Metal Complexes of Mycobactin P and of Desferrisideramines

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Crystalline gallium mycobactin P and chromic mycobactin P have been prepared. The chromic compound, unlike other metallic complexes of mycobactin P, does not detectably exchange its metal with ferric iron; it competitively antagonizes the growth-promoting action of mycobactin P towards *Mycobacterium johnei*. Mycobactin P, desferrioxamine B and desferrichrysin form coloured 1:1 complexes with ammonium vanadate. The vanadyl complexes of the water-soluble desferrisideramines are formed in aqueous solution. Two distinct forms occur at pH 7 and pH 3; these are slowly interconvertible when the pH is changed. The complexes show other changes at lower pH values; unlike other metallic desferrisideramine complexes, the vanadyl compounds do not dissociate even in the strongest acids, but dissociate above pH 9. Their properties have been studied spectrophotometrically, by electrophoresis and by electrometric titration. The affinity of mycobactin for ferric iron is greater than that of desferrioxamine B under two different conditions of measurement.

Sideramines are naturally occurring compounds having structures in which each molecule has a ferric iron atom firmly bound in hexadentate chelation by three hydroxamic acid groups (Bickel *et al.* 1960b). Two groups of sideramines have been chemically characterized so far; these are the ferrioxamines from streptomycetes (Bickel *et al.* 1960a) and compounds related to ferrichrome from the fungi (Zähner, Keller-Schierlein, Hütter, Hess-Leisinger & Deér, 1963). Very low concentrations of these compounds promote the growth of organisms such as *Pilobolus kleinii* or *Arthrobacter terregens* (Neilands, 1953, 1957; Bickel *et al.* 1960a,b). The mycobactins also form stable chelates with ferric iron, but with two hydroxamic acid groups and a third iron-binding centre of a different kind (Snow, 1965). They act as specific growth factors for *Mycobacterium johnei* at concentrations of 5–30 ng./ml. and in a less specific way they can replace the sideramines as growth promoters for the organisms mentioned above. There is therefore some reason for classifying them with the sideramines. Our present results show that under the experimental conditions chosen mycobactin P (I) binds ferric iron even more firmly than does desferrioxamine B (II).

The biological function of the sideramines is still in doubt. The subject has been reviewed by Nüesch & Knüsel (1967) who concluded that they probably act as iron donors in bacterial cells, but in

a relatively non-specific way. They suggest other possible functions, but these remain largely speculative. The problem of the involvement of iron is not easy to approach. Burnham (1962, 1963), working with the sideramine-requiring organism *Arthrobacter* JG-9, showed that ferrichrome (III) stimulated the production of catalase. He also demonstrated the ability of ferrichrome to cause the production of haemin from protoporphyrin IX in the presence of a cell-free extract of *Rhodospseudomonas spheroides*. The haemin produced was measured by a nitrate reductase method and the transfer of iron from ferrichrome to haemin was shown by the use of ^{59}Fe . Unfortunately these experiments are not conclusive because ferrichrome and other sideramines are able to exchange their iron fairly rapidly with unbound iron and with iron chelated to other compounds such as citrate. There is thus no certainty that ferrichrome is a direct iron donor. In an attempt to circumvent this difficulty a search has been made for some other metal or metallic radical that would form a complex with the sideramines having a stability constant high enough to provide effective competition with ferric iron. An acceptable alternative would be a complex, possibly of lower stability, in which the metal exchanges slowly enough to avoid substantial displacement by iron during the course of the experiment. Anderegg, L'Eplattenier & Schwarzenbach (1963) have studied the binding of

Fig. 1. Absorption spectra of metal complexes of mycobactin P. (A) —, Vanadyl complex in ethanol; (B) and (C) ---, chromic complex in ethanol; (D) . . . , gallium complex in methanol. The left-hand ordinate refers to curves A, B and D; the right-hand ordinate to curve C.

the top of the column. The eluate was evaporated. The green residue (67 mg.) was suspended in boiling cyclohexane and brought into solution by dropwise addition of 2-methylpropan-2-ol. The cooled solution gave *chromic mycobactin P* as green hexagonal leaflets (35 mg.), m.p. 225° (decomp.) λ_{max} . 260, 368 and 575 nm. (ϵ 16200, 3570 and 73 respectively); the absorption spectrum is shown in Fig. 1. (Found: C, 61.2; H, 7.75; N, 7.43; Cr, 5.75. $\text{C}_{47}\text{H}_{72}\text{N}_5\text{O}_{10}\text{Cr}$ requires C, 61.4; H, 7.90; N, 7.62; Cr, 5.66%.) T.l.c. (conditions given by White & Snow, 1969) gave a green spot, R_F 0.48 (cf. ferric mycobactin P, R_F 0.52).

Chromic mycobactin P (18 mg.) dissolved in chloroform (2 ml.) was extracted with conc. HCl (3 $\times$ 2 ml.); the faintly yellowish lower layer was washed with water until free from acid, dried over Na_2SO_4 and evaporated. The u.v. spectrum of the residue (15 mg.) dissolved in methanol was identical with that of mycobactin P.

Chromic mycobactin P (2.85 μmoles) and ferric chloride (4.3 μmoles) were dissolved in ethanol (10 ml.). Extinction values were measured at intervals afterwards. The E_{379} declined from 0.600 to 0.542 in 1 day and to 0.420 in 15 days. The E_{525} increased from 0.020 to 0.135 in 15 days. No sign of a peak at 450 nm. was observed over this period. A control in which mycobactin P replaced the chromic complex showed an unchanged peak at 450 nm. throughout this period.

Effect of chromic mycobactin P on the growth of mycobacteria. The tests on *M. johnei* followed method 2 described by Wheeler & Snow (1966). Mycobactin P was added to the medium as a solution in propylene glycol and chromic mycobactin P as a solution in ethanol. Similar additions of solvents were made to all tubes. Chromic mycobactin P produced no growth of *M. johnei* on its own; it antagonized the growth-promoting action of mycobactin P (Table 1). In tests against other mycobacteria four different media

were used: d'Arcy Hart medium (as used in the *M. johnei* experiments) with or without added ferric citrate (1 $\mu\text{g.}$ of Fe/ml.) and medium I (White & Snow, 1968) with or without the same addition of iron. Chromic mycobactin P (5 $\mu\text{g./ml.}$) was added to each of these media in ethanolic solution; controls received the solvent only. Each medium was inoculated with *M. phlei*, *M. smegmatis*, *M. marinum*, *M. terrae*, *M. thermoresistibile* (strains as in White & Snow, 1968, 1969) and *M. tuberculosis* strain B.C.G.; incubation was at the temperature previously found appropriate to the organism. Growth was measured nephelometrically after 3–7 days. In no case was growth in the presence of chromic mycobactin P significantly less than in its absence.

Aluminium desferrioxamine B. Desferrioxamine B methanesulphonate (102 mg.), and anhydrous AlCl_3 (21 mg.) were dissolved in ethanol (7 ml.). After 2 days the solution was evaporated under reduced pressure. The residue was dissolved in water (3 ml.) and the solution was saturated with NaCl. It was extracted with benzyl alcohol (3 $\times$ 2 ml.); the extract was washed with saturated NaCl and clarified. It was diluted with ether (15 ml.) and extracted with water (3 $\times$ 1.5 ml.). The aqueous extract was washed with ether and evaporated under reduced pressure. The residue was dissolved in ethanol, leaving some undissolved NaCl. Evaporation, solution in ethanol and removal of salt were repeated several times. The product was finally dissolved in water (2–3 ml.) and freeze-dried. The almost colourless product (65 mg.) still contained a little NaCl. Its absorption spectrum differed from that of desferrioxamine B in showing an inflexion at 215 nm. and much higher extinction values between 230 and 250 nm. T.l.c. on cellulose (solvent butan-1-ol–propan-1-ol–water, 9:6:5 by vol.) showed aluminium desferrioxamine B, R_F 0.53; ferrioxamine B, R_F 0.57; desferrioxamine B, R_F 0.79.

Repeated recycling of vanadyl desferrioxamine B complexes. Vanadyl desferrioxamine B (23 μmoles) was prepared in 5 mm-phosphate buffer, pH 7.0 (100 ml.); E_{318} was measured and m-HCl (0.5 ml.) was added, bringing the pH to 3.0. The E_{470} was measured at intervals; it reached a maximum in 15 min. m-NaOH (0.5 ml.) was added to restore the pH to 7.0. Ten alternate additions of HCl and NaOH were made with an interval of 20 min. between each addition to allow for equilibration; pH values, checked after the two final additions, were 2.95 and 6.95. The E_{318} values at pH 7 were: initial 0.570; final, corrected for dilution, 0.569; corresponding E_{470} values at pH 3 were 0.654, 0.664.

RESULTS

Complexes of mycobactin P. Mycobactin P reacted with gallium chloride in ethanol to give a crystalline gallium complex closely resembling aluminium mycobactin P in absorption spectrum (Fig. 1) and in other physical properties. Purple-brown complexes of mycobactin P were formed with a number of vanadium compounds, but most readily with vanadates. The characteristic absorption spectrum of the vanadate complex showed five peaks or inflexions above 240 nm. (Fig. 1). The complex was soluble in chloroform and, unlike complexes previously studied, it could not be dissociated by shaking the chloroform solution with

Table 1. *Antagonism by chromic mycobactin P of the growth-promoting effect of mycobactin P towards M. johnei*

Results are given for three separate experiments and show increases in turbidity of *M. johnei* cultures (nephelometer readings) after 12 days (Expts. *a* and *c*) or 14 days (Expt. *b*). Each value is the mean of three replicates.

Chromic mycobactin P ($\mu\text{g./ml.}$)	Mycobactin P ($\mu\text{g./ml.}$) ...	Expt.	Increase in turbidity					
			0	0.01	0.03	0.1	0.3	1.0
0		<i>a</i>	0	46	45	44	46	47
		<i>b</i>	0	41	45	51	50	52
		<i>c</i>			45	47	49	47
0.5		<i>a</i>		34	38	39	39	37
		<i>b</i>		4	13	32	33	
1		<i>a</i>		12	20	34	38	37
		<i>b</i>		1	5	30	39	
		<i>c</i>			9	28	32	32
5		<i>a</i>		1	0	2	7	33
		<i>b</i>		4	3	6	18	

mineral acid; under these conditions the colour of the complex became bluer, but returned to the original hue when the chloroform solution was washed with water. Reaction of mycobactin P with chromic chloride in ethanol occurred slowly when the solution was warmed and was accompanied by some decomposition. The complex purified by chromatography gave green crystals with the absorption spectrum shown in Fig. 1. In chloroform solution chromic mycobactin P was not dissociated by shaking with 5 M-hydrochloric acid but was dissociated by the concentrated acid; the release of mycobactin was confirmed spectroscopically.

The copper, aluminium, gallium and vanadyl complexes of mycobactin P in chloroform or ethanol solution were immediately converted into ferric mycobactin P on addition of ferric chloride. However, chromic mycobactin P showed no spectroscopic evidence of conversion into ferric mycobactin P. Treatment of an ethanolic solution with excess of ferric chloride for several days caused a slow decline in the peak at 379 nm. accompanied by the rise of a low peak at 520 nm. but with no sign of a peak at 450 nm. characteristic of the ferric complex.

Chromic mycobactin P did not promote the growth of *M. johnei* but antagonized the growth-promoting action of mycobactin P (Table 1). The results varied somewhat from one experiment to another, but all showed a competitive effect, higher concentrations of mycobactin P requiring higher concentrations of the chromic complex for antagonism. The presence of the chromic complex also caused some depression of growth unrelieved by mycobactin P at all the concentrations tested. Chromic mycobactin P was tested for the inhibition of growth of six other species of mycobacteria in two

different media, with and without added ferric salts. At 5 $\mu\text{g./ml.}$ (saturation concentration in aqueous media) it did not depress the growth of any.

Complexes of water-soluble sideramines. Most experiments were concerned with desferrioxamine B, but wherever parallel tests were made with desferrichrysin the results were similar. Reaction of desferrioxamine B with aluminium chloride in ethanol gave the aluminium complex, characterized by its behaviour on t.l.c. and by its absorption spectrum.

Like mycobactin P, desferrioxamine gave coloured complexes with vanadium compounds. Although some colour was formed with V^{III} and V^{IV} compounds, reaction was most rapid and complete with vanadate. Two distinct 1:1 complexes were formed, according to the pH of the reaction, pale yellow at pH 7 and brown-purple at pH 3. Reaction with vanadate at pH 7 was very rapid but reaction at pH 3 was appreciably slower. Absorption spectra of the vanadyl complexes of desferrioxamine B were measured at various pH values. A selection of these results is shown in Fig. 2. At pH 7 the first absorption maximum was 318 nm. As the pH was lowered this maximum declined and at pH 3 it was entirely replaced by a maximum of 470 nm. Further lowering of pH caused an increase in this long-wavelength extinction with a shift in the position of the maximum towards longer wavelengths, reaching 540 nm. in concentrated hydrochloric acid. Vanadyl desferrioxamine B did not dissociate in this acid or in 9 M-perchloric acid, but gave deep ink-blue solutions. The extinction of these solutions declined fairly quickly, probably owing to hydrolysis of the ligand. At acid concentrations between 0.1 M and M another maximum at 335 nm. was observed. In the

alkaline range the absorption spectrum was unchanged from pH 6.8 to pH 8.8, but at higher pH values dissociation began and at pH 11 the absorption curve was identical with the sum of the absorptions of desferrioxamine B and ammonium vanadate measured in the same buffer. All the changes caused by varying pH, including the dissociation in alkaline solution, were reversible, but the conversions were not instantaneous. The most clearcut change in the properties of the vanadyl complex occurred between pH 6 and pH 3. An equimolar solution of desferrioxamine B methanesulphonate and ammonium vanadate in water gave

Fig. 2. Absorption spectra of vanadyl desferrioxamine B at various pH values. Solutions (25 ml.) contained desferrioxamine methanesulphonate (5 μ moles) and NH_4VO_3 (5 μ moles) and the appropriate buffer or acid. Absorption spectra were plotted 30 min. after mixing. — pH 8.01;, pH 4.73; -.-.-, pH 2.12; ----, 0.4 M-HCl.

the yellow complex with λ_{max} 318 nm., the pH being about 7.0. Electrometric titration with acid, allowing time for equilibration, gave a typical titration curve for the salt of a monobasic acid having a $\text{p}K$ of 5.1. Measurements of E_{470} plotted against pH showed parallel changes to the pH-titration curve.

The interconversion of the two major forms of vanadyl desferrioxamine B, repeated ten times by alternate addition of acid or alkali to a buffered solution, caused no change in their extinction values. The response of vanadyl desferriochrysin to changes in pH was similar.

The properties of vanadyl desferrioxamine complexes at pH 8.5 and pH 2.0 were also studied by paper chromatography, the corresponding ferric complexes being used for reference purposes. Results are shown in Table 2. At pH 2.0 the vanadyl complexes behaved electrophoretically like the corresponding ferric complexes; those from desferriochrysin did not move significantly, whereas those from desferrioxamine B moved towards the cathode. At pH 8.5 the ferric complexes behaved electrophoretically in the same way as at pH 2.0 except that both showed more movement towards the cathode, probably due to endosmosis. However, the migration of the vanadyl complexes indicated that they carried an extra negative charge; thus vanadyl desferrioxamine B showed only the small movement towards the cathode seen with ferriochrysin whereas vanadyl desferriochrysin moved towards the anode. When vanadyl desferrioxamine B was submitted to electrophoresis at pH 10.5 it gave two spots having migration and

Table 2. Paper electrophoresis of vanadyl and ferric complexes of desferrioxamine B and desferriochrysin

A sample (100 μg .) of each complex was spotted on to the centre of a sheet of Whatman 3MM paper moistened with buffer. (a) Electrophoresis at 50 v/cm. for 45 min. Buffers: 0.02 M-veronal, pH 8.5, or M-acetic acid-formic acid, 0.75 M-, pH 2.0. Distances of migration (in cm.) are given, and signs indicate migration towards the positive or negative pole. (b) Electrophoresis at pH 10.5 in 0.04 M-carbonate-bicarbonate buffer. Desferrioxamine was revealed by a vanadic acid spray. Vanadate was shown by its u.v. absorption at 253 nm. or by spraying with acidified desferrioxamine. The values are the distances of migration (in cm.) in 25 min. at 50 v/cm.

(a)	Desferrioxamine B		Desferriochrysin	
	Vanadyl complex	Ferric complex	Vanadyl complex	Ferric complex
pH				
2.0	-7.4	-7.3	-0.4	-0.5
8.5	-2.2	-10.7	+7.2	-2.3

(b)	Spots		
	Coloured	Reacting as desferrioxamine	Reacting as vanadate
Compound applied			
Ferrioxamine B	-1.0		
Desferrioxamine B		+3.0	
Ammonium vanadate			+11.3
Vanadyl desferrioxamine B		+3.1	+11.0

Table 3. *The competitive binding of ferric iron to mycobactin P and desferrioxamine B*

Solutions contained 20 μ moles of ferric chloride with 40 μ moles of mycobactin P (*m*), 40 μ moles of desferrioxamine B methanesulphonate (*d*), or 40 μ moles of both (*md*) in 4.5 ml. of ethanol. After 4 hr., 1 ml. of each solution was shaken with 20 ml. of chloroform and 20 ml. of 0.05M-phosphate buffer, pH 7.0. The layers were separated and clarified and *E* values measured. A repeat after reaction for 24 hr. gave the same results.

		<i>m</i>	<i>d</i>	<i>md</i>	Fraction of iron in <i>md</i> as ferrioxamine B
Upper layers	Solution ...	5	1	5	
	Cell length (cm.)	0.000	0.606	0.438	0.144
	<i>E</i> ₄₂₅				
Lower layers	Cell length (cm.)	1	5	1	Fraction of iron in <i>md</i> as ferric mycobactin P
	<i>E</i> ₄₅₀	0.912	0.000	0.783	0.859

reaction characteristics of desferrioxamine B and vanadate. At the same pH ferrioxamine B remained undissociated and did not migrate.

At low pH values a competition between ferric and vanadyl ions for chelation with desferrioxamine B could be demonstrated. The extinctions of the complexes at 600 nm. differed sufficiently for the ratio of their concentrations to be determined in a mixture. The extent of binding in the vanadyl form showed a linear relationship to the hydrogen ion concentration over the range examined, about 50% of the ligand being bound in this form at pH 1.0.

Comparison between the binding of ferric iron to mycobactin P and to desferrioxamine B. Ferric chloride solution was allowed to react in ethanol with 2 molar equivalents of mycobactin P and with 2 molar equivalents of desferrioxamine B methanesulphonate added separately or together. After a suitable equilibration period (3 hr.) the solutions were mixed with an excess of buffer, pH 7.0, and shaken with the same volume of chloroform. Extinction measurements were made on both layers. The amounts of ferric mycobactin P passing into the aqueous layer and of desferrioxamine B into the chloroform layer were immeasurably small. Thus with the mixed ligands the amounts of ferric mycobactin P and ferrioxamine B present could be directly determined (Table 3). The equilibration of iron between the complexes was also demonstrated similarly by using equimolar mixtures of ferric mycobactin P and desferrioxamine B, or mycobactin P and ferrioxamine B, in methanolic solution. Both gave the same proportion of iron complexes at equilibrium. In another experiment mycobactin P or its ferric complex dissolved in chloroform was shaken with ferrioxamine B or its desferri form respectively, dissolved in buffer at pH 7.0. Under these conditions the very slow equilibration was incomplete after one month's shaking, but the tendency was strongly towards preferential binding of the iron to mycobactin P; the fraction of the iron bound to mycobactin P was between 0.93 and 0.98.

DISCUSSION

Investigation of the ability of sideramines and mycobactins to form metal complexes has confirmed the uniqueness of the affinity of these chelating agents for ferric iron. The salts of a few trivalent metals readily form complexes in ethanolic solution. Ready chelation appears to be confined to elements of relatively low atomic number; thus indium fails to give complexes under conditions at which those of aluminium and gallium are easily formed. The latter elements are readily displaced from their complexes by ferric iron in aqueous or ethanolic solutions, so these complexes are unsuitable for competitive experiments.

The chromic complex of mycobactin P is formed less readily than those of aluminium or gallium, and its metal is much less easily displaced by acid. It is specially notable because the chromium is resistant to displacement by ferric iron; this is probably due to a slow reaction rate rather than to an exceptionally high stability constant. It is the only metallic mycobactin complex yet found that possesses this property. Chromic mycobactin P antagonizes the growth-promoting action of mycobactin P towards *M. johnei*, the effect being apparently competitive. Since the chromic complex is unlikely to dissociate, its competition with ferric mycobactin P implies interaction of the whole metal complex with some important site in the cell. This opens up a new possibility of studying the action of these iron-chelating compounds uncomplicated by the difficulties caused by the rapid exchangeability of the iron. In spite of its well-marked effect on *M. johnei*, chromic mycobactin P showed no action on the growth of other mycobacteria capable of synthesizing their own mycobactins.

Both mycobactin P and desferrioxamine B form complexes with ammonium vanadate; these are the only complexes other than that with ferric iron that are formed in aqueous solution. All the trivalent metal complexes previously studied dissociate in

mineral acid. In eqn. (1) which exemplifies their formation, DH_3 represents the desferri form of the sideramine carrying the three replaceable hydrogen atoms of the hydroxamic acid groups. For simplicity hydrogen ions are represented as H^+ .

An excess of hydrogen ions will cause the equilibrium to move towards the left-hand side and will result in dissociation of the complex. If the reaction with vanadate gives a vanadyl complex it may be represented by eq. (2).

In this reaction an increase in hydrogen ion concentration should shift the equilibrium towards the right-hand side, and the stability of the complex should increase. In fact the vanadyl complexes of the water-soluble sideramines and the mycobactins persist in the strongest acids and do not dissociate.

At pH values near neutrality the stability constants of the ferric complexes of desferrisideramines greatly exceed those of the corresponding vanadyl complexes, and ferric iron will quantitatively displace vanadate, but at pH 1.0 the stabilities of the ferric and vanadyl complexes of desferrioxamine become equal, and at lower pH values vanadate will displace iron. The interconversion of the two principal forms of vanadyl desferrioxamine B involves the uptake or loss of a proton but does not appear to be accompanied by valency change. Paper electrophoresis shows that the metallic centre is uncharged in the 'acid' form and carries a negative charge in the 'alkaline' form. The interconversion may be tentatively formulated as shown in eq. (3).

Spectroscopic measurements of vanadyl desferrioxamine B over a range of pH values show that other reactions are superimposed on this principal interconversion, especially at lower pH values.

The chelation of ferric iron by the water-soluble sideramines involves the three hydroxamic acid groups and gives rise to hexadentate complexes of exceptional stability. The mycobactins have only two hydroxamic acid groups, but have a third centre comprising the phenolic hydroxyl group and the nitrogen of the oxazoline ring which can engage

the last two co-ordination positions round the iron atom. The low aqueous solubility of the ferric mycobactins is an obstacle to the direct determination of their stability constants. However, measurements were made of the relative affinities of desferrioxamine B and mycobactin P for ferric iron in ethanolic solution, and indications of their relative affinities in a neutral aqueous solution were obtained by a two-phase experiment with chloroform and an aqueous buffer solution. Both results showed that ferric iron has a greater affinity for mycobactin P than for desferrioxamine B. The mycobactins as a class can thus be regarded as entirely parallel to the other sideramines in their extreme selectivity for ferric iron. Exchange experiments with the ferric and desferri forms of mycobactin P and desferrioxamine B demonstrate the rapidity with which iron can be redistributed under some conditions.

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