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O-acetylation affects the binding properties of the carboxyl groups on the Vi bacterial polysaccharide

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Abstract

The capsular polysaccharide of *Salmonella typhi* and *Citrobacter freundii* (Vi) is a linear homopolymer of (1 → 4)-linked 2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid partially *O*-acetylated at the C-3 position. The physico–chemical properties of the carboxyl groups of the Vi polysaccharide, as a function of different degrees of *O*-acetylation, were studied by potentiometric titration, circular dichroism, and their reaction with the bulky nucleophile 2-nitro-phenylhydrazine (NPH). Potentiometric titrations with K^+ and Ca^{2+} hydroxides showed that the difference in the free energy of binding between the two cations (ΔG_K^{Ca}) was inversely proportional to the degree of *O*-acetylation. Similar cationic effects were found when measuring circular dichroism. Moreover there was also an inverse relation between the degree of *O*-acetylation and the extent of binding of NPH to the carboxyl groups. These data all indicate that *O*-acetyl groups hinder the association of carboxyls with cations and nucleophilic reagents. This provides a possible explanation for the importance of the *O*-acetyl and the relative unimportance of the carboxyl groups in contributing to the immunologic properties of the Vi.

Key words: *N*-acetylgalactosaminuronic acid, *O*-acetylation, Potentiometric titration, Binding free energy, Circular dichroism

1. Introduction

Salmonella typhi is the causative agent of typhoid fever. Its capsular polysaccharide (Vi) is both a virulence factor and protective antigen [1]. On the basis of field trials [2,3], the Vi has been certified as a vaccine for typhoid fever by the World Health Organization and in at least 30 countries [4].

The Vi is a linear homopolymer of poly (1 → 4)-linked 2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid [(1 → 4)-α-D-Gal*p*ANAc], partially *O*-acetylated at the *O*-3 position (Fig. 1) [5,6]. Native Vi can be isolated from *S. typhi* or from *Citrobacter freundii* and has 60% to 90% of its hydroxyl groups *O*-acetylated. It was shown that the *O*-acetyl and *N*-acetyl groups stabilize the Vi towards acid hydrolysis and are part of the

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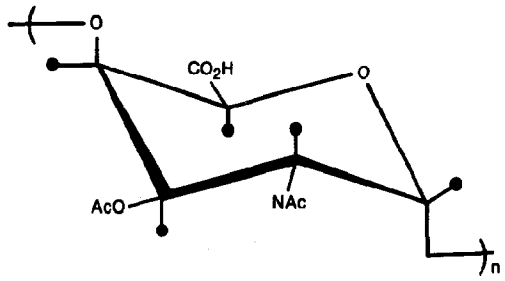

Fig. 1. Structure of the repeating unit of the Vi capsular polysaccharide of *Salmonella typhi*.

immunodominant determinant [7,8]. In contrast, the carboxyl groups have a lesser effect on the immunologic properties of the Vi [9]. Based on a Courtald–Koltun space-filling model, we observed that the bulky *O*- and *N*-acetyl groups dominate the molecular surface of the Vi and could shield the carboxyl groups from interaction with other molecules, present as counter ions in the solvent or as receptors on cell surfaces [7].

The structure of Vi is very similar to the main component of pectin, a common plant polysaccharide [10], which is a poly(1 → 4)-linked α-D-galactopyranosyluronic acid [(1 → 4)-α-D-Gal*p*A] some whose carboxyl groups are esterified. The linear charge densities of Vi and the nonesterified portion of pectin are similar. However, physicochemical characteristics of these two polyacids are different. For example, pectin, but not the Vi, can induce helix formation in poly L-lysine [11,12]. Pectin is not easily solubilized in the presence of chloride ions and forms a gel in the presence of di- or tri-valent cations [13]. The Vi is soluble in chloride and does not form a gel in the presence of multivalent ions [1].

In general, the binding of multivalent cations with polyanions consists of two major types: (1) Electrostatic binding of the cations with adjacent negative charges on the polyanions. This type of binding has the same free energy of binding as the monovalent cation [14]. (2) Intermolecular cooperative binding or the chelating effect [14,15]. In the Vi, the cooperative binding would depend on the accessibility between intermolecular carboxyl groups. Therefore, if carboxyl groups were sterically blocked, the chelating effect would be correspondingly reduced. In order to understand the effects of *O*-acetylation on the carboxyl-accessibility, we used K^+, Ca^{2+} as well as a bulky nucleophilic reagent, 2-nitro-phenylhydrazine (NPH) to characterize binding properties [16]. The difference in free energy of cations binding and dissociation were calculated from potentiometric titrations [17]. The effect of cations on the conformation of the Vi was studied with circular dichroism (CD). The reactivity with NPH was measured by spectrophotometry. These findings were compared with the results obtained when pectin was used instead of Vi.

2. Materials and methods

2.1. Polysaccharides

The Vi, purified from *Citrobacter freundii* WR7011, contained less than 1% w/w of protein, nucleic acid or lipopolysaccharide [7]. The molecular size of Vi, assayed by gel filtration through Sephacryl S-1000, was ≈ 2000 kD using dextrans as standards. The Vi was dialyzed extensively against 5 mM EDTA, deionized water and freeze-dried. The final concentration of Ca^{2+} was less than 1% w/w as determined by elemental analysis [18].

In the native Vi 65% of the hydroxyl groups were acetylated. Vi preparations, possessing various degrees of *O*-acetylation, were obtained by treatment of the native polysaccharide with 0.2 M to 0.5 M NH_4OH at 37°C for 1 and for 18 h [7]. The pH was brought to 7.4 with phosphate-buffered saline to terminate the *O*-deacetylation, and samples were dialyzed at 3°C to 8°C against 0.15 M NaCl, deionized water, and freeze-dried. The *O*-acetyl content was assayed by the Hestrin reaction [19] using an acetylcholine chloride standard (Sigma Chemical Co, MO) and expressed as the percent of *O*-acetyl groups per galactouronic acid. Pectin from citrus (Genu Pectin, Copenhagen Pectin Co., Denmark) containing < 10% neutral sugars and < 1% methyl ester was dissolved in water at 60°C, precipitated with 75% ethanol and freeze-dried.

2.2. Potentiometric titration

Native and *O*-deacetylated Vi were converted to the acidic form by dialysis against H^+ Dowex 50 × 8 ion exchange resin (Fluka AG, Ronkonkoma, NY) in deionized water for 2 days at 3–8°C. The pH of the outer fluid was between 3.5–4.0. This method was chosen over a Dowex resin column because the *O*-acetyl groups were hydrolyzed when in direct contact with the acidic surface of the resin. Residual Cl^- and Ca^{2+} were assayed, respectively, by precipitation with silver nitrate (Mallinckrodt, Paris, KY) and by reaction with the murexide indicator (Sigma Fine Chem., St. Louis, MO) [20]. The Vi in the H^+ form was titrated to neutrality with 0.01 N hydroxides of Ca^{2+} or K^+ (Aldrich Chem. Co., Milwaukee, WI) with a Markson pH meter (Model 88) and Beckman glass electrode (Model S208A). The normality of the hydroxides was standardized with 0.1 N HCl (volumetric standard, Aldrich). The polysaccharide solutions remained clear during the titration. Data were analyzed according to the standard equation for the ionization reaction [17]

$$RCOOH + H_2O \leftrightharpoons RCOO^- + H_3O^+. \quad (1)$$

The pK of the Vi was calculated as $pK = pH - \log[\alpha/(1-\alpha)]$, where α is the degree of dissociation

$$\alpha = \frac{[RCOO^-]}{[RCOOH] + [RCOO^-]}. \quad (2)$$

The electrostatic free energy of proton dissociation (ΔG) during neutralization in relation with the pK is

$$\Delta G = 2.3\, RT \int_{\alpha=0}^{1} (pK - pK_0)\, d\alpha \quad (3)$$

The free energy for Ca^{2+} and K^+ exchange, ΔG_K^{Ca}, was derived from the difference in ΔG, or the area enclosed by the titration curves of the two counter ions [14,17].

2.3. Circular dichroism

Solutions of the Vi as the K^+ or Ca^{2+} salt were obtained by titration of the free acid to the point of equivalence, using the corresponding hydroxides. The concentrations of the polysaccharides used were between 0.5 to 1.5 mg/ml. Samples were equilibrated at room temperature for 24 h prior to measurements to avoid temperature dependent conformational changes [21]. Spectra were measured at room temperature with a JASCO-720 spectropolarimeter (Japan Spectroscopic Co.) from 230 to 185 nm using cells of 0.2 cm path length. Optical density of samples were less than 1.0 (photomultiplier voltage < 0.5 kV) to ensure low level of noise. Data on molar ellipticity were averaged over ten scans and spectra were smoothed by a built-in filtration method.

2.4. Binding with 2-nitro-phenylhydrazine (NPH)

The binding activity of carboxyl groups in the native and *O*-deacetylated Vi with a nucleophilic reagent was measured with NPH [16]. Saccharide solutions (0.1 to 0.5 mg/mL) were mixed with NPH (10 mM in 0.15 M HCl) and the reaction mixture was then activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) (10 mM in 2% pyridine). The pH of the reaction mixture was adjusted to 12 with 1 N NaOH and the absorbance at 530 nm was measured. Reaction of NPH with galacturonic acid and galactose was measured for comparison.

3. Results

3.1. Potentiometric titration and binding free energies

Potentiometric titrations of the native Vi (65% OAc) with KOH and $Ca(OH)_2$ are graphically shown in Fig. 2. The difference in the free energies of binding between Ca^{2+} and K^+ (ΔG_K^{Ca}) were calculated from the area enclosed by the two titration curves and was 0.24 kcal/mol. At a low level of *O*-acetylation (2% O Ac), the titration curve of Ca^{2+} was significantly lowered and thus increased the area enclosed by the two counter ions ($\Delta G_K^{Ca} = 0.68$ kcal/mol) (Fig. 3). Table 1 shows that the values of ΔG_K^{Ca} increased as the degree of *O*-acetylation of the Vi decreased.

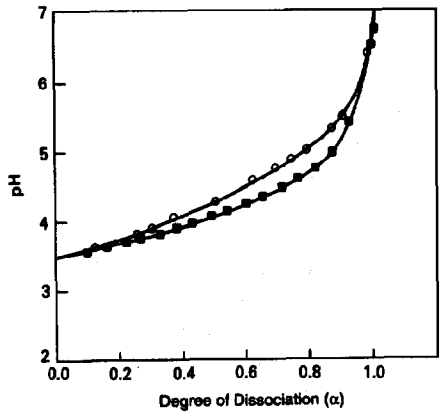

Fig. 2. Titration curves of Vi polysaccharide (65% *O*-acetyl) with (○) KOH or with (■) $Ca(OH)_2$. The pH was plotted against the degree of dissociation α during the neutralization of polysaccharide.

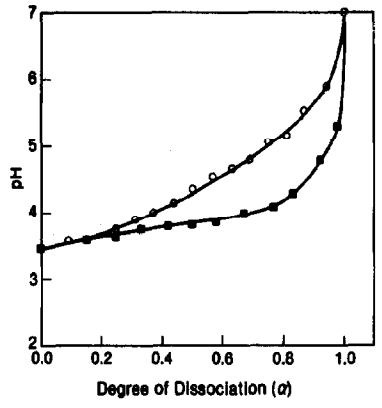

Fig. 3. Titration curves of Vi polysaccharide with 2% *O*-acetyl with (○) KOH or with (■) $Ca(OH)_2$. The pH was plotted against the degree of dissociation α during the neutralization of polysaccharide.

Table 1
Free energy of counterion exchange between Ca^{2+} and K^+ of Vi at various degree of *O*-acetylation

Vi [a] (mM)	*O*-acetyl	ΔG_K^{Ca} [b] (kcal/mol)
1.60	2	0.68
1.70	45	0.50
3.95	62	0.39
1.50	65	0.24

[a] Concentration expressed as moles of *N*-acetyl-2-deoxy-2-amino galacturonic acid.
[b] Difference in binding free energies between Ca^{2+} and K^+ forms calculated from areas enclosed in the potentiometric titration curves.

The titration curves for pectin were similar to those obtained with the *O*-deacetylated Vi (data not shown). The difference between the value of the binding free energy of pectin for K^+ and for Ca^{2+}, $\Delta G_K^{Ca} = 0.9$ kcal/mol, is in the same range as that of the *O*-deacetylated Vi [14].

3.2. Conformational analysis by circular dichroism

The CD spectra for the native Vi (65% *O*-acetylated) in the K^+ and Ca^{2+} forms are shown in Fig. 4. The value of ellipticity at the maximum was lower for the K^+ and Ca^{2+} forms than for the acidic form, due in part to the more symmetric electron distribution of the ionized form of the carboxyl groups [22]. The spectra for Ca^{2+} and K^+ forms were identical. At 1% *O*-acetylation, the ellipticities of all three ionic forms of the Vi were reduced when compared to the CD of the Vi that was 65% *O*-acetylated: 17% for acid, 20% for K^+ and 38% for Ca^{2+} forms (Fig. 5). In contrast to the native Vi, there was an evident difference in the CD spectrum between the Ca^{2+} and K^+ forms ($[\theta]_{Ca}/[\theta]_K = 0.75$ at 192 nm). The decrease in ellipticity shown in the Ca^{2+} form may arise from a more ordered steric arrangement of the complex due to the cooperative binding of the carboxyls groups.

The CD spectra of pectin also showed that the Ca^{2+} form had lower ellipticity than did the K^+

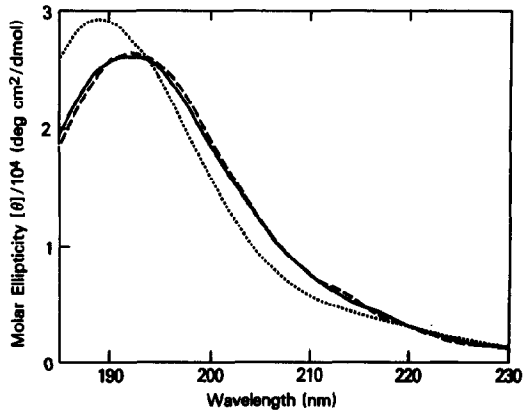

Fig. 4. Circular dichroism spectra of Vi (65% *O*-acetyl) in (·····) acidic form, in (———) Ca^{2+} and (— — —) K^+ form.

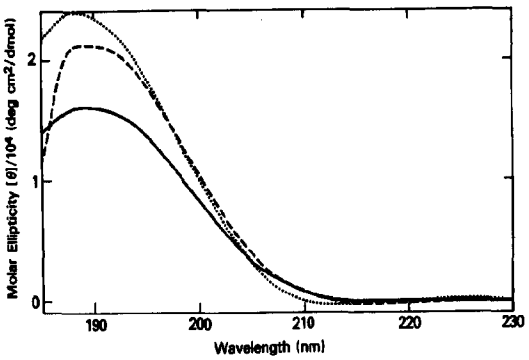

Fig. 5. Circular dichroism spectra of Vi (1% *O*-acetyl) in (·····) acidic form, in (———) Ca^{2+} and (— — —) K^+ form.

form [13]. There was a hypsochromic shift at the maximum: at 201 nm for the K^+ form and at 194 nm for the Ca^{2+} form (data not shown).

3.3. The reactivity of carboxyl groups with 2-nitrophenyl hydrazine (NPH)

The reactivity of the carboxyl groups of Vi at different levels of *O*-acetylation was evaluated by binding studies with NPH (Table 2). The binding of NPH with the native Vi was about 29% of that observed with galacturonic acid (Table 2). As the number of *O*-acetyl groups of the Vi decreased, the amount of NPH bound to the Vi increased: Vi with a 1% *O*-acetyl content showed 52% of the binding activity of that of galacturonic acid, and was similar to the value observed with the pectin.

Table 2
Binding of the native Vi and *O*-deacetylated (De-*O*-Ac) Vi with 2-nitrophenylhydrazine (NPH)

Saccharide	Concentration saccharide [a] (mM)	*O*-acetyl (%)	Absorbance at 530 nm
Vi	0.75	65	0.410
De-*O*-Ac Vi	0.84	1	0.752
pectin	0.93	0	0.696
GalA	0.94	0	1.438
Gal	1.11	0	0.005

[a] Expressed as moles of galacturonic acid.

4. Discussion

All bacterial polysaccharide antigens posses repetitive antigenic sites. Immunologic properties of polysaccharides can be influenced by the substituents such as carboxyl or acetyl groups. Vi polysaccharide is interesting, since it is a homopolymer and possesses a high, linear charge density as well a high content of bulky acetyl substituents. By physico-chemical methods, we have studied the influence of the *O*-acetyl groups on the accessibility to the carboxyl groups on the Vi.

Potentiometric titration showed that when the Vi was highly *O*-acetylated, the binding of the carboxyl groups to Ca^{2+} and K^+ was similar. The binding of the monovalent and the divalent counter ions became different as the level of the *O*-acetylation was decreased. This change indicated that the chelating binding of divalent ions was possible only when the steric hindrance exerted upon the carboxyl groups by the *O*-acetyl groups was removed. A phenomenological description of the dissociation of weak polyacids can be found in Manning's theory [23], where it is shown that counterion binding properties of polyanions are affected by linear charge density. In the case of Vi, where the distance between the charged units is ≈ 0.43 nm [24], the counterions undergo condensation onto the polyanionic chain and accordingly the electrostatic contribution to the free energy of binding depends mainly on the cation concentrations. Structural diversifications due to non-ionic groups are not taken into account. The linear charge density of native and *O*-deacetylated Vi are likely to be the same. Yet we have observed different cationic binding properties. This suggests the possibility that the binding activity of the carboxyl groups may be affected sterically by the acetyl groups that are in close proximity. Issac et al. compared the structure of the *O*-deacetylated Klebsiella K5 polysaccharide, containing glucuronic acid, with the native (acetylated) form. They found that the *O*-deacetylated K5 has the same backbone conformation as the native even thought the interchain packing arrangements are different [25].

Therefore, it is likely that the backbone conformation of the *O*-deacetylated Vi is the same as the native one.

We examined the CD spectra of the native and the *O*-deacetylated Vi. The nature of conformational changes induced by different counter ions is difficult to identify because the CD spectrum of Vi consists of contributions from three chromophoric groups in this region of ultraviolet: *N*-acetyl, *O*-acetyl and carboxyl groups [26,27]. In addition, the ellipticity from cross interactions between these chromophores would be reflected in the same range of wavelengths. Therefore, removing *O*-acetyl groups or modifying the carboxyl environment may influence the CD spectrum in multiple ways. The CD spectra showed that the effects of counter ions on the asymmetric absorption of the Vi chromophores depended on the level of *O*-acetylation. When Vi was highly *O*-acetylated, its conformation was the same when associated with either counterion Ca^{2+} or K^{+}. When the *O*-acetyl groups were removed, the CD for the Ca^{2+} form was reduced when compared with that of K^{+}. The reduction in CD is probably due to changes of the geometric factor in the vicinity of carboxyls induced by the chelating effect from Ca^{2+}. The effect of Ca^{2+} on the conformation of Vi is similar to that of pectin, where it causes chain association and gel formation [13].

The data from potentiometric titration and CD were consistent with the binding activity of Vi with the bulky nucleophilic reagent NPH. There was an inverse relation between the degree of *O*-acetylation and the amount of binding of NPH to the carboxyl groups.

The immunogenicity of the Vi is related to its content of *O*-acetyl groups [18]. Decreasing the *O*-acetyl content to $\approx 1\%$ results in a polysaccharide that fails to elicit antibodies which are reactive with the native Vi [7,28]. In contrast, little or no change in the immunologic properties is observed when the carboxyl groups are reduced [7].

The importance of the *O*-acetyl groups in determining the immunologic properties of the Vi is also observed with pectin. Pectin neither reacts with Vi antiserum nor elicits antibodies reactive with the Vi. Di-*O*-acetylated pectin, which resembles the Vi except that the *N*-acetyl of the latter is replaced with an *O*-acetyl, showed immunochemical properties similar to those of the Vi [9].

The capsular polysaccharides of most pathogenic bacteria (i.e. pneumococci, meningococci, *Haemophilus influenzae*, *Escherichia coli*, group B streptococci) are negatively charged due to either carboxyl or phosphodiester groups [29]. For those polysaccharides that possess them, carboxyl groups dominate the immunologic properties [30,31]. The much lesser role of the carboxyl groups in determining the immunologic properties of the Vi, therefore, is unusual.

The *O*-acetyl group is also an important determinant affecting the immunological properties of other bacterial polysaccharides. For example the immunologic and enzymatic properties of pneumococcal type 1 and *E. coli* K1 were changed upon *O*-deacetylation [32,33]. The physical–chemical characterization of the *O*-acetyl groups and their steric interactions with carboxyl groups in these polysaccharides are important for understanding polysaccharide's immunological properties.

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