Determination of the structure of exochelin MN, the extracellular siderophore from *Mycobacterium neoaurum*

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Background: Siderophores are compounds produced by bacteria to acquire iron. Exochelin MN, the extracellular siderophore from *Mycobacterium neoaurum*, is of particular interest because it has been shown to transport iron into *M. leprae*, which is responsible for the disease leprosy. Exochelins from other species cannot mediate iron transport in *M. leprae*, suggesting a specific uptake mechanism involving exochelin MN. We set out to determine the structure of exochelin MN and identify the features of the molecule that may account for this specificity.

Results: The structure of exochelin MN was elucidated by a combination of techniques including nuclear magnetic resonance, mass spectrometry, derivatization and gas chromatography. Exochelin MN is a peptide, containing the unusual amino acid β-hydroxyhistidine and an unusual *N*-methyl group. The peptide coordinates iron(III) octahedrally using its two *cis*-hydroxamate groups plus the hydroxyl and imidazole nitrogen of the β-hydroxyhistidine. The three-dimensional structure of the hexadentate exochelin/gallium complex was deduced from NMR data.

Conclusions: Exochelin MN has some structural features in common with other siderophores, but has a unique three-dimensional structure, which is presumably important for its specific activity in *M. leprae*. Exochelin MN may be a target for drug design in the fight against infection with this pathogen.

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Introduction

Mycobacterium neoaurum is a non-pathogenic species of a genus that includes both the tubercle and leprosy bacilli: *M. tuberculosis* and *M. leprae*. These bacteria secrete iron-chelating compounds, called siderophores or exochelins; in many cases, siderophore–iron complexes are recognized by a cell-surface receptor and are taken into the cell by active transport. Acquisition of iron is essential for all living cells, because of its role in the function of cytochromes and of various enzymes.

Our interest in *M. neoaurum* arises from the demonstration that the siderophore produced by this species, exochelin MN, not only efficiently transported iron into its own cells, but also could do so with *M. leprae* [1]. Other exochelin siderophores, from *M. smegmatis*, *M. vaccae* and *M. bovis* Bacillus Calmette–Guerin (BCG), did not mediate iron uptake in *M. leprae*. The structures of the exochelins from *M. smegmatis* and *M. avium* have recently been reported; the former is a formylated pentapeptide, N-(δ-*N*-formyl, δ-*N*-hydroxy-D-ornithinyl)-β-alaninyl-δ-*N*-hydroxy-D-ornithinyl-D-allo-threoninyl-δ-*N*-hydroxy-L-ornithine [2], and the latter is a modified mycobactin in which the usual long alkyl side-chain substituent is a short (carboxy) acyl group [3]. The exochelin of *M. avium*, although present extracellularly, is soluble in chloroform, but the exochelins from *M. smegmatis* and other non-pathogens, like that from *M. neoaurum*, are insoluble in most organic solvents, indicating that solubility differences alone cannot account for the specificity of uptake mediated by exochelin MN. The finding that the pathogen *M. leprae* could acquire iron by using the exochelin of a non-pathogen but not by using the modified mycobactin-type exochelin from another pathogenic mycobacterium, suggested a major difference in the mechanism of iron acquisition between *M. leprae* and the tubercle complex of organisms including *M. bovis*. Unfortunately, the exochelin produced by *M. leprae* cannot be directly identified, as the bacillus cannot be grown in laboratory culture, and must be obtained from either armadillos or infected foot-pads of mice. Under such conditions, it is impossible to recover any siderophore. Information on the structure of exochelin MN is therefore important for understanding the mechanism of iron acquisition in *M. leprae*. We report here the structure of exochelin MN, which is a linear peptide that is similar to the exochelin from *M. smegmatis*, but which contains some unusual features.

Results and discussion

Determination of constituent amino acids

Early results from NMR and amino acid analysis had suggested that exochelin MN was composed of three

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δ-*N*-hydroxyornithines, two β-alanines, a β-hydroxyhistidine and an acetyl group. This composition was found to be inconsistent with the mass of the exochelin, however, which was measured at 683 by electrospray mass spectrometry (MS). Furthermore, ^{13}C-NMR revealed six carbonyl resonances whereas seven would be expected for the composition listed above. To resolve these inconsistencies, additional ^{1}H-NMR studies were undertaken. Two-dimensional double-quantum-filtered correlation spectroscopy (DQFCOSY) [4] and total correlation spectroscopy (TOCSY) [5] spectra of the desferri exochelin were recorded in dimethyl sulphoxide (d_6-DMSO) and H_2O. These revealed the presence of spin systems matching with one β-hydroxyhistidine, two β-alanines and two δ-*N*-hydroxyornithines (Fig. 1). One more ornithine-like spin system was found; for this residue, the δ protons were at lower chemical shift by 0.7 parts per million (ppm). This result, together with the presence of a DQFCOSY cross peak between a broad two-proton resonance at 7.8 ppm and the δ protons, suggested that the residue was ornithine and not a third δ-*N*-hydroxyornithine. It was also noted that the methyl singlet, previously assigned to an acetyl group, was sharp in H_2O but rather broad in d_6-DMSO. This could be explained if the methyl group was coupled to a proton that was in intermediate exchange in d_6-DMSO but in fast exchange in water. This strongly suggested that the singlet was an *N*-methyl group and not an acetyl group, also explaining the missing carbonyl resonance in the ^{13}C-NMR. The α proton on one of the δ-*N*-hydroxyornithines showed similar sharpening and broadening in H_2O and d_6-DMSO, respectively, suggesting that the *N*-methyl group was located there. A cross peak between α and methyl protons in the nuclear Overhauser effect spectroscopy (NOESY) spectrum (see below) of the exochelin confirmed this.

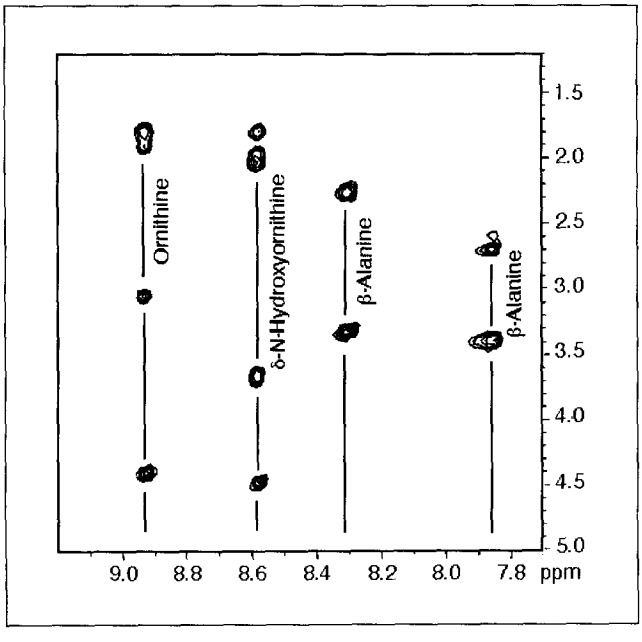

Fig. 1. Part of the TOCSY spectrum of desferri exochelin MN. The spin systems of the two β-alanines and two of the ornithine residues are marked. Note there are no shift differences between diastereotopic protons (see Fig. 3).

Table 1. Results of microderivatization reactions on exochelin MN.

Reaction	Mass	
	Ferriform	Desferri
none	736	683
MeOH/HCl	736	683
Ac_2O/H_2O	862	935
Ac_2O/pyridine	977	977

The mass of desferri and ferriform exochelin were measured by electrospray mass spectrometry after derivatization with the above reagents. Ac_2O, acetic anhydride.

To obtain more information on the functional groups present in the peptide, microderivatization reactions were performed and the products analyzed by mass spectrometry [6–8]. The reactions could be carried out on a nanomole scale, as they produce high yield and involve no tube-to-tube transfers. The results are summarized in Table 1. Treatment of a compound with MeOH/HCl leads to ester formation and hence to the addition of 14 mass units for each carboxylic acid. The mass of exochelin MN was unchanged by such treatment, indicating that no carboxylic acid group was present. Since no blocking group was detected by NMR, we deduced that the peptide must contain a ring.

Reaction with Ac_2O/H_2O results in acetylation of amino groups, adding 42 mass units for every amino group in the molecule. Under these conditions, four acetyl groups were added to ferriform exochelin. For a linear peptide of the composition given above, five acetyl groups would be expected (one for the histidine, one for each of the three ornithines and one for the amino-terminus). The presence of four groups is entirely consistent with a peptide containing a ring, however. When the desferri exochelin was reacted under the same conditions, six acetyl groups were added. Previous work [2] had shown that under these conditions, the *N*-hydroxyl groups of δ-*N*-hydroxyornithine are also acetylated in desferri exochelins. It therefore seemed that there were only two δ-*N*-hydroxyornithines, in agreement with the NMR evidence above.

The amino and hydroxyl groups of a compound are acetylated by reaction with acetic anhydride and pyridine. Under these conditions, seven acetyl groups were added, for both the desferri and ferri form. This is consistent with the presence of one β-hydroxyhistidine. This reaction resulted in a loss of affinity for iron, indicating that the extra three acetyl groups added in the pyridine reaction were blocking iron binding. These acetyl groups were added to the hydroxyls of δ-*N*-hydroxyornithine and β-hydroxyhistidine, suggesting that these hydroxyl groups were involved in iron chelation.

The spectroscopic evidence suggested that exochelin MN is a peptide composed of two δ-*N*-hydroxyornithines, one ornithine, one β-hydroxyhistidine and two β-alanines. With an *N*-methyl group and a ring in the structure, the mass of such a peptide would be 683 — the mass found by electrospray MS. We concluded that the composition we had determined for the peptide was correct, and set out to determine the the order and manner in which the residues are linked.

Determination of sequence

The first pieces of information on the sequence of exochelin MN came from DQFCOSY and TOCSY spectra, which revealed no amide proton for β-hydroxyhistidine, indicating that this was the amino-terminal residue. They also showed no amide proton for one of the δ-*N*-hydroxyornithines, and no protons assignable to the δ-NHs of either δ-*N*-hydroxyornithine. This suggested that both δ-*N*-hydroxyornithines were linked via their side chains, one with an additional link to its α-amino group and the other free at this position. As well as accounting for the observed NMR data, this would provide two hydroxamate linkages, which are common in siderophores and are known to be good bidentate ligands for iron. More information was provided by two-dimensional NOESY [9] and rotating-frame Overhauser effect spectroscopy (ROESY) [10] experiments, which were performed on the desferri exochelin in both H_2O and d_6-DMSO. NOE enhancements indicating close spatial contacts were found between the α proton of β-hydroxyhistidine and the amide proton of one β-alanine. The α protons of this β-alanine showed an NOE to the amide of the other β-alanine, indicating that the first three residues were β-hydroxyhistidine, β-alanine, β-alanine. The α protons of the second β-alanine showed an NOE to the δ protons of a δ-*N*-hydroxyornithine, confirming that this δ-*N*-hydroxyornithine was linked via its side chain. No amide proton was found for this residue, suggesting a free α-amino group. Consistent with this, the chemical shift of this α-proton was notably upfield of those for the other ornithines. The α-proton did, however, show an NOE to the methyl group, indicating that this was in fact an α-*N*-methyl amino group, as predicted by mass spectrometry and NMR (see above). The α-proton also showed an NOE cross peak to the amide proton of ornithine, the α-proton of which gave an NOE to the amide of the final residue, δ-*N*-hydroxyornithine. As this δ-*N*-hydroxyornithine had a side chain linkage, and as the exochelin had no free carboxyl terminus, it was clear that the side chain of this residue was cyclized onto the carboxyl group. The complete covalent structure is shown in Figure 2a.

The sequence shown in Figure 2a was further supported by studying fragmentation of the exochelin by electrospray MS. Electrospray MS normally gives little or no fragmentation, but can be induced to do so by raising the cone voltage on the instrument [11]. The best results were obtained using the acetylated exochelin, for which two series of fragment ions were observed, arising from cleavage of the peptide bonds. Charge retention by an amino-terminal fragment to give an acylium ion explained one series of results (Fig. 2b). The other series could be explained by cleavage of the peptide bond, followed by migration of hydrogen onto the carboxy-terminal fragment, which would also carry an extra proton and would therefore be charged (Fig. 2b). The fragments observed are entirely consistent with the sequence proposed from NMR data (Fig. 2c).

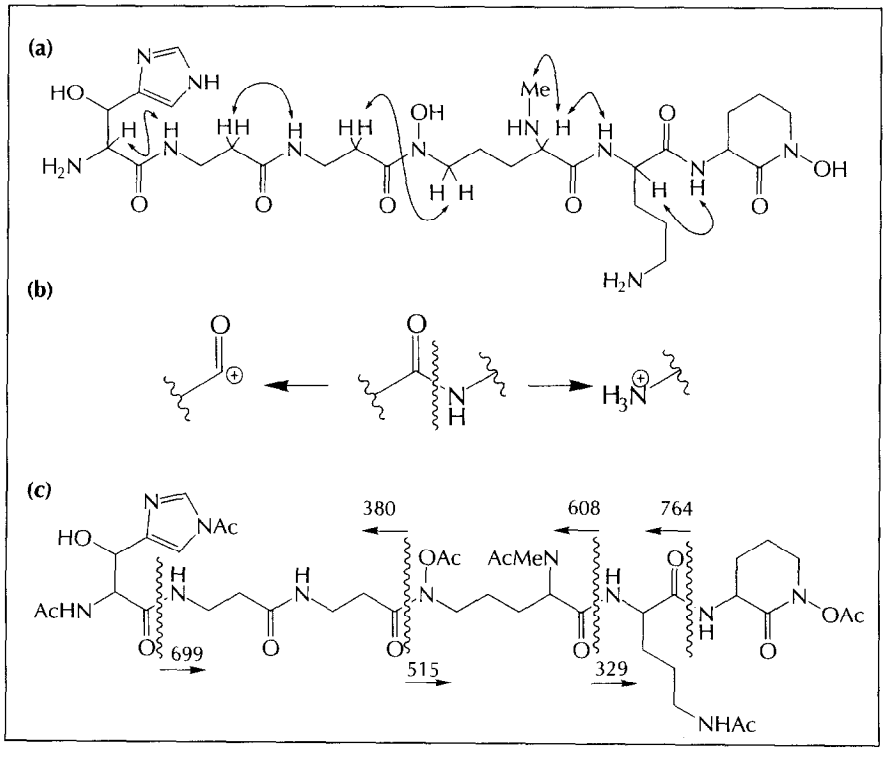

Fig. 2. Structures of exochelin MN and fragment ions. **(a)** Covalent structure of exochelin MN. The arrows show observed NOEs, which allowed the sequence to be deduced. **(b)** Fragment ions that can be formed by cleavage of amide bonds in the mass spectrometer. Left, acylium ion, formed by charge retention by an amino-terminal fragment; right, carboxy-terminal fragment. **(c)** Fragment ions observed by electrospray MS of exochelin MN. These were entirely consistent with the sequence deduced by NMR. Arrows pointing to the right indicate that an ion of the noted molecular mass is formed by the carboxy-terminal fragment when the peptide bond breaks. Arrows pointing left indicate formation of an acylium ion of the noted molecular mass.

Determination of amino acid stereochemistry

The absolute stereochemistry of the five chiral centres in the exochelin remained to be determined. The stereochemistry of the ornithine was determined first, using chiral gas chromatography (GC) and standards of authentic D- and L-ornithine derivatized as the *N*-pentafluoropropionyl methyl esters [12]. The exochelin was hydrolyzed with HCl, and the resulting mixture of amino acids derivatized in the same way. Comparison with the standards showed that the ornithine in exochelin MN was of the L configuration. A similar assay for δ-*N*-hydroxyornithine was impossible because standards were unavailable. The exochelin was therefore instead hydrolyzed using concentrated HI, to convert δ-*N*-hydroxyornithine to ornithine [13]. Only L-ornithine was detected after this procedure, showing that the δ-*N*-hydroxyornithine was also of the L configuration. The α-*N*-methyl-δ-*N*-hydroxyornithine was also shown to be of L stereochemistry by considering the NOE enhancements during the process of determining the three-dimensional structure of the exochelin (see below).

The β-hydroxyhistidine residue contains two chiral centres, so there were four possible isomers to distinguish. The question of whether the erythro or threo diastereomer was present was addressed first. Initial attempts to prepare a volatile histidine derivative suitable for GC failed. However, ozone converts histidine to aspartic acid which does form volatile derivatives [14], and it seemed probable that β-hydroxyhistidine would be converted to β-hydroxyaspartic acid by a similar treatment. A sample containing both diastereomers of β-hydroxyhistidine was treated with ozone and subsequently derivatized as the trifluoroacetyl methyl esters. Two peaks were observed by GC. One of these matched a pure sample of threo β-hydroxyaspartic acid and the other matched a sample of ozonolyzed erythro β-hydroxyhistidine. Exochelin MN was then treated with ozone, hydrolyzed and derivatized as above. Comparison of the GC peak obtained with the standards already run showed that the threo isomer was present. To determine whether this was of L or D absolute stereochemistry, amino acid oxidase enzymes were used. A sample of exochelin MN was ozonolyzed and hydrolyzed and divided into two equal portions that were incubated separately with D- and L-amino-acid oxidase enzymes. The mixtures were derivatized and analyzed as above. The sample incubated with the L-oxidase enzyme gave a much smaller peak, showing that the exochelin contains L-threo-β-hydroxyhistidine. An internal check was provided by the β-alanine which was unaffected by the enzyme. A large decrease (~90 %) in the amount of β-hydroxyhistidine relative to β-alanine was observed after incubation with the L-oxidase.

Determination of three-dimensional structure

We wished to examine the three-dimensional structure of the exochelin MN-iron complex using NMR. Iron-bound exochelins give very poor NMR spectra, however, due to the presence of paramagnetic iron. We therefore examined the spectrum of the complex of exochelin MN with gallium(III) which is of similar size to iron(III) but is not paramagnetic. When gallium sulphate was added to the desferri NMR sample, no changes in the ^{1}H NMR spectrum were at first observed; however, when the pH of the sample was raised above 6, the spectrum abruptly changed, indicating that binding had occurred. This is consistent with binding via the histidine and hydroxamate groups, for which protonation at lower pH would suppress binding of the metal.

The gallium complex of the exochelin was examined by two-dimensional DQFCOSY and TOCSY in water at 278 K, 283 K, 288 K and 308 K. The different temperatures were used to resolve signals that were coincident. These spectra allowed complete assignment of the proton-spin systems. In this respect the TOCSY proved particularly useful, as it directly gave the chemical shifts of all resonances in those residues with amide protons simply by examining the amide–aliphatic cross peak region (Fig. 3). A number of differences were apparent between the spectra of the gallium and desferri exochelins. Perhaps most notably, many of the diastereotopic protons of the two β-alanines and the δ-*N*-hydroxyornithines, which had been equivalent for the desferri exochelin, became non-equivalent for the gallium complex (compare Fig. 1 with Fig. 3). Some of the chemical shift differences involved were large; for example, the β-protons of β-alanine-2, which appeared at 3.38 ppm for the desferri exochelin, appeared at 3.74 ppm and 3.37 ppm for the gallium complex. Strong two-bond couplings were apparent in the DQFCOSY spectrum for these and the other diastereotopic protons (Fig. 4).

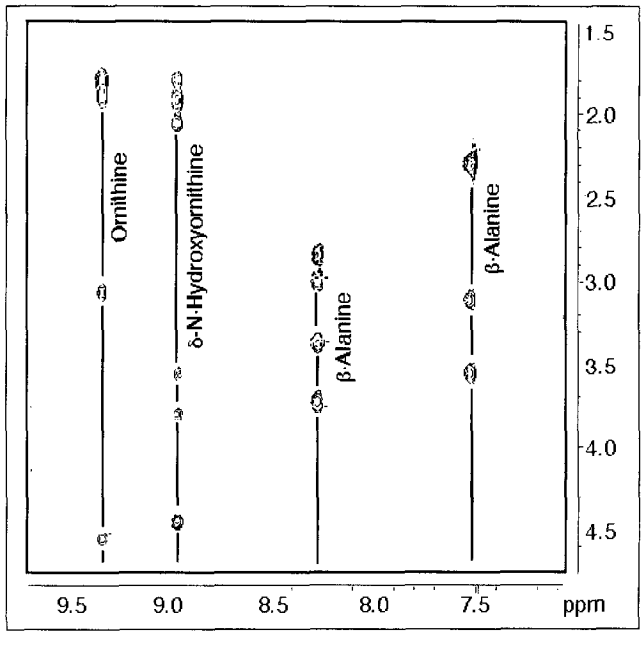

Fig. 3. Part of the TOCSY spectrum of the gallium complex of exochelin MN. The spin systems of the residues with amide protons are marked. Note the shift changes and large diastereotopic shift differences compared to Figure 1.

Many other chemical shift changes were also observed upon gallium complexation. For example, the amide proton of β-alanine-1 shifted upfield by 0.75 ppm, whereas that of β-alanine-2 shifted downfield by 0.44 ppm. Changes were also observed for the β-hydroxyhistidine aromatic protons (8.74 ppm to 7.95 ppm and 7.70 ppm to 7.40 ppm) and β-proton (4.75 ppm to 4.91 ppm).

No peaks that would indicate the presence of some minor conformer were observed; together with the changes in chemical shift and non-equivalence, this result suggested a change from a random-coil desferri peptide to a specific conformation in the gallium complex. NOESY experiments were performed to investigate the details of the three-dimensional structure further (Fig 5). It was anticipated that the molecular weight of the exochelin complex would place it very close to the zero crossing point where NOEs change from positive to negative enhancements. Indeed, whereas the NOEs in the spectrum recorded at 278K were negative, those at 308K were positive. ROESY experiments were also performed with shorter mixing times. From these spectra several NOEs providing spatial constraints were collected. These NOEs were all fairly weak due to the size of the molecule, and therefore were not classified further. They included an NOE enhancement between the histidine C-4 aromatic proton and the α-proton of the terminal δ-*N*-hydroxyornithine — protons at opposite ends of the molecule but close in space in the complex. An NOE enhancement was also observed between the α-proton of cyclo-δ-*N*-hydroxyornithine and its amide, suggesting a *cis* arrangement (Fig. 6). The coupling constant of 7.2 Hz was consistent with this. The amide

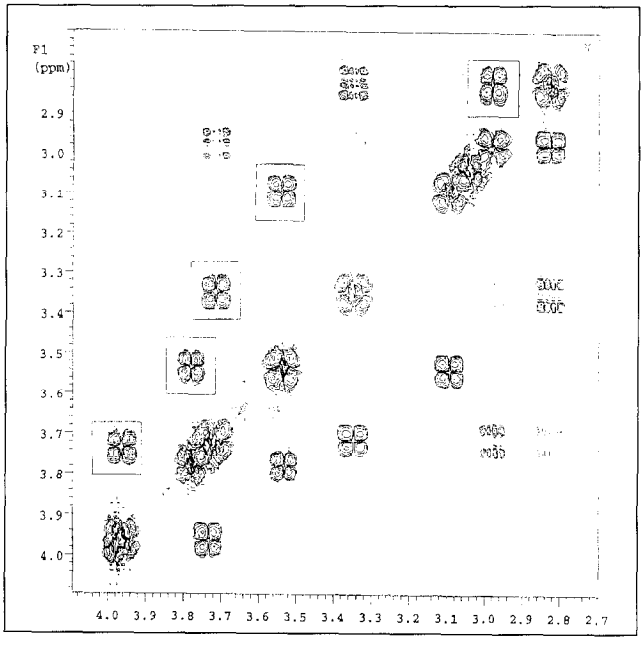

Fig. 4. Expansion of the DQF-COSY spectrum of the gallium complex of exochelin MN. The strong cross peaks marked with boxes arise from the large geminal coupling between non-equivalent protons on the same carbon.

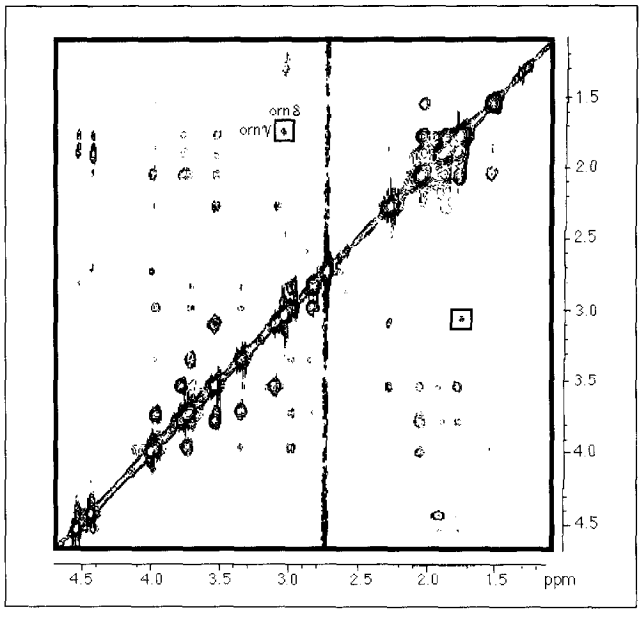

Fig. 5. Expansion of the NOESY spectrum of the gallium complex of exochelin MN at 283 K. The marked cross peak is between δ and γ protons of the ornithine residue. Whereas other NOEs go from negative to positive as the temperature increases, this NOE remains positive, showing that more motion is present in this side chain.

proton also gave an NOE enhancement to the α-proton of the preceding ornithine residue, showing that these atoms are also close in space. The ornithine α-proton did not give an NOE to its own amide, suggesting a *trans*-like conformation. The coupling constant was 6.6 Hz, suggesting a diaxial angle of about 140°.

Similar considerations of NOE data, the requirement for coordination of the metal by the hydroxamates and β-hydroxyhistidine, and the use of Corey–Pauling–Koltun (CPK) models allowed the general, overall folding of the structure to be defined. In total, over 20 informative NOE constraints were used, representing most of the expected close contacts in the molecule. Some of these NOEs are shown in Figure 6. During this process, it also became clear that the α-*N*-methyl-δ-*N*-hydroxyornithine had to be of the L configuration. The D-isomer could not be made to fit the observed NOEs and coordinate the metal ion. Notably, there were no NOEs to suggest a specific conformation in the side chain of the ornithine residue, which was expected to be reasonably free to rotate in solution, as it is neither a part of the peptide framework nor one of the bidentate ligand groups. No diastereotopic shift differences were noted in this side chain, and the NOE enhancement between δ- and γ-protons was positive even at 278 K, again suggesting a greater degree of movement (Fig. 5). The β-alanine region of the peptide gave more NOEs than could be explained by one structure; however, the model suggested a degree of flexibility in this region of the peptide, and it was clear that a simple rocking motion of the amide bond could account for the extra crosspeaks (Fig. 7). The fact that the β-alanine amides appear as

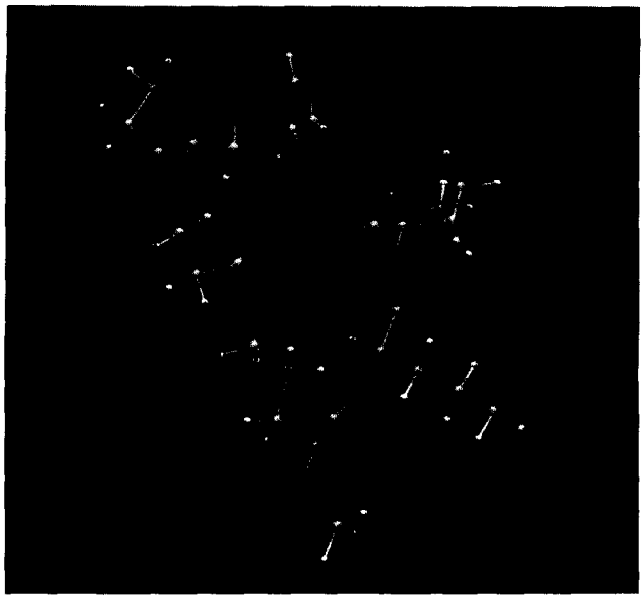

Fig. 6. Three-dimensional structure of exochelin MN deduced from NOE constraints. The dotted green lines show some of the NOEs that defined the overall structure.

broad triplets, which indicates that they have identical coupling constants to both β-protons, also suggests some degree of rotational oscillation.

The structure was entered into the Macromodel [15] molecular modelling package and minimized, then subjected to a 100 ps dynamics run during which the temperature was gradually decreased from 600 K to 0 K. Samples taken during the run overlaid well, except for the anticipated freedom in the ornithine side chain, and the restricted motion in the β-alanine region suggested above. The final structure was energy-minimized again (Fig. 8). No constraints were applied during any of these computational procedures. Thus, although the initial structure was defined from experimental data, it was refined purely by energetics. The final structure still satisfied all of the experimental constraints, however. It is, of course, a static, energy minimum structure; the NMR and dynamics data show that the structure 'breathes', a point that should always be kept in mind when looking at crystal structures of biomolecules and weak interactions between them.

Examining the structure, it is clear that the amide proton of β-alanine-1 lies above the face of the histidine aromatic ring, which could explain the large upfield shift described above. The structure also clearly shows the octahedral coordination of the central metal ion by the two hydroxamates and the β-hydroxyhistidine. Although hydroxamates are extremely common in siderophores, nitrogen ligands are very rare, and L-threo-β-hydroxyhistidine has only been reported once before [16]. The arrangement of the two hydroxamate groups is such that the two *N*-hydroxyl groups are *cis* to each other. The hydroxyl of β-hydroxyhistidine is also *cis* to both of these groups, such that all three hydroxyls are next to each other (in a *fac* arrangement). For all trihydroxamate structures for which the three dimensional structure is known, this same *fac* arrangement is observed such that the central atom has a three fold axis of symmetry [17,18]. The presence of one nitrogen ligand here means that the same symmetry cannot exist, but it is interesting that the arrangement of the ligands follows the same general pattern. Presumably, this reflects the greater underlying stability of this particular geometric isomer.

Significance

The ability to acquire iron is important to all living organisms. Mycobacteria, a group which includes the pathogens *M. leprae* and *M. tuberculosis*, acquire iron using compounds called exochelins, and the ability to acquire iron efficiently has been linked to virulence. Exochelin MN, the extracellular siderophore from *M. neoaurum*, can transport iron into cells of the pathogen *M. leprae*, whereas exochelins from other mycobacteria cannot. This suggests a close similarity of structure between the siderophores of these two organisms, and, together with the fact that it has been impossible to isolate the exochelin from the leprosy bacterium, makes the structure of exochelin MN of great interest.

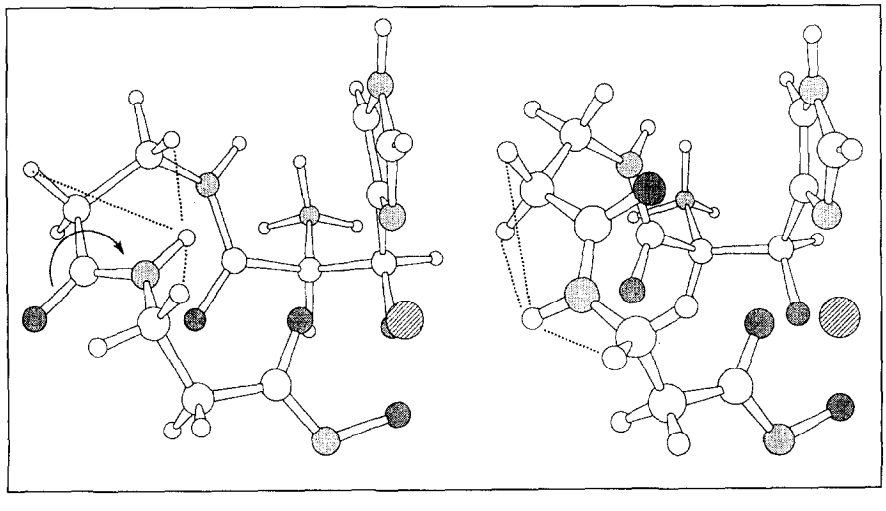

Fig. 7. Two sample structures from a dynamics run on exochelin MN. The β-alanine amide rocks through an angle of ~180° (see arrow). Together the two structures account for the observed NOEs in this region of the molecule.

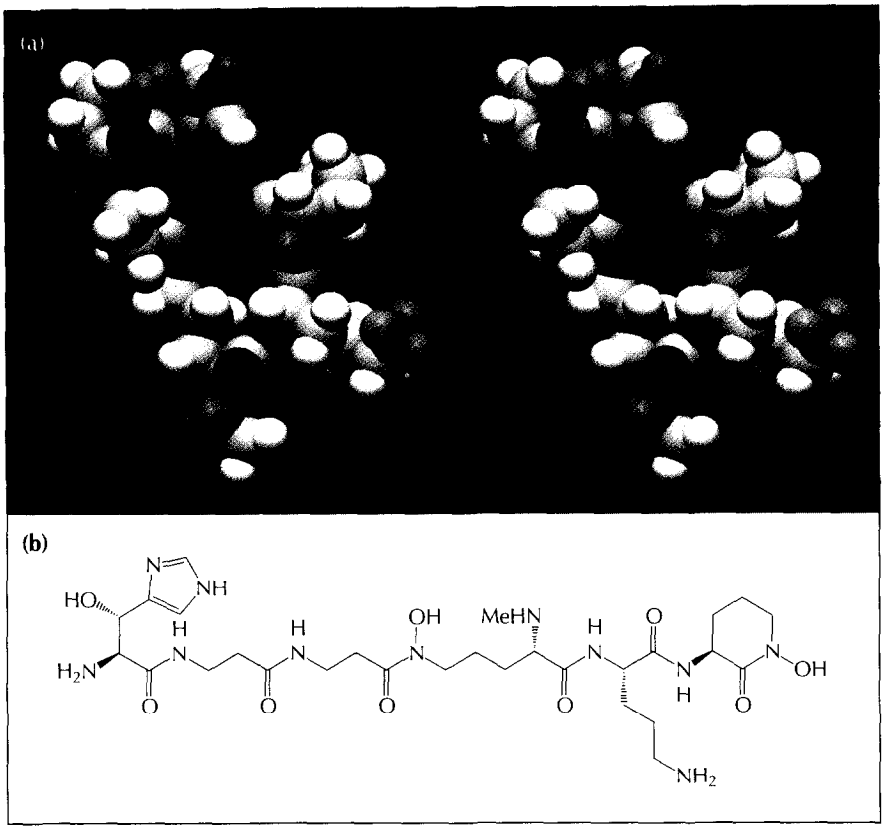

Fig. 8. Structure of exochelin MN. **(a)** Space-filling representation of the three-dimensional structure of exochelin MN. Note how the peptide folds around the central metal ion, co-ordinating it octahedrally. The three hydroxyl groups are all *cis* to each other (at the back in this picture), an apparently stable arrangement for hydroxamate ligands. **(b)** Stereochemistry of exochelin MN.

The exochelin is a hexapeptide which coordinates iron octahedrally. Some aspects of the structure of exochelin MN are common to many siderophores. The presence of two hydroxamate groups, formed from the unusual amino acid δ-*N*-hydroxyornithine and their involvement in the octahedral coordination of iron(III) is found in many siderophores including the exochelin from *M. smegmatis*. The overall structure of the exochelin is unique and contains unusual structural elements, however. The most striking of these is the β-hydroxyhistidine residue, so far found in only one other siderophore. Together, these unusual features could be responsible for the specific molecular recognition of the siderophore in *M. neoaurum* and *M. leprae*, suggesting possibilities for the design of drugs that could be useful for treating leprosy.

Materials and methods

Isolation and purification of exochelin MN

Mycobacterium neoaurum NCTC 10439 was grown on a glucose/asparagine medium under iron-deficient conditions (0.02 μg Fe ml^{-1}) to promote exochelin formation. Cultures (20 x 100 ml) were shaken continuously at 37 °C for 6 days, then centrifuged to remove cells. The supernatant solution was adjusted to pH 7.0, $FeCl_3$ solution was added dropwise until a precipitate began to form and the solution was stirred for a further 1 h. The solution was centrifuged (5000 x g for 15 min at 4 °C) and the clear orange liquid freeze-dried. The residue was dissolved in 50 to 60 ml distilled water and passed through an ultrafiltration membrane with a cut-off of 5 kDa using a stirred ultrafiltration cell (Amicon Corp., U.S.A.). The orange-red filtrate was evaporated under reduced pressure at 30 °C to ~2–3 ml and passed through a gel-permeation column (Sephadex G-10; 60 cm x 3 cm) to remove residual salts. The conductivity of the eluate was measured to determine the point of salt elution and the exochelin was collected before this occurred. The exochelin fraction (one peak) was collected and evaporated as before to ~2–3 ml, then fractionated using a high-resolution cation exchange resin (Bio-Rad AG 50W-X4, 200400 mesh, NH_4^+-form, 40 x 2.5 cm) eluting with a linear gradient of 0.1 M NH_4OH/acetic acid, pH 6.0, to 2.0 M NH_4OH/acetic acid, pH 9.0 at a flow rate of 1 ml min^{-1} and the eluate monitored at 430 nm. A minor peak (~5 % total) of coloured material eluted after ~650–700 ml with the main exochelin emerging between 960 ml to 1150 ml. The fractions containing the exochelin were pooled, and the pH adjusted to 7.0 with 1 M HCl; the pool was evaporated to ~2–3 ml and de-salted by passage through a Sephadex G-10 column as before. The final exochelin solution was evaporated to ~1 ml and finally purified in lots of 0.2 ml by semi-preparative HPLC (Lichrosorb RP8, 10 μ, 250 x 10 mm) using a linear gradient of 0.1 % aq. trifluoroacetic acid (TFA) to 0.1 % TFA in methanol/water (10:90 v/v) at 1 ml min^{-1} over 30 min. A major peak of exochelin (>90 % of total eluate monitored at 220 nm) eluted between 12 and 20 min. A sample of the final material was re-run under similar conditions on an analytical column and no impurities were detected. The overall yield of exochelin was 5–10 mg per litre of culture.

Preparation of desferri exochelin MN

The procedure used was similar to that described previously [2] to prepare desferri exochelin from *M. smegmatis*, except that desferri mycobactin was used followed by extraction into chloroform. Care was taken to use iron-free water and glassware. The final aqueous solution was evaporated to ~1 ml and freeze-dried.

NMR spectroscopy

NMR experiments were performed on desferri and gallium exochelins in H_2O/D_2O (9:1) and d_6-DMSO (desferri).

Samples were all of ~5 mM. All experiments were performed on either a Varian Unity Plus 500 or Bruker DRX500. One-dimensional spectra were typically recorded with 16K data points, zero-filled to 32K. For two-dimensional experiments (DQFCOSY, NOESY, ROESY and TOCSY), 2K data points were collected in F2, using 512 increments in F1. 16 or 32 transients were acquired for each value of t_1. Data was collected in phase sensitive mode using either TPPI (Bruker) or States-Hackerborn (Varian) to achieve quadrature detection in F1. The data was zero-filled in F1 prior to transformation using a shifted sine bell-squared weighting function in both dimensions.

For the experiments performed in water, the intense solvent resonance was suppressed by presaturation or WATERGATE. TOCSY and ROESY spectra were acquired using spin locking fields of 5 kHz and 2 kHz respectively. NOESY experiments employed a mixing time of 400 ms; for ROESY the mixing time was 100 ms.

The gallium complex was prepared by adding approximately two equivalents of gallium sulphate to the desferri exochelin NMR sample dissolved in water. The pH was raised using NaOH until the spectrum showed a significant change (pH 6), which was taken as indicating that binding had occurred. The sample was removed from the NMR tube and spun down between additions of alkali when solid material precipitated out.

Microderivatization reactions and mass spectrometry: Esterification

0.1 M HCl in anhydrous methanol was prepared by blowing a gentle stream of HCl gas over the alcohol until the appropriate gain in mass was achieved. This solution (50 μl) was added to ~25 nmol of exochelin in a 0.3 ml vial. The mixture was left to stand for 24 h, after which excess reagents were removed under vacuum and the residue dissolved in 50:50 acetonitrile:water to give a 50 μM solution for analysis by electrospray MS.

Acetylation I

A 50:50 mixture of acetic anhydride and pH 8.4 aqueous ammonium acetate (50 μl) was added to a small sample (25 nmol) of the exochelin and the mixture sonicated for 2 h at room temperature. Excess reagents were removed under vacuum and a solution of the residue prepared as above for analysis by electrospray MS.

Acetylation II

A 2:1 mixture of pyridine and acetic anhydride (50 μl) was added to small samples of the exochelin. The mixture was left at room temperature for 4 h; excess reagents were then removed under vacuum and the residue analyzed by electrospray MS.

Electrospray mass spectrometry

All experiments were performed on VG BioQ instrument in positive ion mode using a source temperature of 70 °C and a capillary potential of 4 kV. The mobile phase used was 50:50 acetonitrile:water flowing at 4 μl min^{-1}. Typically 10 μl of 50 μM solution was injected, data was collected over 20 scans, smoothed and peak top mass assignment was carried out. Calibration was achieved using polyethylene glycol. For the standard experiments a B1 voltage of 65 V was used, but this was raised to either 100 V or 130 V to induce fragmentation.

GC analysis of amino acid stereochemistry: ornithine

Concentrated HCl or HI (100 μl) was added to a small sample (100 nmol) of the exochelin and the mixture heated at 100 °C for 24 h. Excess reagents were removed under vacuum. 5 M HCl in methanol was prepared by adding 0.5 ml of acetyl chloride to 1.5 ml anhydrous methanol with stirring and 50 μl of this reagent was added to the hydrolyzed exochelin. The mixture was heated at 90 °C for 30 min, after which excess reagents were again removed under vacuum. Pentafluoropropionic anhydride (50 μl) was then added and the mixture left to stand for 30 min. Excess reagents were removed under a gentle stream of argon. Dichloromethane (50 μl) was added and the resulting solution analyzed by GC using a Carlo Erba 4130 instrument fitted with a Chiralsil-Val capillary column (0.2 mm x 50 m). Helium was used as carrier gas with the oven temperature fixed at 175 °C; both injector and detector were set to 250 °C. 0.1 μl of the derivative was injected (no splitting), and the stereochemistry deduced by comparison with standard samples of D and L ornithine derivatized in the same way.

Determination of β-hydroxyhistidine stereochemistry

Ozone was bubbled through a small sample (1 μmol) of the exochelin, dissolved in 100 μl of water, for about 15 min. The water was removed under vacuum and 100 μl of 6 M HCl was added. The mixture was then heated at 100 °C for 6 h. After removal of the HCl under vacuum, 50 μl of 5 M HCl in methanol was added and the mixture was heated for 30 min at 100 °C. Again excess reagents were removed under vacuum. A 1:1 mixture of trifluoroacetic anhydride and dichloromethane (50 μl) was then added and the mixture was heated at 60 °C for 30 min, after which the derivative was dried under a stream of argon and dissolved in 50 μl of dichloromethane. Standards were prepared as above from pure erythro-β-hydroxyhistidine and from a mixture of the erythro and threo isomers (samples provided by Professor Sidney Hecht — the pure threo isomer was unavailable). A standard of threo-β-hydroxyaspartic acid (Aldrich) was also prepared by reacting with methanol:HCl and trifluoroacetic anhydride. The standards were analyzed by GC using the above instrument fitted with a BP5 column held at 110 °C. The erythro and threo peaks were well resolved, and the threo peak matched with that of the threo β-hydroxyaspartic acid, showing ozone conversion had been successful. The stereochemistry of the derivative from the exochelin was deduced to be threo by comparison to these standards.

A further sample (2 μmol) of the exochelin was ozonolyzed and hydrolyzed as above. It was then dissolved in 100 μl of pH 8.0 phosphate buffer. 45 μl of this solution was added to 50 ml of a solution containing catalase (bovine, 0.3 mg ml^{-1}) and D-amino acid oxidase (from pig kidney, 0.2 mg ml^{-1}) in the same buffer. The second 45-μl aliquot was added to a solution containing L-amino acid oxidase (from *Crotalus adamanteus*, 0.2 mg ml^{-1}). Both solutions were incubated for 7 days at 35 °C, then derivatized as the trifluoroacetyl methyl esters and analyzed by GC as above.

Molecular modelling

All modelling was performed using Macromodel on a Silicon Graphics Iris Indigo R4000 computer. The AMBER force field was used throughout with water solvent modelling turned on. Parameters for iron(III) were created by changing the charge and radius of Ba^{2+} to +3 and 0.69 Å respectively. Parameters for $N–O^-$ stretch were approximated as being equal to those of N–OH. Since the only forces between ligand and metal were electrostatic, it was necessary to average the default partial charges for the six atoms involved in chelation. This resulted in good octahedral geometry on minimization. All minimizations were run to convergence (typically < 1000 iterations) using

PRCG. For the molecular dynamics, an initial bath temperature of 600 K was used, decreasing to 0 K at the end of the 100 ps run. The SHAKE algorithm was used and samples were taken every 5 ps.

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