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Characterization of colominic acid by circular dichroism and viscosity analysis

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Abstract

Conformations of oligo- and poly-($\alpha(2 \rightarrow 8)$-*D*-Neu*p*NAc) (colominic acid) and its derivatives were studied by circular dichroism (CD) spectroscopy and viscometry to understand the molecular basis of their unusual antigenic properties. No temperature-dependent conformational transition between 5 and 70 °C or divalent salt effect of Ca^{2+} or Mg^{2+} was observed in colominic acid or its *N*-deacetylated form by CD spectroscopy. However, CD spectroscopy indicated that the distribution of conformers in oligocolominic acid changes continuously from $n = 2$ to octamer, and there was no further change of the conformer distribution for $n > 9$. Colominic acid exhibited a much lower intrinsic viscosity compared with the values for other polyelectrolytes of similar linear charge density, such as polynucleic acids. The apparent absence of induced conformational transition by salt or temperature, and the high flexibility indicated that the binding of colominic acid to its antibodies may not contain a significant amount of specific conformationally controlled determinant. Instead, our data suggest that more than nine saccharide units are needed for a cooperative binding process.

Keywords: Circular dichroism; Colominic acid; Viscosity analysis

1. Introduction

The capsular polysaccharides of *Escherichia coli* K1, Group B *Neisseria meningitidis*, *Pasteurella haemolytica* A-2 and *Moraxella nonliquefaciens* are all composed of a linear homopolymer of colominic acid ($\alpha(2 \rightarrow 8)$-*D*-Neu*p*NAc) [1,2]. The capsules are both virulence factors and protective antigens for these pathogens. Colominic acid of various lengths is found in fetal brain tissues and tumors of neural origin [3]. It is also a common 'glyco-component' of glycoproteins and glycolipids [4].

The relationship of conformation and binding properties—such as those with its antibodies—of this commonly found sugar have been studied extensively [5–9]. The antigen–antibody binding properties of colominic acid differ from those of other polysaccharides. In general, hexasaccharides can accomplish maximal inhibition. However, for colominic acid, more than 14 units are necessary to reach

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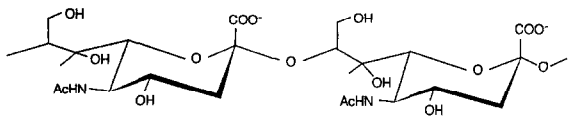

Fig. 1. Repeating structure of colominic acid.

maximal inhibition (Fig. 1) [10]. Furthermore, Mandrell and Zollinger [11] found that the amount of antibody needed for maximal binding of the antigen at 37 °C was five times higher than that required at 4 °C in the case of colominic acid, but that only 1.2 times as much antibody was required in the case of meningococcal C polysaccharide ($\alpha(2 \rightarrow 9)$-Neu*p*NAc). Similar differential results were obtained with polysaccharide–tetanus toxoid conjugates [12]. Such data supported the hypotheses that the antigenic properties of colominic acid are related to a temperature-sensitive conformational epitope [13].

From nuclear magnetic resonance (NMR) study, Yamasaki and Bacon suggested that colominic acid adopted a helical structure [7]. Using potential energy calculations and NMR, Brisson et al. [8] suggested that, although mostly in the form of a random coil in nature, colominic acid in solution can exist in high-order local helix—but not highly populated. These local helixes might be responsible for antibody binding. However, Brisson et al. could not find a unique conformer that would correctly simulate the NMR experimental data. Instead, they suggested that a wide range of conformations are needed to assemble a statistical distribution to interpret the NMR data. The crystal structure of a monoclonal IgG2a kapa murine antibody with specificity for colominic acid revealed a groove-shaped binding site with a geometry that could accommodate 'helices' with a narrow range of pitches, such that the surface charges would be in close contact with the binding site of the antibody [14].

We studied a series of colominic acid oligosaccharides by CD and viscometry at different temperatures and salt concentrations to elucidate conformational transitions and the degree of flexibility of colominic acid. The effect of *N*-deacetylation on these properties of colominic acid was also investigated.

2. Materials and methods

2.1. Chemicals

$Ca(OH)_2$, $Mg(OH)_2$ and volumetric standards, including HCl (0.1 N) and KOH (0.1 N), were obtained from Aldrich; NaOH and NaCl were obtained from J.T. Baker, Phillipsburg, NJ; H^+ Dowex ion exchange resin was obtained from Fluka, Switzerland; dextrans as molecular weight references were obtained from Pharmacia, Uppsala, Sweden; colominic acid ($n > 50$) was obtained from Sigma Fine Chem., St. Louis, MO; oligomers ($n = 2$–6) were obtained from E.Y. Laboratories Inc. All the chemicals were of analytical or higher grade. The oligosaccharides ($n = 4$–17) were prepared by mild acid hydrolysis of colominic acid [15]: colominic acid solution (500 mg per 180 ml Na–acetate buffer; 0.15 M, pH 5.0) was hydrolyzed at 70 °C for 30 min and then neutralized with 0.2 M NaOH. The products were fractionated according to size, through a DEAE-Sephadex A-25. The fractions were desalted through a Sephadex G10 column and freeze dried.

N-deacetylated colominic acid was prepared by treatment with 10 N NaOH at 80 °C for 3 h [16]. The product was dialyzed against deionized water and freeze dried.

Solutions of colominic acid in K+, Ca^{2+} and Mg^{2+} forms were prepared as follows: 10 mg ml^{-1} of the Na^+ form was passed through the H^+ Dowex resin column (8 mm × 50 mm) and converted to the H^+ form (pH 2.5). The lactone formed at low pH values [17] was restored to the carboxyl group by the addition of the hydroxides (0.1 N solution of KOH, $Ca(OH)_2$ or suspension of $Mg(OH)_2$) to pH 10.5 at room temperature overnight. The solutions were then titrated to pH 7.0 using 0.1 N HCl. The absence of lactone was verified by Fourier transform IR (FTIR) spectroscopy (Model FTS 40 A, Bio-Rad) in 10 mg ml^{-1} solution at a wavenumber of 1740 cm^{-1}.

2.2. Measurement of molecular size

The molecular size of the colominic acid or its derivatives was estimated using high performance liquid chromatography (HPLC) with a Superose 12 column (Pharmacia, Piscataway, NJ) (1 cm × 30

cm) in 0.15 M phosphate buffer (pH 6.8), and using dextrans as molecular weight references. The degree of polymerization of oligocolominic acid ($n = 2$–17) was determined by HPLC using a Mono Q column (Pharmacia) in an NaCl gradient [18].

2.3. Viscometry

The viscosity of the colominic acid in the Na^+ form was measured using an Oswald viscometer (Sargent) in 0.04–1.0 M NaCl with the concentration of the molar residue charge well below that of the solvent. The intrinsic viscosity $[\eta]$ values were calculated by extrapolation of $\eta/c = f(c)$, where c is the concentrations of the colominic acid. $[\eta]$ was plotted against $I^{-1/2}$, where I is the ionic strength of the solvent, yielding a linear relationship from which the slope S was calculated.

A chain flexibility parameter B was calculated according to

$$B = S[\eta_{0.1}]^{-1.3} \quad (1)$$

where $[\eta_{0.1}]$ is the intrinsic viscosity at the ionic strength of 0.1 M NaCl [19]. The viscosity of the colominic acid was measured in water and in 0.15 M NaCl from 20 to 70 °C, at intervals of 10 °C.

2.4. CD spectroscopy

CD spectra were measured using a Jasco 720 spectrometer (Japan Spectroscopic Co.) with an optical path length of 2 mm and spectral range from 185 to 250 nm. The samples (0.2–1.0 mg ml^{-1}) were dissolved in different concentration of NaCl (0.04–1.0 M NaCl). The effect of the chain length on the conformation of oligosaccharides was analyzed according to the principle of optical superposition [20,21]. The ellipticity of an n-member polymer $[\theta]_n$ can be expressed as the sum of the ellipticities of the terminal units $[\theta]_t$ and central units $[\theta]$. In these calculation, three terminal units were chosen for $[\theta]_t$, because the NMR spectra showed that the end-effect begins to diminishes at $n = 4$ [6].

The total ellipticity was expressed as

$$[\theta]_n = [\theta]_3 + (n-3)[\theta]$$

$$\frac{[\theta]_n}{n} = \frac{[\theta]_3}{3} + \frac{([\theta] - [\theta]_3/3)(n-3)}{n} \quad (2)$$

The effect of the temperature on the CD spectra of the saccharides was measured between 5 and 70 °C (increasing in increments of 5 °C) in 0.04–1.0 M NaCl.

3. Results

3.1. Viscosity

The specific viscosity of the colominic acid was linear over a range of concentration from 0.1 to 2.0 mg ml^{-1} at 0.04–1.0 M NaCl, and yielded the intrinsic viscosities by extrapolation (data not shown). The intrinsic viscosity of the colominic acid was plotted against $I^{-1/2}$, where I is the ionic strength of the NaCl (Fig. 2). The slope S, estimated from fitting a straight line with linear regression, was 0.02. Using S and Eq. (1), the flexibility parameter B was calculated to be 0.62 (compared with values of 0.02 for Na^+ pectinate, 0.065 for hyaluronate and 0.23 for dextran sulfate). There was no detectable conformational transition induced by NaCl in the concentration range 0.04–1.0 M NaCl. Furthermore, the specific viscosities showed a steady decrease on increasing the temperature from 5 to 70 °C (data not shown), indicating that no temperature-induced conformational transition had occurred.

3.2. Circular dichroism

3.2.1. Resolution of dichroic bands

Colominic acid has two chromophoric groups, i.e. *N*-acetyl and carboxyl, both of which exhibit n → π^*

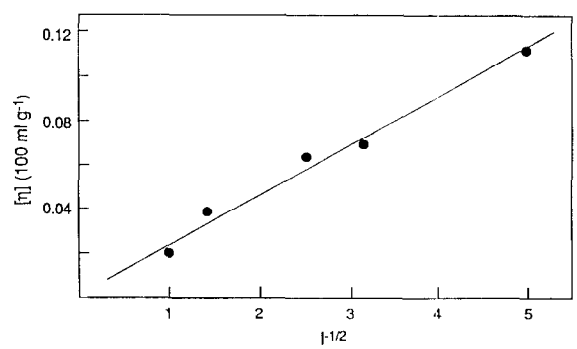

Fig. 2. Dependence of the intrinsic viscosity $[\eta]$ of the sodium salt of colominic acid on the ionic strength I of NaCl. Apparent mass, 22000.

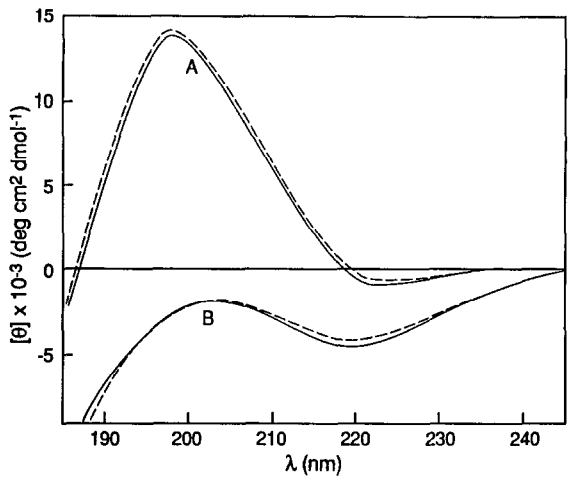

Fig. 3. CD spectra of (a) sodium colominic acid and (b) *N*-deacetylated polysaccharide at 5 °C (————) and 70 °C (– – –) in water.

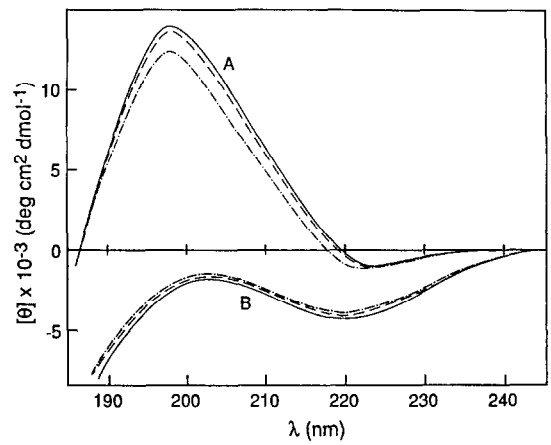

Fig. 4. CD spectra of (a) colominic acid and (b) *N*-deacetylated polysaccharide in Na+ (————), Mg^{2+} (– – –) and Ca^{2+} (– · – · –) forms.

electron transitions in the region 205–225 nm. The dichroic absorption of carboxyls generally occurs at a higher wavelength than is the case for the *N*-acetyl groups, which usually exhibit peaks of stronger intensity. To study the optical properties of the carboxyl group, the CD spectra of the *N*-deacetylated polysaccharides and of the colominic acid were measured and compared at temperatures from 5 to 70 °C (Fig. 3). The spectrum of *N*-deacetylated polysaccharide showed a negative first dichroic band, which represents the chiral environment of the carboxyl chromophore. In the colominic acid, the carboxyl band overlaps with the *N*-acetyl band. The resultant positive spectrum reflects a strong positive band that arise from the N–Ac group, which appears to be centered near 210 nm. The temperature change between 5 and 70 °C does not affect the general CD pattern of either polysaccharide.

3.2.2. *Effect of counterions*

The CD spectra of the colominic acid measured at different ionic strengths (0.04–1.0 M NaCl) did not exhibit any change (data not shown). The CD spectra of the native and *N*-deacetylated polysaccharides with Na^+ Ca^{2+} or Mg^{2+} as counterions are shown in Fig. 4. There is a small diminution in intensity between 210 and 250 nm for the Ca^{2+} form of colominic acid when compared with the Mg^{2+} or Na^+ forms. The CD spectrum of the *N*-deacetylated colominic acid was the same in the presence of the three different counterions.

3.2.3. *Effect of chain length*

The spectra of the oligomeric form of colominic acid ($n = 2$–17) are shown in Fig. 5. The monosaccharide, prevalently in the beta form, exhibits mainly a trailing positive end in the region 220–230 nm [22]. The CD spectrum of the $\alpha(2 \rightarrow 8)$ dimer has a low intensity, negative first dichroic band near 220 nm, which is characteristic of the chiral environment of the C′1 carboxyl group in the vicinity of the glycosidic bond. The intensity of this negative ellipticity increased with the degree of polymerization up

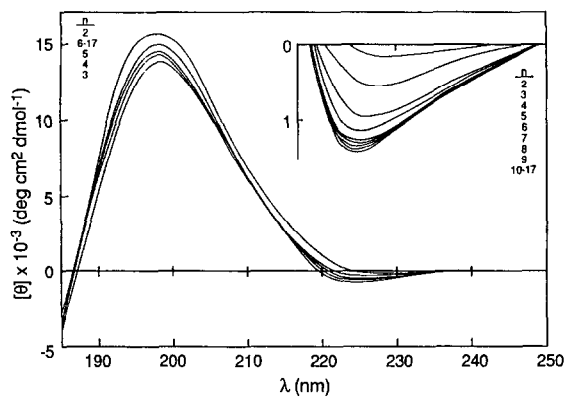

Fig. 5. CD spectra of sodium oligomeric colomonic acid ($n = 2$–17).

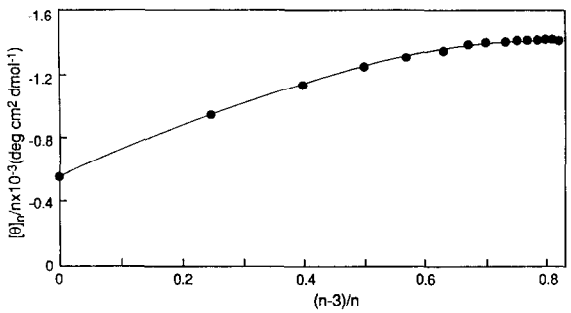

Fig. 6. Molar ellipticity $[\theta]_n/n$ plotted against $(n-3)/n$ for sodium oligomeric colominic acid ($n = 3$–17).

to $n = 9$. The molar ellipticities remain stable with increasing chain length from $n = 10$ to $n = 17$. According to the principle of optical superposition, the optical activity of polymers obeys the rule of additivity [21].

In the present study, the dependence of the molar ellipticity on the chain length n was analyzed as follows. To eliminate the effect of end-units, the analysis started with $n = 3$. The molar ellipticity was assessed at 225 nm to examine mainly the dichroic absorption of the carboxylic group. The dependence of $[\theta]_n/n$ on $(n-3)/n$ for sodium oligosaccharides and polysaccharides ($n = 3$–17) is shown in Fig. 6. For the linear Eq. (2), the intercept on the ordinate of the plot represents the mean value of the ellipticity of the terminal units. The slope expresses the difference between the average ellipticity of the internal and terminal units. For polymers with ordered structures, the slope should be constant for the length of the ordered structure [23]. For colominic acid, however, the slope of the plot showed a negative deviation from linearity, indicating that there is no extension or formation of an ordered structure as the chain length increases. As the length of the chain increases, the slope of the curve reaches zero, indicating that the distribution of conformations of internal units slowly reaches stability ($n \approx 9$).

4. Discussion

From the CD measurements, we have found that the colominic acid has no conformational transition over a wide range of salt concentrations. Furthermore, there is no cooperative (chelate) binding induced by divalent ions. In general, for a polyelectrolyte with every residue charged, various counterions could induce different binding states [24]. The insensitivity of the colominic acid to the type and strength of the ionosphere is probably because of the large charge spacing (about 0.6 nm) compared with other polyanions, such as polygalacturonic acid or nucleic acids. Also, there was no thermally induced conformational transition over a broad temperature range.

The presence of helical structures in colominic acid has been proposed from various measurements and energy calculations to provide a molecular basis for the recognition of the acid's antigenic binding. Here, from the CD measurements of the colominic acid at low concentrations (less than 4 mM), we have found that the spectra vary significantly from the monosaccharide to trisaccharides, and the variation diminishes when the chain length is longer than nine units: this is an indication that the distribution of conformers stabilizes at this length. This observation is consistent with the NMR measurements, which suggested that the relative distributions of conformers in the solution differ between oligomers and polymers [6]. This is also consistent with the observed minimum length required for this antigen to bind the antibodies successfully [10].

Brisson et al. [8] suggested that the length dependence for binding of the colominic acid oligosaccharides to specific antibodies resulted from the helical structure formed by internal units only when the chain is longer than a minimum of nine units. However, the monotonic trend of the intrinsic viscosity and the lack of change in the CD spectra over broad ranges of salt concentrations and temperatures observed here excludes the possibility of cooperative order–disorder conformational transition, such as helix–coil transition, induced by salt or by heat. Nevertheless, this observation does not exclude the possibility of having a family of local helices that have a different number of residues per turn with similar energy and that exhibit transitions between the helices [8]. It is probable that there is no prevailing conformation in colominic acid for binding but that the bound state is instead selected from more than one of the many energetically accessible conformations that bind the antibodies.

The intrinsic viscosity measurements indicated that colominic acid is more flexible than most of the highly charged polysaccharides. Calorimetric analyses of colominic acid binding to IgG monoclonal antibody [14] indicated that the entropy term compensates for more than half of the enthalpy of association. It was also shown that the association constants are strongly temperature dependent [11,12]. There is an entropy lost on decreasing the conformational freedom, as a result of the association of the two molecules. Because the colominic acid is highly flexible, the conformational change induced by binding does not require much energy. However, the release of solvent from the binding site could bring additional 'favorable' entropy. Overall, the weak binding at higher temperature can be attributed to the strong thermal motion of polysaccharide molecules, which gives the unfavorable increase in binding entropy.

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