ENERGY-INDEPENDENT BINDING OF IRON COMPLEXED TO SMALL ORGANIC CHELANTS BY *NEISSERIA MENINGITIDIS*

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Fe³⁺-dicitrate binding and iron uptake from citrate have been characterized in three carrier and three case isolates of *Neisseria meningitidis*. Culture in Fe³⁺-dicitrate rich medium did not induce subsequent Fe³⁺-dicitrate binding as it does for *Escherichia coli* and other bacteria. Growth in iron-restricted conditions slightly stimulated the Fe³⁺-dicitrate binding system in all the strains studied. Total binding of citrate as Fe³⁺-dicitrate was 2.5 times that of citrate in the absence of iron. Affinity for Fe³⁺-dicitrate ranged from 6.3×10^4 to 2.6×10^5 l/mol, and the number of receptors per cell ranged from 8,500 to 64,800. The binding system was highly specific for Fe³⁺-dicitrate (it did not bind other iron complexes with isocitrate, lactate, pyruvate or EDDA). Fe uptake efficiency was significantly higher in the case strains but, due to the higher number of receptors per cell in the carrier strains, the resulting iron uptake rate is similar in both groups.

Iron, despite its importance as an essential metal for all organisms and its abundance and ubiquity in nature, has very limited availability for microorganisms because of both its tendency to form highly insoluble complexes and its being sequestered by specialized molecules such as transferrin and lactoferrin. In order to increase their iron supply, many microorganisms, when grown under iron restriction conditions, synthesize siderophores, low molecular weight chelants with very high affinity for iron, that are able to extract this metal from sequestering macromolecules. Iron-siderophore complexes are trapped by specialized microbial outer-membrane macromolecules and usually are taken up in an energy-coupled process across the outer and cytoplasmic membranes.

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Abbreviations: NDM, *Neisseria* defined medium; EDDA, ethylene-diamine-di-hydroxy-phenyl-acetic acid; MH, Mueller-Hinton broth; K_a , affinity constant.

Neisseria meningitidis grows readily in a wide range of environments as a result of being able to extract iron from various sequestering agents (5). West and Sparling (21) demonstrated that this ability is not mediated by the production of siderophores, but by a receptor system located in the outer membrane which only acts under iron restriction. Iron uptake from transferrin, for example, needs direct contact between transferrin and the bacterial wall (18) and involves iron-regulated outer membrane proteins that function as specific receptors (1, 6, 10) and shows a highly variable mass (7). The binding process is stable and highly specific (17) and the transferrin is not internalized (14). So far, however, the biochemical mechanisms responsible for the extraction of iron from transferrin and its transport through the outer and inner bacterial membranes have not been elucidated in depth.

Serum and other biological fluids such as cerebrospinal fluid contain other iron sources besides transferrin and lactoferrin; citric acid and lactic acid are efficient iron chelators, and bacteria able to obtain iron from them have obvious advantages over those forced to secrete their own siderophores. Moreover, iron bound to these organic acids, particularly citric acid, is taken up much more easily than that carried by transferrin, and constitutes an important source of this metal for pathogens such as *N. meningitidis*. The meningococcal system for uptake of iron from citrate is based on the presence of specific outer membrane receptors which bind iron borne in Fe^{3+} -dicitrate complexes which have not been yet characterized (3, 19). However, citrate is not used as a source of carbon or energy, as in *Escherichia coli* (19, 20), but only as a shuttle for iron, the dissociation of metal and ligand taking place at the bacterial envelope and being followed by the internalization of iron alone (12, 19).

In *N. meningitidis* iron uptake from Fe^{3+} -dicitrate is not inducible by the presence of the complex as it is in *E. coli* (9). Probably, an initial energy-independent phase in which intense non-specific, concentration-dependent binding takes place is followed by an energy-dependent phase in which iron uptake occurs via a high-affinity binding mechanism like uptake from ferric chloride (3) or transferrin (18).

In this paper we report the characteristics of energy-independent Fe^{3+} -dicitrate iron binding system in various *N. meningitidis* strains, as well as the measurement of energy-dependent iron uptake in these microorganisms.

MATERIALS AND METHODS

Bacteria and growth conditions. *Neisseria meningitidis* strains P000, P391 and P636 were obtained from the oropharynx of healthy carriers. Strains V002, V019 and V021 were isolated from the cerebrospinal fluid of patients in our region. All strains are serogroup B, but differ in serotype. Serogrouping and serotyping were performed in the Centro Nacional de Microbiología, Virología e Inmunología at Majadahonda (Spain). An *Escherichia coli* K12 strain was used as a reference for positive control of ferric citrate receptors induction. All strains were stored at

–80°C in 5% sodium glutamate–5% bovine albumin (16). For experiments, the strains were cultured as described previously (8). Briefly, one isolated colony from Choc-Iso agar plates incubated for 24 h at 37°C in a 5% CO₂ atmosphere, was cultured for 8 h under the same conditions as above before inoculation in 100 ml of MH (in iron sufficiency). Iron restriction medium was MH containing the iron chelant EDDA at a final concentration of 39 µM (MH-EDDA); medium for receptor induction experiments (MH-Fe) was MH containing 1 µM Fe³⁺-dicitrate (1 mM sodium citrate:1 µM FeCl₃). All final cultures were incubated for 14 h at 37°C with constant shaking at 100 rpm. For Fe uptake measurements, the bacteria were cultured in NDM modified as described in Table 1 and deferrated by treatment with Chelex-100 (5); all material used was washed with 6N HCl and thoroughly rinsed with distilled deionized water. Briefly, 2 ml of a preinoculum cultured in MH-EDDA were inoculated into Erlenmeyer flasks containing 100 ml of NDM or NDM with 20 µM ferric citrate (controls) and incubated for 8–10 h at 37°C and 100 rpm constant shaking in a water bath.

Chemicals. [⁵⁵Fe]Ferric chloride (1–50 mCi/mg Fe) and [¹⁴C]citric acid (100–120 mCi/mmol) were obtained from Amersham International plc (Buckinghamshire, England). Isocitric, lactic, pyruvic and citric acids and EDDA were purchased from Merck, Darmstadt, Germany. MOPS was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and the scintillation cocktail (Ready-Protein) was obtained from Beckman Instruments Inc. (Fullerton, CA, U.S.A.). Complexes of iron with the organic acids were always prepared by mixing solutions of FeCl₃ and organic acid at a 1/100 molar ratio, except in the case of EDDA in which molar ratio was 1/2.

Table 1. Composition of the *Neisseria* defined medium (NDM).

Components	Chemicals	Concentration (mM)
Stock solution A (25 ml/l)	NaCl	100.00
	K ₂ SO ₄	5.70
	NH ₄ Cl	4.11
	Na ₂ EDTA	0.01
	MgCl ₂	1.07
Stock solution B (20 ml/l)	K ₂ HPO ₄	2.00
	KH ₂ PO ₄	2.00
	HEPES	21.00
Stock solution C (1 ml/l)	CaCl ₂	0.25
Stock solution D (250 ml/l)	L-Arginine	0.50
	L-Cysteine	0.06
	L-Glycine	2.00
	L-Serine	0.20
	L-Glutamine	8.00
Stock solution E (25 ml/l)	Sodium lactate	60.00
Stock solution F (25 ml/l)	Glucose	28.00

All stock solutions, except sol. C, are added together at the indicated quantities and adjusted to 1 l with distilled, deionized water (pH must be adjusted to 7.45±0.1). The medium is then sterilized by filtration and sterile sol. C added after cooling.

[⁵⁵Fe]Fe³⁺-dicitrate binding assays. Binding of [⁵⁵Fe]Fe³⁺-dicitrate to killed meningococci was determined by a modification of the methods described by Simonson et al. (19) and Harding and Royt (11). Briefly, bacteria grown in MH or MH-EDDA were obtained by centrifugation at 10,000 × *g* for 10 min, washed in 50 mM MOPS (pH 7.5) and adjusted to 10¹⁰ cfu/ml in the same buffer. KCN was then added to 60 mM and the bacteria incubated for 20 min at room temperature (the concentration of KCN needed to kill all the bacteria in the suspension was determined as 60 mM by incubation for 20 min in serial dilutions of KCN followed by inoculation onto MH agar plates to determine viability). A 0.1 ml sample of the suspension (10⁹ bacteria) was then mixed in 1.5 ml Eppendorf tubes with 0.1 ml of [⁵⁵Fe]Fe³⁺-dicitrate complex (specific activity 180 ct/min/pmol) and incubated at 20°C. After incubation, bacteria were harvested by centrifugation at 15,000 × *g* for 2 min and washed three times in 0.2 ml of MOPS containing 2 μM KCN and 2.5 mM sodium citrate. The bacterial pellet was finally suspended in 0.1 ml of distilled water and the associated radioactivity measured in a scintillation counter. Non-specific binding was determined in parallel experiments in which Fe³⁺-dicitrate was added at a concentration 100 times higher than that of [⁵⁵Fe]Fe³⁺-dicitrate. All experiments were repeated three times.

Radioactivity counts were analyzed using a commercial radioligand binding analysis program (EBDA; Elsevier-Biosoft, Cambridge, U.K.) based on non-linear curve fitting techniques (15). Differences between groups were analyzed by the Mann-Whitney U test.

For calculation of saturation times, concentration of the [⁵⁵Fe]Fe³⁺-dicitrate complex was 2.5 μM (referred to Fe; 0.5 μCi/μg) and several incubation times, from 0 to 60 min, were assayed. For calculation of the number of Fe³⁺-dicitrate receptors and their affinity constants (*K_a*), several concentrations of the [⁵⁵Fe]-Fe³⁺-dicitrate complex (0.5, 1, 2, 3, 5, 10, 20 and 30 μM Fe; specific activity 180 ct/min/pmol) and a 20 min incubation time were used.

Binding of ⁵⁵Fe chelated by other organic acids. The binding of ⁵⁵Fe chelated by isocitrate, lactate, pyruvate and EDDA was studied as described for citrate.

[¹⁴C]Citrate binding assays. Citrate binding was determined using Fe³⁺-[¹⁴C]dicitrate as described for [⁵⁵Fe]Fe³⁺-dicitrate, with the same Fe:citrate molar ratio and a specific activity of 247 ct/min/pmol. In this case, concentrations of Fe³⁺-dicitrate complex are referred to citrate.

⁵⁵Fe uptake measurement. Uptake of ⁵⁵Fe was measured by a modification of the method described by Harding and Royt (11). Briefly, bacteria cultivated in NDM as described above were suspended in deferrated MOPS (by Chelex-100 treatment) to 10⁶ CFU/ml. Control bacteria were killed by incubation with 60 mM KCN for 20 min. One hundred microliters of the suspensions were then incubated with 100 μl of 60 μM [⁵⁵Fe]Fe³⁺-dicitrate in Eppendorf vials, and washed twice with deferrated MOPS after 1, 15, 30, 60, 120 and 180 min. Counts associated to the bacteria were determined by liquid scintillation and converted to uptake rates as pmol/min/10⁹ cells. Uptake efficiencies of the receptors, here defined as uptake rate

per receptor, were calculated dividing the total uptake by the time and the number of receptors in the 10^9 cells in the assay.

RESULTS

$[^{55}\text{Fe}]\text{Fe}^{3+}$ -dicitrate binding assays

Figure 1 shows the time dependence of $[^{55}\text{Fe}]\text{Fe}^{3+}$ -dicitrate binding by *N. meningitidis* strain P000 (used as standard) in normal and in iron-restricted media. Binding increased with time, reaching saturation after about 10 min. The same was observed for other strains (data not shown), and 20 min was considered time enough for saturation of the Fe^{3+} -dicitrate receptor system.

The presence of Fe^{3+} -dicitrate in the growth medium increased Fe^{3+} -dicitrate binding by *E. coli* K12 about 20 fold (Fig. 2), implying an increase from a low number of receptors per cell (about 400) to about 14,300 ($K_a = 1.7 \times 10^6 \text{ l/mol}$). There was no statistically significant effect on binding by *N. meningitidis* ($K_a = 6.3 \times 10^4$ to 2.6×10^5 ; Table 2).

Figure 3 compares $[^{14}\text{C}]$ citrate binding by *N. meningitidis* P000 cells in the presence and absence of Fe^{3+} . About 14,400 receptors per cell ($K_a = 1.6 \times 10^4 \text{ l/mol}$) were calculated for sodium citrate and 21,000 receptors per cell ($K_a = 1 \times 10^4 \text{ l/mol}$) for Fe^{3+} -dicitrate complex. Although this difference is not statistically significant, it seems that the efficiency of citrate binding may be enhanced in the case of Fe^{3+} complexes.

Figures 4 and 5 show saturation and Scatchard plots for six *N. meningitidis* strains cultured both under normal and iron-restricted conditions. The strains differed mutually both in the number of receptors per cell and in the affinity

Fig. 1. Saturation time of $[^{55}\text{Fe}]\text{Fe}^{3+}$ -dicitrate complex binding in *Neisseria meningitidis* P000.

Squares indicate bacteria cultivated in Mueller-Hinton broth (MH), and circles indicate bacteria cultivated in iron-restricted medium (MH with $39 \mu\text{M}$ EDDA). Open symbols show the corresponding non-specific binding.

Fig. 2. Induction of ^{55}Fe Fe^{3+} -dicitrate receptors in *Escherichia coli* K12 (squares) and *Neisseria meningitidis* P000 (circles).

Open symbols correspond to cells grown without Fe^{3+} -dicitrate. Solid symbols are from cells grown with ferric citrate (1 mM sodium citrate : 1 μM ferric chloride).

Fig. 3. Binding of Fe^{3+} - ^{14}C dicitrate complex (●) and sodium ^{14}C citrate (○) to *Neisseria meningitidis* P000 cells.

Fe^{3+} -dicitrate was prepared at an ferric chloride : sodium citrate ratio of 1 : 100.

constant for Fe^{3+} -dicitrate complex. The number of receptors depended on whether normal or iron-restricted culture conditions were used, especially for strain P391, but the affinity constants were practically unaffected by iron restriction. Table 2 lists, for the six strains, the values of the above parameters together with the maximum binding of Fe^{3+} -dicitrate complex.

Binding of ^{55}Fe chelated by other organic acids

Figure 6 shows the results of binding experiments using different organic acids to carry iron. The binding kinetics were linear reflecting non-specific binding, and in keeping with this statistical analysis failed to afford the parameters corresponding

Fig. 4. Saturation (left) and Scatchard (right) plot of $[^{55}\text{Fe}]\text{Fe}^{3+}$ -dicitrate binding to three *Neisseria meningitidis* strains obtained from healthy carriers cultured under normal and iron-restricted culture.

A, strain P000; B, strain P391; C, strain P636. Open symbols corresponds to cells grown in iron sufficiency (MH broth) and solid symbols are from cells grown in iron restriction (MH with $39\ \mu\text{M}$ EDDA).

Fig. 5. Saturation (left) and Scatchard (right) plot of [⁵⁵Fe]Fe³⁺-dicitrate binding to three *Neisseria meningitidis* invasive strains cultured under normal and iron-restricted culture.

A, strain V002; B, strain V019; C, strain V021. Open symbols correspond to cells grown in iron sufficiency (MH broth) and solid symbols are from cells grown in iron restriction (MH with 39 μM EDDA).

Table 2. Characteristics of the ferric dicitrate receptor systems present in six *Neisseria meningitidis* strains.

Strain	Normal culture conditions			Iron restriction				
	Binding (pmol/10 ⁹)	K _a (l/mol)	Receptors/ Cell	Binding (pmol/10 ⁹)	K _a (l/mol)	Receptors/ Cell	Fe uptake rate (pmol/ min/10 ⁹)	Fe uptake efficiency (pmol/ min/receptor)
P000	24.6	9.3 · 10 ⁴	22,500	29.5	6.3 · 10 ⁴	29,400	0.91	31 · 10 ⁻¹⁵
P391	17.8	6.3 · 10 ⁴	24,600	85.5	2.0 · 10 ⁵	64,800	1.35	20 · 10 ⁻¹⁵
P636	31.2	2.4 · 10 ⁵	21,700	64.3	1.5 · 10 ⁵	33,700	1.63	48 · 10 ⁻¹⁵
V002	13.5	2.6 · 10 ⁵	14,500	20.0	1.1 · 10 ⁵	21,400	1.46	68 · 10 ⁻¹⁵
V019	8.6	2.6 · 10 ⁵	6,200	11.7	2.1 · 10 ⁵	8,500	0.47	55 · 10 ⁻¹⁵
V021	13.9	1.4 · 10 ⁵	15,300	26.1	1.5 · 10 ⁵	19,700	1.00	51 · 10 ⁻¹⁵

Fig. 6. Binding of iron chelated to various low molecular weight organic acids by *Neisseria meningitidis* cells grown in iron-restricted conditions.

to the assumption of a saturation kinetics (in contrast to the case of citrate). The extent of iron binding depended on the carrier; it was greatest for lactate and very low for EDDA. These results suggest that there are no specific receptors for iron bound to these organic acids as there are for citrate.

Measurement of ^{55}Fe uptake

Table 2 shows the iron uptake rate and uptake efficiency from iron-citrate complexes in the six *N. meningitidis* strains used in this study, cultured under iron restriction (in NDM). As can be seen, there were found no differences in the uptake rates in carrier and invasive strains ($p=0.5$; Mann Whitney U test), whereas uptake efficiency was significantly higher ($p<0.05$; Mann Whitney U test) in the last group.

DISCUSSION

Many microorganisms, including *N. meningitidis* (3), *E. coli* (20) and *Pseudomonas aeruginosa* (4) are capable of taking up iron directly from naturally occurring iron-binding acidic siderophores such as citrate, *N. meningitidis* being able to use also malate, pyruvate and isocitrate for that purpose (2). This phenomenon has been most intensively studied in the case of *E. coli*; studies with *N. meningitidis* have hitherto mainly focused on quantifying iron uptake rather than on characterizing the uptake system. It is known, however, that in both *E. coli* and *N. meningitidis* the uptake system involves the production of outer membrane receptors that recognize extracellular iron-citrate complexes (9,19,20). Iron and citrate are dissociated at the cell envelope (12) by an energy-independent mechanism (19).

In *E. coli*, citrate-dependent Fe^{3+} binding is enhanced by prior growth in the presence of citrate. That this does not occur in the case of *N. meningitidis* has been shown by Simonson et al. (19) and is confirmed by the results of this work (Fig. 2). We did, however, find that citrate-dependent iron binding was increased by culture in iron-restricted conditions, especially in the case of strain P391 (Table 2).

Archibald et al. (3) and Simonson et al. (19) reported that citrate was not bound by KCN-treated *N. meningitidis* either in the presence or in the absence of iron. Citrate was thus regarded as acting as a shuttle transporting iron to the cell from the extracellular medium (in particular, it did not itself act as a source of carbon or energy). In our experiments, on the other hand, we found that [^{14}C]-citrate was bound both as Fe^{3+} -dicitrate complex and as sodium citrate, though binding was less in the latter case (Fig. 3). The possibility that binding of citrate could be non-specific would appear to be ruled out by the fact that, although the binding of iron borne by isocitrate, lactate, pyruvate and EDDA was estimated as linear by the analysis software used, which clearly indicates its non-specificity, whereas the binding of dicitrate-borne iron exhibited saturable kinetics. The linear kinetics of the non-citrate carriers likewise appear to rule out the possibility that the difference between citrate binding in the presence and absence of iron may have been due to some of the Fe^{3+} -dicitrate having bound to Fe receptors not requiring the presence of citrate, whose existence has been suggested by Archibald et al. (3). We therefore propose that in *N. meningitidis* there is an iron uptake system that involves receptors that are specific for citrate, especially for citrate complexed to

iron; these receptors are not induced by the presence of Fe^{3+} -dicitrate.

It has been suggested that the metabolically efficient uptake of iron from iron-citrate complexes may be of importance for microbial pathogenicity (19) and for the in vivo survival of micro-organisms in disseminated disease (13). In our experiments, invasive and carrier strains of *N. meningitidis* did not differ significantly as regards total binding, the affinity constant for citrate-dependent iron binding, and the iron uptake rate, being the differences in receptor numbers compensated by those in uptake efficiency. We accordingly believe that uptake of iron from Fe^{3+} -dicitrate cannot be considered a marker of virulence in *N. meningitidis*, and that this source of iron is not as important for *N. meningitidis* as it is for bacteria such as *E. coli* or *P. aeruginosa*. Uptake experiments are being continued to draw conclusions about the possible role of iron-dicitrate uptake in pathogenicity.

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