton's iron-deficient broth medium (see above). The bacterial cells were recovered, washed twice, and resuspended in the same medium but without iron. Equal aliquots of the bacterial suspension (1 g of wet cells) were dispensed in sterile tubes and incubated for 3 h at room temperature with pure

[1] *Abbreviation used in this paper:* MS, mass spectrometry.

ferri-exochelin, human transferrin (40 or 95% iron-saturated), or ferric chloride, all containing 20 μM Fe, or with no iron. Bacteria were then washed twice with iron-deficient broth medium, and the mycobactins were extracted, purified, and quantitated by reverse phase HPLC, as described above.

Assay for Capacity of Exochelins to Exchange Iron with Each Other. Ferri-exochelins 758C and 772C (molecular weights of the Fe^{3+} adduct 758 and 772, respectively), and their desferri-forms were dissolved in ultrapure HPLC-grade water. Desferri-exochelin 758C was mixed with ferri-exochelin 772C at equimolar ratios (40 μM) and incubated overnight at room temperature. Similarly, desferri-exochelin 772C was mixed with ferri-exochelin 758C. The amount of ferri- and desferri-exochelin of each exochelin species in the reaction vessel was quantitated by reverse phase HPLC as described in the previous section. All these experiments were performed in iron-free glassware.

Results

Desferri-Exochelins Remove Iron from Iron-binding Proteins. To determine if exochelins can acquire iron from human high-affinity iron-binding proteins, we first purified two major exochelins from *M. tuberculosis* Erdman strain: exochelins 758C and 772C (Fig. 1). Each exochelin was highly pure as evidenced by a single peak eluting off the alkyl phenyl column in the final reverse-phase HPLC purification step and by the demonstration of a single iron-loaded species of exochelin on subsequent MS analysis (6). We converted the purified exochelins to their desferri form, incubated them with either human transferrin or human lactoferrin at 4:1 and 1:1 molar ratios of iron/exochelin, and assayed the conversion of desferri-exochelin to ferri-exochelin as described in Materials and Methods. Exochelins rapidly removed iron from iron transferrin. By 1 min after incubation with 95% iron-saturated transferrin, the exochelins were saturated with iron from the transferrin, and the exochelins remained saturated with iron throughout the experiment (Fig. 2). Exochelins also rapidly acquired iron from 40% iron-saturated transferrin, but at a slower rate than from 95% iron-saturated transferrin, reaching saturation by 3 h. No conversion of desferri- to ferri-exochelin was detected when exochelins were incubated with apotransferrin as a control ($n = 4$).

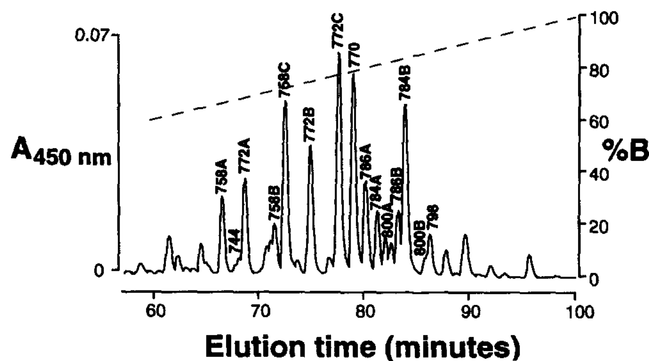

Figure 1. *M. tuberculosis* Erdman exochelin family. The graph shows the elution profile of exochelins in the filtrate of a 6-wk culture on a reverse-phase HPLC C-18 column. Each 450-nm absorbance peak contains an exochelin in its iron-loaded form. The molecular mass of each exochelin species, determined by liquid secondary ion ms, is indicated above each peak. Some exochelins have the same molecular mass but a different structure (see text). The letter (*A, B, C*) following their mass differentiates them by the order in which they elute from the column, with A coming off first, then B, and then C. The same figure except for the letter designations was previously published (6). The dashed line represents the concentration of buffer B.

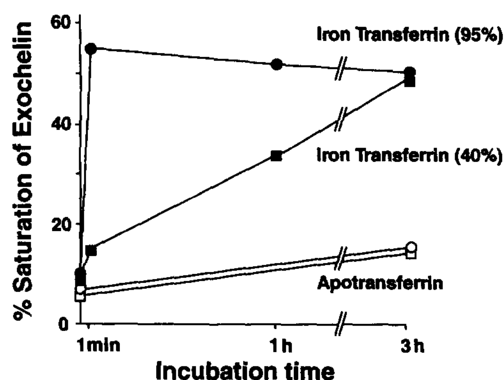

Figure 2. Exochelins remove iron from human transferrin. Desferri-exochelin 758C was incubated with 95% (●) or 40% (■) iron-saturated transferrin at an iron/exochelin ratio of 1:1 or with apotransferrin at a protein concentration equivalent to that of the 95% (○) or 40% (□) iron-saturated transferrin. The percentage of iron saturation of the exochelin was assayed at the indicated times.

Similarly, exochelins rapidly removed iron from iron lactoferrin. By 1 min after incubation with 90% iron-saturated lactoferrin, exochelins were heavily saturated, and by 1 h, they were fully saturated (Fig. 3; $n = 3$).

To determine if exochelins can acquire iron from the iron storage protein ferritin, we incubated desferri-exochelins (7.5 μM) with either 1 or 0.1 μg/ml of horse ferritin. The exochelins also acquired iron from this protein, but the rate of acquisition was much slower than from transferrin and lactoferrin; 2 d were required to reach saturation (Fig. 4; $n = 2$). Exochelins did not acquire iron from apoferritin, the negative control.

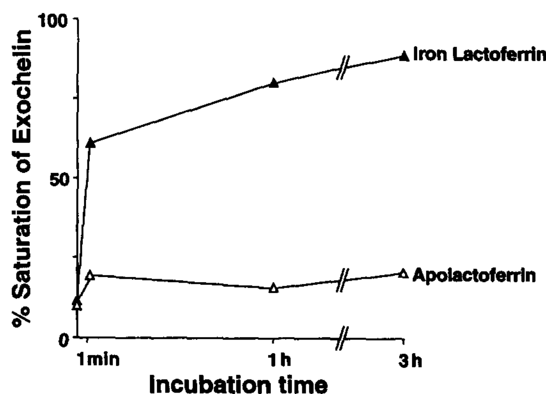

Figure 3. Exochelins remove iron from human lactoferrin. Desferri-exochelin 758C was incubated with 90% iron-saturated lactoferrin or with an equivalent protein concentration of apolactoferrin. The percentage of iron saturation of the exochelin was assayed at the indicated times.

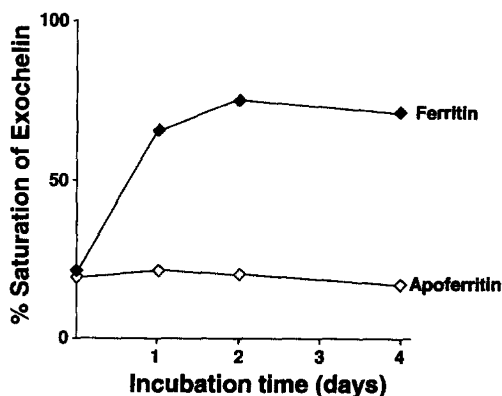

Figure 4. Exochelins remove iron from ferritin. Desferri-exochelin 758C was incubated with ferritin or an equivalent protein concentration of apoferritin. The percentage of iron saturation of the exochelin was assayed at the indicated times.

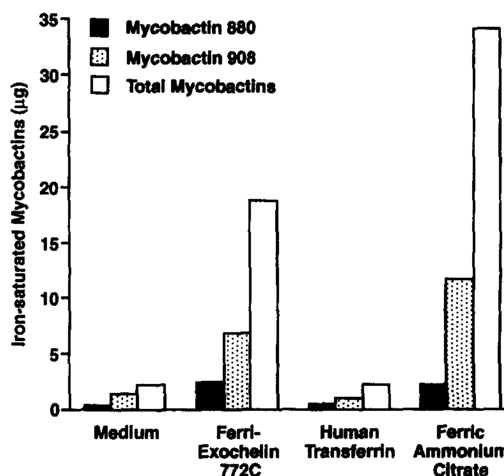

Figure 5. Ferri-exochelins transfer iron to desferri-mycobactins in the cell wall of live bacteria. *M. tuberculosis* cells were cultured under iron-deficient conditions to enhance mycobactin production and then incubated with ferri-exochelin 772C, 40% iron-saturated human transferrin, or ferric ammonium citrate, all containing 20 µM iron, or with iron-free medium. The mycobactins were then extracted, and the total amount of ferri-mycobactin and the amount of the iron-loaded form of the two most abundant mycobactin species (mycobactin 880 and 908) were assayed.

Ferri-Exochelins Donate Iron to Mycobactins in the Cell Wall of Live Bacteria. To explore the hypothesis that exochelins function by transferring iron to mycobactins, we examined whether ferri-exochelins of *M. tuberculosis* have the capacity to donate iron to desferri-mycobactins in the cell wall of live bacteria. To enhance mycobactin production, we cultured *M. tuberculosis* under iron-deficient conditions. We then incubated the bacteria with a pure ferri-exochelin or, as positive and negative controls, with ferric ammonium citrate, human transferrin, or medium alone. Finally, we extracted the mycobactins and measured the amount in the ferri form. Mycobactins readily acquired iron from ferri-exochelins. The total amount of ferri-mycobactins recovered after incubating the bacteria with ferri-exochelin was eight times higher than after incubating the bacteria in iron-free medium (Fig. 5). Mycobactins also readily acquired iron from ferric ammonium citrate. Interestingly, however, mycobactins did not acquire iron from 40% iron-saturated transferrin. The amount of iron acquired by the two most abundant mycobactins, mycobactin 880 and 908 (mass of the iron-loaded species 880 and 908 daltons, respectively), parallelled that of the total mycobactin population (Fig. 5). Similar results were obtained using other exochelin species (exochelins 772A and 770C) as the iron donor.

Exochelins of Different Polarity Exchange Iron with Each Other. The experiments described above demonstrating that ferri-exochelins transfer iron to desferri-mycobactins in the bacterial cell wall showed that the relatively polar exochelins can transfer iron to the relatively nonpolar mycobactins. This raised the possibility that the polarity of these molecules influences their iron-binding affinity. To explore the potential influence of polarity on iron-binding affinity, we studied the capacity of two different species of exochelin, one relatively polar and one relatively nonpolar, to exchange iron with each other. Because even the relatively nonpolar exochelins are soluble in water, we could carry out these experiments under conditions in which both of the molecules competing for iron were pure and in aqueous solution. This was not feasible in the studies of iron exchange between the water-soluble exochelins and the water-insoluble mycobactins described in the previous section.

The polarity of exochelins is reflected by their elution pattern off of the reverse-phase HPLC column, with the relatively polar exochelins eluting early and the relatively nonpolar exochelins eluting late in the acetonitrile gradient (Fig. 1). We first studied the capacity of the relatively polar exochelin 758C to compete for iron with the relatively nonpolar exochelin 772C. When we incubated desferri-exochelin 758C with ferri-exochelin 772C, the iron saturation of exochelin 758C increased from 26 to 64%, whereas the iron saturation of exochelin 772C decreased from 100 to 68% (Fig. 6 *A*). In the reverse experiment, when we incubated ferri-exochelin 758C with desferri-exochelin 772C, the final outcome was very similar. The iron saturation of exochelin 758C decreased from 100 to 53%, whereas the iron saturation for exochelin 772C increased from 42 to 58% (Fig. 6 *B*). This experiment was repeated three times with the exochelins cited above and once with exochelins 772A and 772C with similar results.

Discussion

This work demonstrates that purified *M. tuberculosis* desferri-exochelins have the capacity to remove iron from high-affinity host iron-binding molecules. The desferri-exochelins rapidly acquire iron from human transferrin, whether it is saturated with iron to 95 or 40%, its approximate percent saturation in human serum, and from human lactoferrin. In addition, exochelins remove iron, although

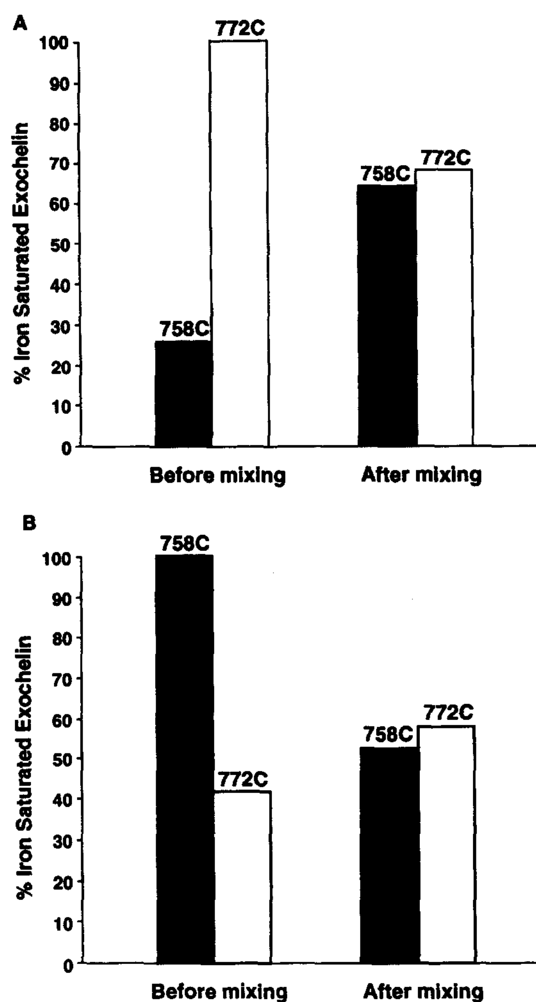

Figure 6. Relatively polar and nonpolar species of exochelins exchange iron with each other. (*A*) Desferri-exochelin 758C was mixed with ferri-exochelin 772C. (*B*) Desferri-exochelin 772C was mixed with ferri-exochelin 758C. Afterwards, the percentage of iron saturation of the two exochelin species was assayed.

less rapidly, from ferritin, the major iron storage protein of mononuclear phagocytes. The biological relevance of this latter observation is uncertain since a phagosomal membrane separates intracellular *M. tuberculosis* from ferritin in the cytoplasm of the host cell. However, some ferritin is found extracellularly, potentially providing *M. tuberculosis* with an additional iron source. In any case, the capacity of desferri-exochelins to remove iron from three different iron binding molecules and ones as structurally disparate as ferritin on the one hand and transferrin and lactoferrin on the other demonstrates the iron scavenging power of these remarkable molecules.

Our study provides evidence to support the hypothesis that *M. tuberculosis* exochelins function by transferring iron to mycobactins, their more lipophilic sister molecules in the cell wall of the organism. According to this hypothesis, the mycobactins in turn transfer the iron to the interior of the bacterial cell (8). As exochelins are both water and lipid soluble, it is not clear why a second siderophore is necessary for iron transport across the mycobacterial cell wall. Possibly, the greater lipophilicity of the mycobactins renders them more efficient iron transporters than exochelins in the unusually lipid-rich environment of the mycobacterial cell wall.

Exochelins differ from each other and from mycobactins in polarity, which is dependent primarily upon the length of the alkyl side chain and whether or not this side chain terminates in a methyl ester or sometimes a carboxylic acid group. The polarity of these molecules greatly influences their water solubility. However, our study shows that polarity has little if any influence on their iron affinity. This finding argues against the possibility that iron is transferred from exochelins to mycobactins along an affinity gradient. Rather, it indicates that exochelins and mycobactins have comparable affinities for iron and that the exchange of iron between them is primarily dependent upon their relative concentrations at the cell surface.

Our finding that exochelins but not transferrin transfer iron to mycobactins in the cell wall of *M. tuberculosis* underscores the importance of exochelins in iron acquisition. It suggests that the water insolubility of mycobactins and their consequent confinement in the mycobacterial cell wall restricts their access to the sites where iron is held by the transferrin molecule. The rapidity with which the water soluble exochelins remove iron from transferrin and lactoferrin suggests that exochelins readily access the sites where iron is bound by these host proteins and remove the iron from them.

Finally, our study demonstrates that exochelins retain their biologic activity after purification and conversion to the desferri form using the methodology described in this and our previous study (6). Hence, exochelins obtained using this methodology are suitable for further studies requiring biologically active material, including studies of their role in *M. tuberculosis* pathogenesis, their potential as therapeutic iron-chelating agents, and the efficacy of new drugs targeted against them.

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