On the pH-induced conformational transition of the exocellular polysaccharide from *Rhizobium trifolii* strain TA-1*

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The pH-induced conformational transition of the exocellular polysaccharide from *Rhizobium trifolii* strain TA-1 (TA1EPS) has been studied by chiroptical, calorimetric and potentiometric methods. By lowering the pH of neutral aqueous TA1EPS solutions, the specific rotation shows an abrupt change from positive to negative values of $[\alpha]$. The same trend is also observed in the molar ellipticity. No evidence of a pH-induced change in the chain conformation of TA1EPS has been noticed using aqueous 40 mM NaCl as solvent. From isothermal calorimetric and differential scanning calorimetric experiments, the value of the enthalpy change for the pH-induced ordering process is $-6\,kJ\,equiv.^{-1}$. Potentiometric titration data suggest that the pH-induced transition involves only a small change in the overall charge density. The possible differences between the ordered form at low pH and that previously found in salt solution are discussed.

(Keywords: microbial polysaccharides; *Rhizobium trifolii* exocellular polysaccharide; solution thermodynamics; calorimetry; chiroptical properties; potentiometric titrations; conformational transition)

INTRODUCTION

The repeat unit (*Figure 1*) of the exocellular polysaccharide from *Rhizobium trifolii* strain TA-1 (TA1EPS) comprises eight sugar residues, with four glucosidic units in the backbone. It features two consecutive D-GlcA*p* residues linked $\beta(1\rightarrow4)$ in the backbone and two more charged centres (two pyruvyl groups) in the side arm[1–5]. TA1EPS chains thus have a rather high 'charge density', built up by two different types of regularly positioned carboxylate groups of slightly different acid strength. This may explain the high solubility of TA1EPS in water and in aqueous salt solutions, as well as evidence indicating that in water (pH 7, room temperature) the charged polysaccharide has an expanded, coil-like conformation[6–8].

However, reduction of Coulombic repulsions along TA1EPS chains—either by protonation or the screening exerted by added, excess counterions—can induce the chains to assume an ordered, helical conformation as allowed by the regular enchainment of the constituent sugar residues. The influence of added NaCl on the conformational state of TA1EPS in dilute aqueous solution has already been studied in detail[6–8]. We wish to report here the results obtained by investigating the solution behaviour of TA1EPS as a function of pH by means of chiroptical, calorimetric and potentiometric experiments.

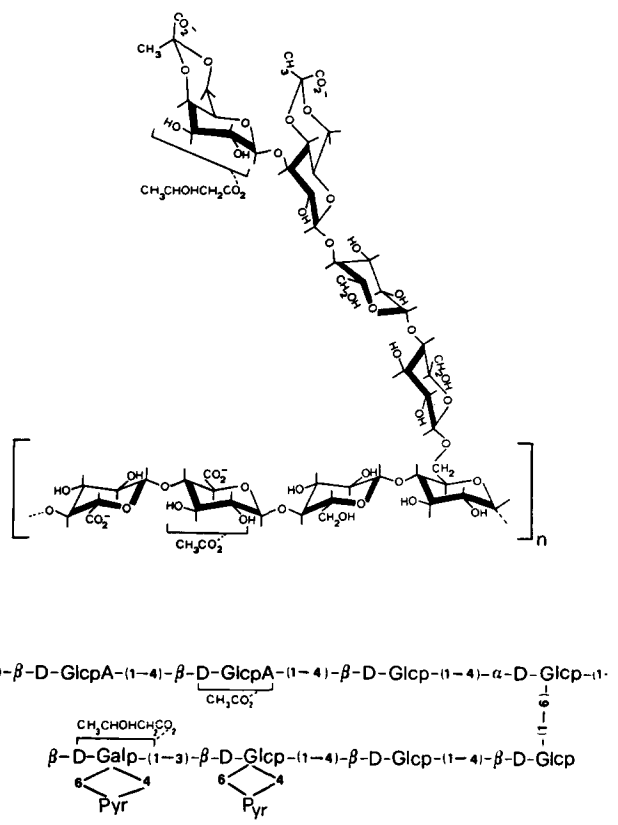

Figure 1 Repeat unit of TA1EPS (acetyl and hydroxybutyryl substitution is also shown)

* Dedicated to Professor Walther Burchard on the occasion of his 60th birthday

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EXPERIMENTAL

The exocellular polysaccharide from *Rhizobium trifolii* strain TA-1 (TA1EPS) has been isolated at the Department of Microbiology of the University of Wageningen, The Netherlands, by Professor L. P. T. M. Zevenhuizen[1]. The equivalent weight of TA1EPS, determined by potentiometric titration data, is 396, in agreement with structural data.

Optical activity measurements were performed with a Perkin–Elmer 241 polarimeter using a 10 cm path-length, thermostatted cell. Circular dichroism spectra were recorded by using a Jasco (model J500) dichrograph. The molar ellipticity $[\theta]$ was calculated on the basis of the polymer concentration, expressed in equivalent per litre. In the chiroptical experiments, solutions of the polysaccharide at different degree of protonation were prepared by mixing polymer and $HClO_4$ solutions and waiting a suitable time before reading the equilibrium value. In all the experiments, the pH of the solution was measured in order to evaluate the actual degree of protonation α_p after correcting for the amount of free protons.

Isothermal calorimetric heat-of-mixing experiments were performed with a heat-flow LKB batch microcalorimeter (equipped with golden twin cells) at 25°C. All calorimetric measurements were experimentally corrected for the dilution effects by matching in the reference cell dilution of the $HClO_4$ solution. The heat of dilution of the polysaccharide solution was determined in a separate experiment. Integration of the thermograms was made on a timescale sufficiently long to ensure complete evolution of the reaction, as suggested by the polarimetric kinetic data.

Potentiometric titrations were carried out by using a Radiometer PHM62 pH meter and a Radiometer combination calomel–glass electrode (type GK2401C), calibrated with standard buffers at pH 4, 7 and 10. Solutions of TA1EPS, in the protonated acid form, were titrated potentiometrically with 0.1 N NaOH at 25°C.

The results of the potentiometric titrations were analysed to obtain the actual degree of ionization α and the apparent $pK_a(\alpha)$ by using the relations:

$$\alpha = \alpha_n + [H^+]/C_p$$

and

$$pK_a(\alpha) = pH - \log[\alpha/(1-\alpha)]$$

where α_n is the degree of neutralization and C_p the polymer concentration (equiv.l^{-1}).

However, a cursory inspection of the titration curve reveals a 'bump' in the higher range of degree of neutralization (α_n) values, which was taken as a possible indication of a partial separation of the titration of the two different classes of acidic groups along the polysaccharide chains. This possibility was not excluded *a priori*, although pyruvyl groups and glucuronic residues have only slightly different intrinsic pK_0 values (2.5 and 3.2, respectively, for the monomeric analogues[9]).

Therefore, treatment of the titration data has first been attempted by using the approach outlined by Dubin and Strauss[10] for a poly(diprotic) acid. The degree of dissociation α' of a poly(diprotic) acid is then defined to reach the value 1 when the first set of acidic groups (pyruvic) are titrated and to reach the value 2 at the equivalence point. Extrapolation of the $pK_a(\alpha)$ smooth function to $\alpha'=0$ and to $\alpha'=1$, that is to the midpoint of the titration curve, gives for the intrinsic pK_0 of pyruvyl and glucuronic carboxylic groups the values of 2.3 and 3.2, respectively. These figures are consistent with those reported for the corresponding 'monomers'[9]. However, simulation pH curves for a diprotic and a poly(diprotic) acid, calculated with equation (2) reported by Dubin and Strauss[10], systematically give for $0<\alpha'<2$ a single inflection whenever the difference $(pK_2 - pK_1)$ is less than 1.5. Consequently, the analysis of the experimental data has been made by assuming that the acidity of the two carboxylate groups is not resolvable potentiometrically.

RESULTS

Chiroptical data

In order to appreciate better the TA1EPS protonation/dissociation data to be discussed in the following, it is expedient to recall briefly that the polysaccharide (Na^+ salt) in water is in a disordered chain state, but that upon addition of NaCl it assumes even for salt concentrations below 30 mM an ordered conformation characterized by a specific rotatory power $[\alpha]_{302} = -130°$, which remains unchanged by further increase in ionic strength at 25°C[6–8].

Protonation data of *Figure 2* then clearly show that with increasing degree of protonation α_p (by addition of $HClO_4$), in the range $0.14<\alpha_p<0.30$, the specific rotatory power at 302 nm of TA1EPS in water (at 25°C) changes abruptly, reaching a plateau value of −104°. This demonstrates the occurrence of a rather cooperative, pH-promoted conformational change of the polysaccharidic chains (in the absence of added electrolyte), qualitatively resembling the salt-promoted process[6–8].

When the same kind of experiment is carried out in 40 mM NaCl (25°C) the initial optical activity value ($[\alpha]_{302} = -130°$) is only little affected by $HClO_4$ additions. From what was said above, this indicates that the salt-stabilized ordered state of TA1EPS chains would be insensitive to changes in pH.

We have also found that for α_p values within the above critical range the optical activity change is rather slow (in water, 25°C). In fact, the phenomenon can be easily followed by pH jump, conventional polarimetric measurements, as shown by the example illustrated in *Figure 3*. In view of this, care was taken in all experiments to collect data at equilibrium. On the other hand, for $\alpha_p>0.4$, the optical activity changes become quite fast, suggesting that chain ordering becomes distinctly faster the lower the final pH.

The circular dichroism spectra reported in *Figure 4a* show that protonation data collected in 40 mM NaCl can be simply related to carboxyl-group ionization equilibria, as indicated by the isodichroic point at 223 nm and by the linear relationship between the ellipticity at 209 nm and α_p (insert). Data collected for a similar experiment in water (*Figure 4b*) exhibit a more complex, irregular trend. Not only do the chiroptical features of carboxylic chromophores change upon protonation, but it is also clear from the absence of an isodichroic point that more than two states are involved in the overall equilibrium. In line with the optical activity evidence mentioned above, we ascribe these c.d. findings to the pH-induced conformational change of the polysaccharidic chains. Such a change, however, appears less clearly

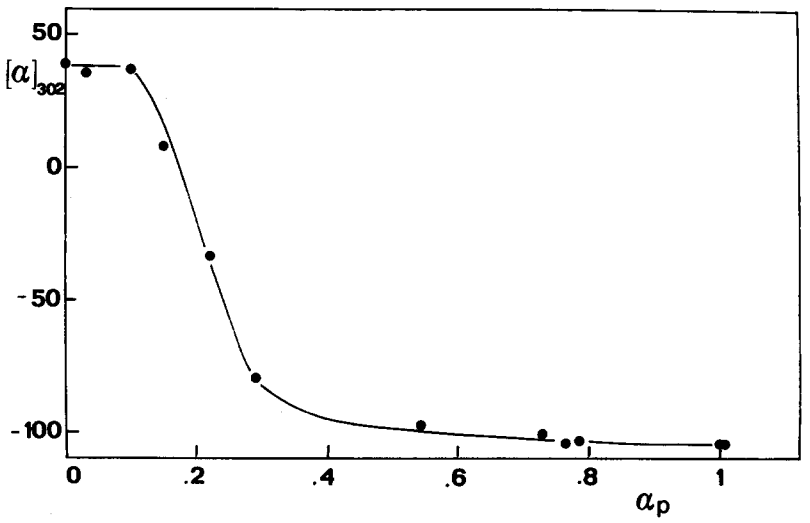

Figure 2 Specific optical activity $[\alpha]_{302}$ of TA1EPS aqueous solutions at 25°C as a function of the degree of protonation α_p obtained by adding $HClO_4$. Polysaccharide concentration, $C_p = 1.2 \times 10^{-3}$ g cm^{-3}

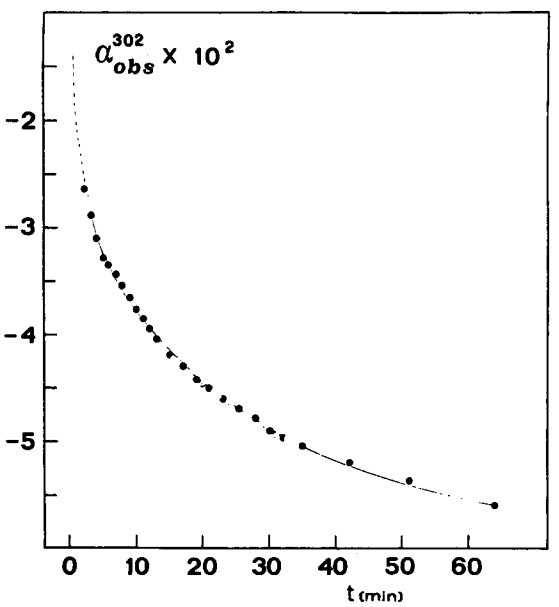

Figure 3 Variation of optical activity with time of TA1EPS aqueous solutions subsequent to the addition of $HClO_4$ to reach $\alpha_p = 0.20$. $C_p = 1.3 \times 10^{-3}$ g cm^{-3}

defined by c.d. data owing to the fact that in water pyruvyl groups and glucuronic residues give overlapping contributions to the observed spectrum as a function of α_p.

Calorimetric data

In 40 mM NaCl at 25°C the integral enthalpy of protonation of TA1EPS is always positive and an increasing function of α_p (*Figure 5*). On the basis of all other data the enthalpy change must arise almost exclusively from the protonation of carboxylic groups. The behaviour of TA1EPS in water (*Figure 5*) is quite different. Superimposed on the endothermic protonation process, there occurs an exothermic process, i.e. a conformational change. The calorimetric data also indicate that the latter would be complete at $\alpha_p \simeq 0.4$, and permit one to evaluate for the associated enthalpy change a value of $\Delta H_{298K} \simeq -6.5$ kJ equiv.$^{-1}$.

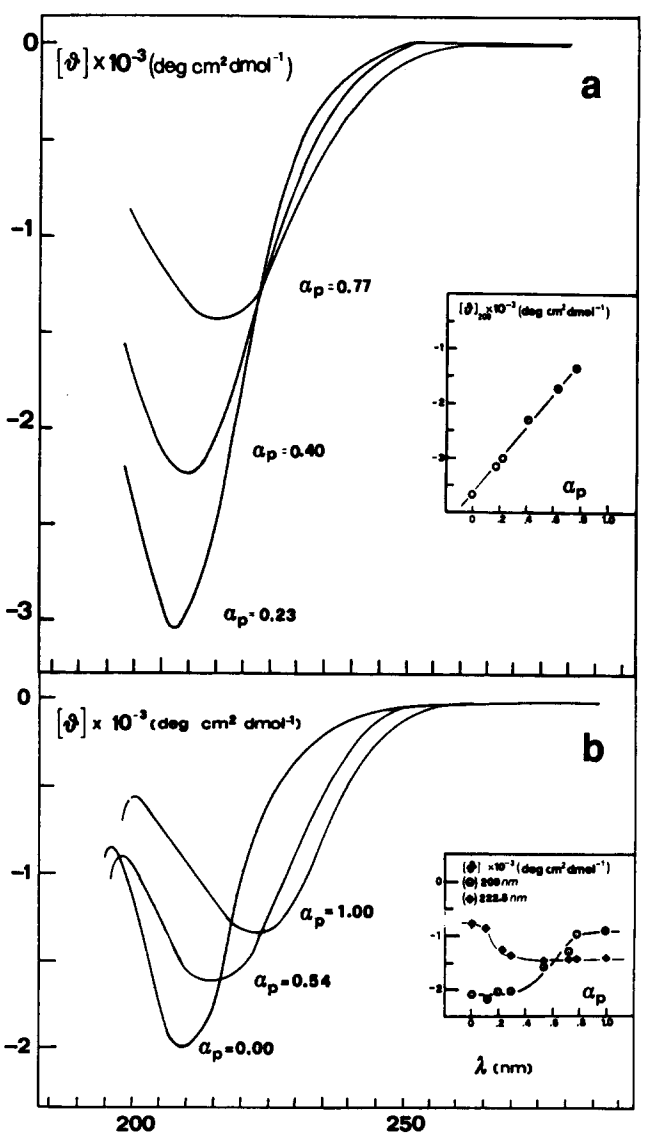

Figure 4 Circular dichroism spectra of TA1EPS aqueous solutions at different values of α_p in (a) 40 mM NaCl and (b) water. Inserts: molar ellipticity $[\theta]$ at (a) 209 nm and (b) 209 and 222.5 nm as functions of α_p. $C_p = 1.2 \times 10^{-3}$ g cm^{-3}

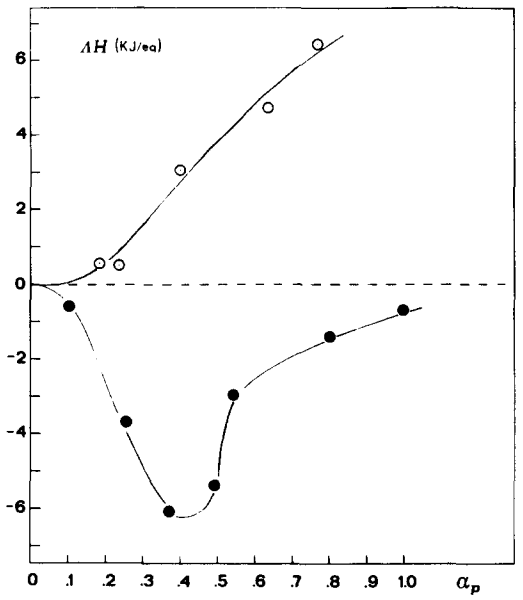

Figure 5 Isothermal, integral, enthalpy change (at 25°C) on mixing aqueous TA1EPS solution with aqueous $HClO_4$ as a function of the final α_p value: (○) in 40 mM NaCl; (●) in water. $C_p = 1.3 \times 10^{-3}$ g cm^{-3}

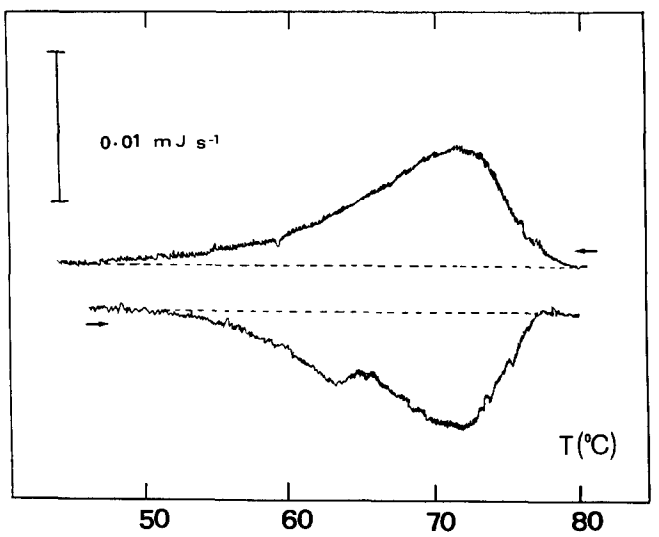

Figure 6 Heating and cooling thermograms obtained by differential scanning microcalorimetry of TA1EPS aqueous solution in 1.9×10^{-3} M $HClO_4$. Scan rate 0.2°C min^{-1}. $C_p = 2 \times 10^{-3}$ g cm^{-3} (Micro DSC, Setaram)

The occurrence of a temperature-induced disordering process was followed by differential scanning calorimetry. The d.s.c. thermograms of *Figure 6* obtained by using a high-sensitivity scanning microcalorimeter demonstrate that the ordered chain state of TA1EPS at pH 3 is disrupted upon heating with a characteristic peak temperature of 72°C. The thermal transition is totally reversible, with no hysteresis on cooling, while the latter phenomenon was always observed[6] in the thermal cycles in the presence of NaCl. From the area integrated under the thermogram, the transition enthalpy $\Delta H_{345K} = -6$ kJ equiv.$^{-1}$ (the negative sign is relative to the ordering cooling run). This value is very close to that reported for ΔH_{298K}, in view of the possible contribution of a ΔC_p term and of the overall experimental uncertainty.

Potentiometric data

The potentiometric titration data for TA1EPS in water at 25°C are typical of a weak polyacid and show a continuous variation of pH from 3 to 5 before the steep rise at the equivalence point. However, the pK_a *versus* α curve of TA1EPS in water (*Figure 7a*) shows a peculiar broad curvature in the range between $\alpha = 0.7$ and 0.9 (before rising near $\alpha = 1$ due to the obvious inapplicability of the pK_a equation). Evidence afforded by chiroptical and calorimetric data lead us to ascribe the above-mentioned curvature to a charge-induced conformational change, which relaxes the TA1EPS chain in terms of charge density, when the total fraction of charged groups rises above 0.7. Evaluation of the distribution function of the dissociated forms of pyruvyl and of glucuronic carboxylic groups indicates that the transition process starts approximately after half of the glucuronic residues in the main chain are dissociated.

To the contrary, the same type of pK_a plot for TA1EPS in 40 mM NaCl (25°C) exhibits no anomalies, in agreement with the finding that, in such conditions and at neutral pH, TA1EPS chains assume a stable, ordered conformation, which may be destabilized only by increasing the temperature[6–8].

In an attempt to characterize the two different conformational states, the excess electrostatic free energy (corresponding to the difference $pK_a - pK_0$) has been calculated for the ordered structure proposed from the X-ray fibre diffraction studies[11]. The description of the theoretical model of semiflexible polyelectrolytic chains

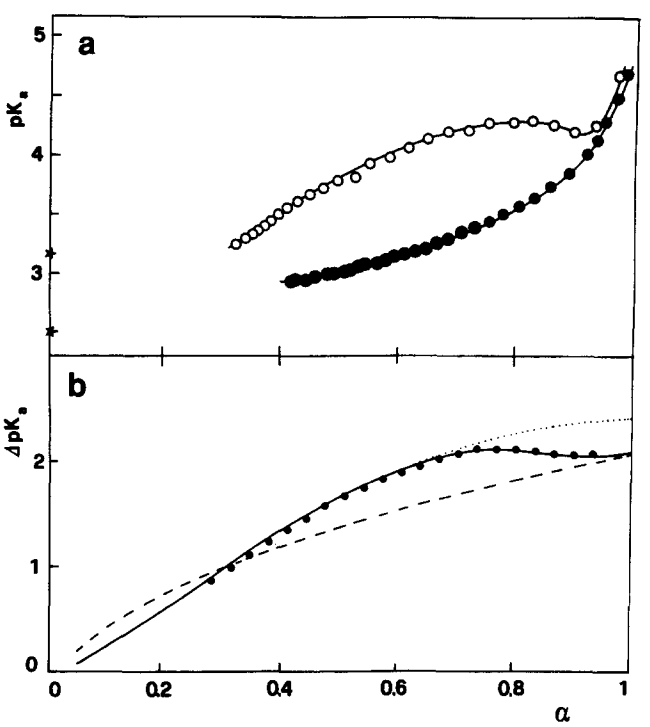

Figure 7 (a) Experimental pK_a curves as a function of the degree of dissociation α. Data refer to proton potentiometric titration in water (○) and in 40 mM aqueous NaCl (●). The pK_0 values of pyruvyl and glucuronic monomers are also shown (*). (b) Theoretical modelling (according to ref. 12) of the ΔpK_a curve of TA1EPS in water. Points are the interpolated experimental data; curves refer to the ΔpK_a curve of disordered form (– – –), of ordered form (· · · ·) and of the polyacid undergoing the conformational transition (——). The simulated curve has been obtained with a free-energy difference of 1.2 kJ equiv.$^{-1}$ and a cooperative length of four repeat units

and the derivation of the equations are given elsewhere[12]. No allowance for the chemical difference of the two charged groups is made in the theoretical approach, since only the excess electrostatic contribution is predicted.

The full curve reported in *Figure 7b* is the only possible fit of the experimental data of TA1EPS in water. It has been obtained by imposing that the chain backbone is quite rigid (as given by the conformational rigidity factor[12], $k_0 = 0.2\,\text{kcal mol}^{-1}\,\text{Å}^{-2}$) and that the side-chains do not contribute significantly to the excess polyelectrolytic properties. Although this may seem surprising at first sight, it may simply mean that charging the pyruvyl groups of side-chains moves them apart from the chain axis to a distance where their charge repulsions can easily be screened. Consideration of all four charges per repeat unit in the polyelectrolytic model always gives a very poor fit (not reported). More structural detail on the ordered and the random conformations would be necessary for the analysis of the energetics of the transition process.

In conclusion, the presence of two different structures in water, at low and high pH, can be considered as established also from the pK_a data, even if the anomaly in the experimental curve of *Figure 7* does not provide a clear-cut α range for the occurrence of the transition. This anomaly is, however, more pronounced than that of poly(galacturonic acid)[13], so far the only reported case of a polysaccharide with pK_a *versus* α curves showing a pH-induced shape transition.

DISCUSSION

The set of data reported above convincingly show that at low pH and at 25°C the chains of TA1EPS polysaccharide in dilute aqueous solution can assume an ordered conformation in the absence of added salts. The pH-induced transition from the disordered to the ordered state is characterized by the following main features:

(i) The process starts at the early stages of protonation and would be complete at ~40% protonation (chiroptical and calorimetric data).

(ii) It takes place at a relatively low rate when the pH value is in the middle portion of the process.

(iii) It is accompanied by a large change in specific rotatory power from +33° to −104° (equilibrium values at 302 nm).

(iv) The associated enthalpy change is $-6\,\text{kJ equiv.}^{-1}$.

Moreover, the final ordered state can 'melt' reversibly without hysteresis at around 72°C.

The conformational transition shown in the pK_a curve leads to a thermodynamic state (disordered state at high α values), which cannot be fully characterized, in view of the scarce number of data available for such a state. Either an increased chain backbone flexibility (five times larger than that of the ordered structure) or a structural change (which increases the average distance between charged groups on the backbone by about 20%) will give a satisfactory fitting of the experimental data around $\alpha = 0.9$.

Notwithstanding the above-mentioned limitations, a free-energy change of $1.2\,\text{kJ equiv.}^{-1}$ (and $\Delta S = 18\,\text{J K}^{-1}\,\text{equiv.}^{-1}$) associated with the order→disorder conformational transition can be tentatively calculated by using the pK_a data of the theoretical fit of *Figure 7b*.

At this point, it is interesting to compare the features discussed above with those characterizing the salt-induced, isothermal disorder→order process (at 25°C) and with the thermal stability of the ordered state for the same polysaccharide sample in 0.1 M NaCl[6].

The first two features appear, qualitatively speaking, to be similar, i.e. the salt-induced transition takes place in a narrow range of added NaCl concentration and, in the middle portion, at a rate comparable to that of the pH-promoted transition. Probably, at the root of such kinetic behaviour there is a concomitant action due, on one hand to the tendency of the polysaccharidic stereoregular backbone to assume a helical conformation (an intrinsically fast process) and, on the other hand, to the severe steric hindrance of the side-chains, opposing, at least kinetically, the former process. Moreover, it is highly probable that even in dilute aqueous solution the side-chains themselves have to adjust synchronously their conformation and their orientation with respect to the backbone in order to minimize the energy of the system. In fact, energetically speaking, no difference in the former electrostatic interactions would exist whether charges are screened by sodium counterions or by bound protons.

However, the salt-induced transition is accompanied by a larger change in optical activity (from +33° to −130°, at $\lambda = 302$ nm) and in enthalpy ($-8\,\text{kJ equiv.}^{-1}$), always at 25°C. These differences could be assigned, in the first instance, to the local difference of chromophore conformation, but they may even derive from a different level of structure on a chain scale. The latter explanation is more appealing in view of the previous finding that, according to light scattering data[8], the ordered state of TA1EPS chains in 0.1 M NaCl (pH 7) features an average of three chains engaged in a stiff, highly elongated structure. In the hypothesis that the ordered conformations taken up locally by TA1EPS chains in 0.1 M NaCl and at pH ~3 in water are identical, a tentative interpretation of the apparently conflicting issues enumerated above is qualitatively formulated by assuming that the stiffness and chain aggregation are higher in 0.1 M NaCl (pH 7) than at pH 3.

This corresponds to saying that in the former case (0.1 M NaCl) longer stretches of the chains are conformationally ordered (and rigid) and hence that the entropy of the system is lower. This would more than counterbalance the slightly higher enthalpy, yielding a lower 'melting temperature' (about 40°C).

The absence of hysteresis in the cooling thermogram of *Figure 6* might then be traced to the minor complexity of chain ordering in acidic condition than in salt condition. However, chain aggregation could equally occur at pH < 3 and it might qualitatively explain the smearing out of pK_a curves at very low α, from back-titration experiments.

Evidently, even if the simple hypotheses set out above validly explain the whole set of experimental data, one major point still remains to be elucidated, namely which helical conformation is taken up by TA1EPS chains in dilute aqueous media. Unsolved controversies raised by a similar question in the case of xanthan[14–18] and carrageenans[19,20] suggest that for TA1EPS structurally more complex chains the answer must await additional experimental/theoretical evidence. As a first step in this direction, work is being done in our laboratory in collaboration with Dr R. Chandrasekaran's group at Purdue University by matching the results of conformational analysis calculations[21] with those of X-ray fibre diffraction studies.

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REFERENCES

1 Zevenhuizen, L. P. T. M. and van Nerven, A. R. W. *Carbohydr. Res.* 1983, **124**, 166
2 McNeil, M., Darvill, J., Darvill, A. and Albersheim, P. *Carbohydr. Res.* 1986, **146**, 307
3 Zevenhuizen, L. P. T. M. in 'Industrial Polysaccharides', (Eds. S. S. Stivala, V. Crescenzi and I. C. M. Dea), Gordon & Breach, New York, 1987, p. 45
4 Hollingsworth, R. I. and Dazzo, F. E. *Carbohydr. Res.* 1988, **172**, 97
5 Zevenhuizen, L. P. T. M. in 'Industrial Polysaccharides: Biomedical and Biotechnology Advances', (Eds. V. Crescenzi, I. C. M. Dea, S. Paoletti, S. S. Stivala and I. W. Sutherland), Gordon & Breach, New York, 1989, p. 301
6 Crescenzi, V., Dentini, M., Coviello, T., Paoletti, S., Cesàro, A. and Delben, F. *Gazz. Chim. Ital.* 1987, **117**, 611
7 Crescenzi, V., Dentini, M. and Coviello, T. in 'New Developments in Industrial Polysaccharides', (Eds. V. Crescenzi, I. C. M. Dea and S. S. Stivala), Gordon & Breach, New York, 1987, p. 69
8 Dentini, M., Coviello, T., Burchard, W. and Crescenzi, V. *Macromolecules* 1988, **21**, 3312
9 Kortum, G., Vogel, W. and Andrussow, K. 'Dissociation Constants of Organic Acids in Aqueous Solution', IUPAC Publication, Butterworths, London, 1961
10 Dubin, P. L. and Strauss, U. P. *J. Phys. Chem.* 1970, **74**, 2842
11 Chandrasekaran, R., Personal communication
12 Cesàro, A., Paoletti, S., Urbani, R. and Benegas, J. *Int. J. Biol. Macromol.* 1989, **11**, 66
13 Cesàro, A., Ciana, A., Delben, F., Manzini, G. and Paoletti, S. *Biopolymers* 1982, **21**, 431
14 Norton, I. T., Goodall, D. M., Morris, E. R. and Rees, D. A. *J. Chem. Soc., Chem. Commun.* 1980, 545
15 Liu, W., Sato, T., Norisuye, T. and Fujita, H. *Carbohydr. Res.* 1987, **160**, 267
16 Paradossi, G. and Brant, D. A. *Macromolecules* 1982, **15**, 874
17 Liu, W. and Norisuye, T. *Biopolymers* 1988, **27**, 1641
18 Coviello, T., Kajiwara, K., Burchard, W., Dentini, M. and Crescenzi, V. *Macromolecules* 1986, **19**, 2826
19 Paoletti, S., Delben, F., Cesàro, A. and Grasdalen, H. *Macromolecules* 1985, **18**, 1834
20 Paoletti, S., Smidsrod, O. and Grasdalen, H. *Biopolymers* 1984, **23**, 1771
21 Palleschi, A., Crescenzi, V., Dentini, M. and Coviello, T. (in preparation