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Sorption of metals by extracellular polymers from the cyanobacterium *Microcystis aeruginosa* f. *flos-aquae* strain C3-40

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Abstract

The sorption of cadmium (II), copper (II), lead (II), manganese (II), and zinc (II) by purified capsular polysaccharide from the cyanobacterium *Microcystis aeruginosa* fo. *flos-aquae* strain C3-40 was examined by four methods: equilibrium dialysis, metal removal from solution as detected by voltammetry, metal accumulation by capsule-containing alginate beads, and calorimetry. The polysaccharide's saturation binding capacities for these metals ranged from 1.2 to 4 mmol of metal g^{-1} of capsule, which corresponds to 1 metal equivalent per 2 to 4 saccharide subunits of the polymer. Competition between paired metals was tested with simultaneous and sequential additions of metal. Cadmium (II) and lead (II), as well as lead (II) and zinc (II), competed relatively equally and reciprocally for polymer binding sites. In contrast, manganese (II) strongly inhibited the binding of cadmium (II) and lead (II), but itself was not substantially inhibited by either the prior or simultaneous adsorption of cadmium (II) or lead (II).

The data are interpreted with respect to overlap of binding sites and possibilities of altered polymer conformation or solvation. Calorimetric studies of lead (II) and cadmium (II) association reactions with the polysaccharide suggest that the enthalpies are small and that the reactions may be driven by entropy.

Introduction

The cyanobacterium *Microcystis aeruginosa* fo. *flos-aquae* strain C3-40 produces an extracellular capsule that strongly accumulates metals (Plude et al., 1991; Parker et al., 1996). Unlike most *M. aeruginosa* cultures, strain C3-40 is not toxic when assayed by either ELISA or phosphatase inhibition assays for microcystin, consistent with its lack of the N-methyl transferase domain of the *mcyA* gene of microcystin synthetase (Tillet, Parker & Nielan, pers. comm.). The capsule of strain C3-40 also has a simpler chemical composition (Plude et al., 1991) than that of certain other *M. aeruginosa* isolates (Nakagawa et al., 1987) and is more easily released from the cells.

The purified capsule of strain C3-40 contains 83% (wt/wt) galacturonate, 5.5% rhamnose, 5% mannose, 3% xylose, 2% glucose, 1.5% galactose, and no detectable protein (Plude et al., 1991). The molecular mass is heterogeneous, but greater than 10 000 daltons (Parker et al., 1996). The sugar linkages and degree of branching are unknown. Most of the carboxylic acid residues in the galacturonate subunit are not esterified (Parker et al., 1996), in contrast to pectin, which has a similar sugar composition (Walter, 1991). The capsule from strain C3-40 is the only pectin-like polysacchar-

ide reported from a microbe. As such, it may have applications as a texturizer, gelling agent that does not require heating, immobilizer for the localization or timed release of metals, and material that can remove toxic metals from polluted waters. An advantage is that the capsule can bind many metal species at high pH.

This paper examines selected properties of purified capsular polysaccharide from strain C3-40, including: (a) the saturation binding of divalent metals when they are added singly, in pairs, or sequentially, and (b) the relative binding of various metals at non-saturating conditions. To investigate the role of negatively-charged galacturonate residues in the capsular polysaccharide, the enthalpies of metal associations with capsular polysaccharide are compared to those with polygalacturonate.

Materials and methods

Capsule and reagents

Capsular polysaccharide was purified by the method of Plude et al. (1991), modified to include precipitation with cetyltrimethylammonium bromide (CTAB) and ethanol (Isobe et al., 1992). One gram of capsular polysaccharide equaled 3.95 μmol equivalents of galacturonate subunits or 4.93 μmol equivalents of total subunits of all sugar types. All metal salts were divalent chlorides, except for $Pb(NO_3)_2$.

Voltammetry

A 50 mg capsule L^{-1} solution was titrated into each 0.1–0.5 mM metal salt solution in 0.1 M NH_4 tartrate, pH 9.0. Reciprocal titrations of metal into capsule also were performed in most cases. The residual dissolved metal was assayed by square wave voltammetry with an EG&G/Princeton potentiostat (model 263A) and an EG&G/PARC Standard Mercury Dropping Electrode (model 303A). Anaerobic measurements were made at 30 s intervals after mixing with bubbled N_2 gas. Metal precipitation or adsorption to the siliconized chamber was not found within the experimental timeframe. The endpoints of duplicate titrations did not differ by more than 6% of each other; 4–6% variability was also indicated by preliminary controls and a combination-of-errors analysis. In mixtures of metal salts, each was 0.12 mM.

Equilibrium dialysis

Microdialysis chambers (Orr et al., 1995), each containing 31 μg of capsule in 75 μL, were dialyzed for 48 h with a 100 times greater volume of each 30–50 μM metal solution in 10 mM n-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid (EPPS) buffer, pH 8.8. Metal inside the chambers was assayed with a Varian SpectrAA 200 atomic absorption (AA) flame spectrophotometer. Standard deviations of triplicate or quadruplicate chambers were 3–9% of the means. EPPS has an exceptionally low affinity for metals (Perrin & Dempsey, 1974).

Alginate beads

Alginate beads that contained or lacked capsule were prepared by standard methods (Mattiasson, 1983). One mL of beads was agitated for 24 h in 100 mL of each 43–157 μM metal solution in 10 mM EPPS buffer, pH 8.0. Before and after treatment, metal in the liquid phase was assayed by AA.

Calorimetry

Procedures, instruments, and data analysis methods have been described (Mihalick et al., 1999). Each metal solution was injected into a 100 mL aqueous solution of polysaccharide. The enthalpy of dilution of the metal solution was measured in a separate experiment. The amount of added metal was in the range of complete binding as determined by voltammetry. For polygalacturonate, the molar ratio for lead:galacturonic acid units was 1:5, whereas that for lead and capsule was 1:3.

Results

Saturation metal-binding of capsular polysaccharide, for metals tested singly

The maximum amount of each metal that was bound by capsular polysaccharide at pH 8 to 9 was determined by three methods: equilibrium dialysis, metal accumulation by capsule-containing alginate beads, and metal removal from solution as detected by voltammetry (Table 1). Depending on the metal and assay method, the maximum amount of bound metal at pH 8 to 9 ranged from 1.2 to 4.1 mmoles of metal per gram of polysaccharide (Table 1), which equates with 0.23 to 0.82 mole of bound metal per mole equivalent of

Table 1. Metal-binding capacities of capsular polysaccharide from strain C3-40

Metal	Metal bound at saturation (mmol metal g^{-1} capsule)	Metal bound at saturation (mol metal mol equiv.$^{-1}$ sugar subunits)	pH	Method
Cu(II)	1.7	0.35	pH 9.0	Voltammetry
	3.3	0.68	pH 8.8	Equilib dialysis
	4.1	0.82	pH 8.0	Alginate beads
Cd(II)	1.23	0.25	pH 9.0	Voltammetry
	1.15	0.23	pH 8.8	Equilib dialysis
Mn(II)	1.72	0.35	pH 9.0	Voltammetry
	2.84	0.58	pH 8.0	Alginate beads
Pb(II)	1.36	0.28	pH 9.0	Voltammetry
	1.50	0.30	pH 8.8	Equilib dialysis
Zn(II)	1.23	0.25	pH 9.0	Voltammetry

Table 2. Saturation metal binding by capsular polysaccharide exposed to pair-wise mixtures of metal ions at equimolar concentrations

Metal mixture		Saturation binding in metal mixtures (% of saturation binding of each metal alone)		
Metal 1	Metal 2	Metal 1	Metal 2	Sum
Pb(II)	Cd(II)	51	62	113
Pb(II)	Zn(II)	50	64	114
Mn(II)	Cu(II)	70	31	101
Pb(II)	Cu(II)	116	61	177
Mn(II)	Cd(II)	82	67	149
Mn(II)	Pb(II)	82	56	138

saccharide subunits and 0.3 to 1 mole of bound metal per mole equivalent of negatively-charged galacturonate subunits. All determinations were performed in the presence of excess monovalent ions from the pH buffers (Table 1).

Saturation metal-binding of capsular polysaccharide, for metals tested in pairs

Table 2 summarizes voltammetric data for the maximum metal binding by capsular polysaccharide that was reacted with equimolar pairs of metals. The data revealed two distinct patterns of competitive metal binding.

For the first group of paired metals, the sum of the amounts of both metals bound at saturation was close to the saturation amount of each metal when titrated alone to its saturation value (Table 2). The mixtures of lead(II) with cadmium(II), lead(II) with zinc(II), and manganese(II) with copper(II) were in this group. The molar amounts of bound lead, cadmium, and zinc were approximately equal and were close to 50% of the amount of each metal that was bound when tested alone. In contrast, more Mn than Cu was bound by a molar ratio of 70:31.

For the second group of paired metals, one member of the pair was bound at approximately 50% of its saturation value when tested alone, whereas much more than 50% of the other metal was bound (Table 2). This group included lead(II) with copper(II), as well as manganese(II) with either cadmium(II) or lead(II). The saturation amounts of lead and manganese that were bound in these mixed metal conditions were, respectively, 116% and 82% of their saturation binding alone.

Table 3. Effect of order of addition on the saturation binding by capsule of manganese(II), cadmium(II) and lead(II)

	Bound metal (%)	
	$MnCl_2$	$Pb(NO_3)_2$
Added first (100%)	100	100
Added second	87	9
Added simultaneously	92	31
	$MnCl_2$	$CdCl_2$
Added first (100%)	100	100
Added second	88	7
Added simultaneously	96	87

Effect of order of metal addition on the saturation metal binding of capsule

One metal was first titrated to saturation before a second metal was added to its saturation of the capsule solution. When manganese(II) was added first, the saturation binding of either lead(II) and cadmium(II) was severely decreased (Table 3). If lead(II) or cadmium(II) was added first, very little inhibition of manganese(II) binding was observed (Table 3).

Metal binding by capsule at non-saturating conditions

The relative binding of manganese(II) and copper(II) by capsule was examined in an alginate bead system that was exposed to a mixture of 0.08 mM $CuCl_2$ and 0.09 mM $MnCl_2$, low enough metal concentrations that saturation binding by capsule did not occur. The bound Cu and Mn were, respectively, 2.5 and 0.06 moles of bound metal per gram of polysaccharide, which correspond to roughly 63% and 2% of saturation in this system. Although the molar ratio of added copper to manganese was only 0.87, the ratio of bound copper to manganese was 4.1.

Capsule-containing alginate beads were agitated in cadmium(II) and manganese(II) solutions of similar concentrations (43 μM $CdCl_2$; 47 μM $MnCl_2$). The amounts of bound Cd and Mn were, respectively, 0.5 and 0.007 mole of metal per gram of polysaccharide, or about 44% and 6% of saturation. Although the molar ratio of added cadmium to manganese was only 0.91, 4.4 times more cadmium than manganese was bound by capsule.

Metal sorption by polygalacturonate, as compared to capsule

Although both capsule and polygalacturonate are comprised predominantly of galacturonate subunits, the associations of these two polysaccharides with Cd(II) and Pb(II) differed considerably. Capsular polysaccharide bound lead and cadmium at approximately equal levels of 0.35 and 0.3 equivalents of metal per galacturonate residue, respectively, in voltammetric experiments. In contrast, polygalacturonate did not detectably bind cadmium, although it bound nearly as much lead (0.2 metal equivalents per galacturonate) as did capsule. Calorimetric data also suggested the lack of a substantial cadmium:polygalacturonate association, since no temperature change was detected when $CdCl_2$ was added to polygalacturonate (data not shown). The reaction of $Pb(NO_3)_2$ with polygalacturonate was exothermic (–10 kJ mol^{-1}), while that of $Pb(NO_3)_2$ with capsule was slightly endothermic (+5 kJ mol^{-1}).

Discussion

The capsule of *M. aeruginosa* is unusual in that it more efficiently adsorbs certain metals at high than at low pH (Pradhan et al., 1998). This property suggests various applications at alkaline conditions, including a pH-controlled release or uptake of metals. High pH is also relevant to environmental issues, since *M. aeruginosa* forms thick growths in alkaline lakes, where it is sufficiently abundant to influence the solubilization, chelation, precipitation, bioavailability, and cycling of metals. However, experiments at high pH are difficult because metal speciation is complicated (Martell & Hancock, 1996), metal solubilities can decrease, and chances of metal oxidation can increase (Stumm & Morgan, 1995). We have therefore focused on rapid studies that were performed entirely within the anaerobic environment of a voltammetric chamber. Voltammetry allows identification of a metal's oxidation state, and only divalent forms were seen during the experimental timeframe.

Based on voltammetric data, saturation occurred at a molar ratio of one metal equivalent per 3 to 4 saccharide subunits of the capsular polysaccharide. This stoichiometry corresponds to one metal equivalent per 2.3 to 3.2 galacturonate subunits, which are the only negatively-charged moieties in purified capsule (Plude et al., 1991). A metal:galacturonate ratio of 2 would

theoretically represent full reaction for an ideal system in which the carboxylates of galacturonate were fully ionized and completely associated with divalent metal ions. Most of the carboxylates were probably ionized, since the pK_a of polygalacturonate is 3.6 (Cesaro et al., 1982). However, various metal species were possibly present in addition to simple ions, a phenomenon that is currently being investigated.

Three lines of evidence suggest that lead(II) and cadmium(II) compete fairly equally for similar or overlapping sites on the capsular polysaccharide at saturation conditions. First, titrations of the polysaccharide into an equimolar mixture of the two metals yielded a saturation amount of each metal that was close to 50% of its binding in parallel titrations with that metal alone (Table 2). Second, both lead and cadmium exhibited similar patterns of competition with manganese (Tables 2, 3). Finally, the two metals saturated at similar metal to capsule ratios when tested singly (Table 1).

Roughly 80% of the manganese(II) sites on capsule can not be the same as, or overlap substantially with, those of lead(II) or cadmium(II) because manganese binding was only slightly decreased by prior saturation of the polymer with either lead or cadmium, which remained bound after the addition of manganese (Table 3). In contrast, the prior binding of manganese(II) almost completely blocked the subsequent binding of lead(II) or cadmium(II) by capsule (Table 3). This paradoxical result suggests that manganese can indirectly affect the access of lead and cadmium to their sites, perhaps via altered polymer conformation or cross-linking. Similar phenomena involving conformational changes, cation bridging, and modified solvation have been invoked to explain the effects of these same metals on the viscosity of capsular polysaccharide from strain C3-40 (Parker et al., 1996). Calorimetric studies of metal binding by capsule also suggest a primacy of conformation factors and solvent effects. These calorimetric measurements indicate that the enthalpies associated with metal binding by capsule are small or endothermic (data presented here), whereas the equilibrium constants and entropy increases are large and positive (J. Mihalick, pers. comm.). This relationship implies that the metal-polymer reactions are driven by the increase in entropy. In light of these viscometric and calorimetric observations, it is plausible that manganese sorption alters the solvation, folding, or chain-chain interactions of the polysaccharide in such a way that the sites of the larger cadmium and lead atoms are deformed or rendered inaccessible.

An alternative interpretation that manganese has higher affinities for most of its sites than lead or cadmium have for theirs is contradicted by the relative binding of cadmium and manganese at non-saturating conditions (that is, conditions of polymer excess). In contrast to non-saturation, the relative binding of different metals at saturation is insensitive to metal-polymer affinities because the metals are added in excess until all available polymer sites, including low affinity ones, are filled. It is therefore not surprising that the capsule's relative binding of certain metal pairs at non-saturating conditions differed substantially from those at saturation. For example, capsule bound 4.4 times more cadmium than manganese at polymer excess, but slightly more manganese than cadmium at metal excess. A similar situation was observed with copper and manganese. In these two cases, the cadmium and copper had reached 44 or 63% of saturation, respectively, while manganese that was added in equimolar amounts only achieved 2 to 6% of saturation. The simplest way to explain these discrepancies is to hypothesize that copper and cadmium have higher affinities (association constants) for at least 40% of their sites than manganese has for its sites, but that manganese can occupy additional or different sites.

Although the carboxylate groups of uronic acid salts are known to be involved in metal sorption by many acidic polysaccharides (Geddie & Sutherland, 1993; Geesey & Jang, 1990), the function of galacturonate in the capsule of strain C3-40 is unknown. The galacturonate probably plays some role in metal binding, since it comprises 83% of the capsule by weight and since the amounts of bound metal per saccharide subunit are quite large (Table 1). However, both voltammetric and calorimetric data indicate that lead(II) and cadmium(II) interact differently with capsule than with polygalacturonate. Some of these disparities between capsule and polygalacturonate may result from differing linkages between galacturonate residues, since the sugar linkages in capsule are still unknown. However, it is also possible that the five neutral sugars in the capsule (Plude et al., 1991) enhance metal binding.

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