Double-Stranded Structure for Hyaluronic Acid in Ethanol-Aqueous Solution As Revealed by Circular Dichroism of Oligomers†

Paul W. Staskus and W. Curtis Johnson, Jr.*
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331
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ABSTRACT: The sigmoidal nature of circular dichroism (CD) changes for hyaluronic acid solutions as a function of solvent composition or temperature is studied as a function of chain length by using oligomers. We find a chain length effect with approximately nine disaccharides required for the structural transition as a function of organic solvent, which proves that the transition is cooperative with large transition enthalpy and entropy. The transition also depends on sample concentration as expected for strand association, and this was investigated in detail for oligomers 12 and 16 disaccharides long. Indeed, it was possible to prevent completely the transition in mixed solvent with sufficient dilution of these oligomers, which demonstrates strand association. The CD data in mixed solvent as a function of oligomer concentration were fit with various models for association of two and more strands. Simplex methods were used to investigate the vector space of unknowns for the models, and two-strand models were shown to consistently give a better fit. A cooperative two-strand zipper model which allows relative sliding of the chains had the smallest fitting error and produced the following thermodynamic parameters (in terms of a duplex of disaccharide units) for the ordered structure in an aqueous solution containing 45% v/v ethanol, 12.5 mM NaH_2PO_4, and 7.5 mM H_3PO_4: enthalpy of growth, -1.0 ± 0.3 kcal mol^{-1}; entropy of growth, -2.3 ± 1.3 eu mol^{-1}; enthalpy of initiation, -20 ± 3 kcal mol^{-1}; entropy of initiation, -71 ± 15 eu mol^{-1}. The results are consistent with a double-stranded and helical structure for hyaluronic acid in solutions of reduced dielectric constant.

The chiroptical transition of hyaluronic acid (HA)[1] polymer in aqueous-organic solvent at low pH as a function of temperature or solvent composition possesses considerable sigmoidal character, as described in the preceding paper (Staskus & Johnson, 1988). This is common in cooperative transitions of polymers, which typically involve large enthalpies and entropies as many residues essentially change conformation as a single unit. The van't Hoff analysis discussed in the preceding paper (Staskus & Johnson, 1988) yields large enthalpy and entropy for the HA transition. The sharpness of cooperative transitions is due to the reduced energy required to propagate a conformational change through a polymer, relative to that needed for initiation of the change.

Cooperativity in a polymer transition can be verified through studies of corresponding oligomers, as has been done in the case of model polypeptides (Zimm et al., 1959; Nakamoto et al., 1974; Shaw & Schurr, 1975) and nucleic acids (Applequist & Damle, 1965; Martin et al., 1971; Nelson et al., 1981). We show here that the same approach can be used with polysaccharides. Studies of oligomers in acidic organic-aqueous solvent show that the transition of HA is a cooperative function of chain length. Moreover, the oligomer studies reveal a sensitivity of the chiroptical transition to molecular concentration for shorter oligomers.

Since lower pH and decreased dielectric constant of the solution are two conditions that favor network formation in HA (Morris et al., 1980a), we suggest that the conformational transition for HA in a mixed solvent system reflects chain association, with formation of a structure such as that predicted from analysis of X-ray diffraction patterns obtained from fibers (Arnott et al., 1983).

To pursue our analysis of cooperativity in the HA chiroptical transition, we have used an endo-β-hexosaminidase to cleave HA into a range of oligomers with the acetylated amino sugar at their reducing ends. Gel exclusion chromatography was then used to separate the species of various sizes. Polyacrylamide gel electrophoresis was the method of choice not only for determining chain lengths and polydispersity of HA in column fractions but also for judging purity of sample materials regarding potential glycosaminoglycan contamination and assessing sample integrity during the course of our spectroscopic measurements.

Data describing chiroptical transitions of various HA oligomer species were analyzed in terms of two-state (infinite cooperativity) and (finite) cooperative transition models. Our approach involves the use of a directed search algorithm known as a simplex (Nelder & Mead, 1965; Deming & Morgan, 1973) to determine those unknown model parameters that minimize the fitting error to the data. We are able to seek local as well as global solutions, and changing the error function helps us avoid erroneous results due to spurious data. From our analysis, we conclude that the chiroptical transition of HA in aqueous-organic solvent reflects a cooperative association of two strands, consistent with rheological (Welsh et al., 1980) and X-ray diffraction (Arnott et al., 1983) results.

Experimental Procedures

Oligomer Preparation. HA isolated from *Streptococcus* was purchased as the sodium salt from Calbiochem-Behring. Listed purity was greater than 99%, free of chondroitin,

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[1] Abbreviations: HA, hyaluronic acid; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; L2, least-squares error; L1, least absolute value error; ΔH_{ind}, length-independent/initiation enthalpy for transition; ΔS_{ind}, length-independent/initiation entropy; ΔH_{res}, per duplex or triplex of disaccharide residue/growth enthalpy; ΔS_{res}, per duplex or triplex of disaccharide residue/growth entropy; CD_{com}, per disaccharide residue ordered structure circular dichroism; Tris, tris(hydroxymethyl)-aminomethane.

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dermatan, and keratan, and with protein contamination of less than 0.1%. The purity of this material is crucial, since bovine testicular hyaluronidase (Worthington; hyaluronate 4-glycanohydrolase, EC 3.2.1.35) is used to cleave the polymer into oligomers, and the hyaluronidase is capable of degrading chondroitin as well as HA. The presence of contaminating chondroitin in the HA substrate will result in the creation of hybrid molecules. Oligomers of hyaluronic acid were prepared according to the method of Cowman et al. (1981). The digest was evaporated to dryness, solvated in 2 mL of 0.5 M pyridine–acetic acid solution, and chromatographed in the same solvent on a 950-mL packed bed volume of Bio-Gel P60, −400 mesh (Bio-Rad). Fractions were screened for glucuronic acid content by using the Bitter and Muir (1962) modifications of the colorimetric assay of Dische (1947). Size heterogeneity of material in the fractions was assessed by PAGE, and appropriate fractions were pooled, evaporated to dryness, solvated in distilled water, lyophilized, and stored at room temperature over phosphorus pentoxide.

Electrophoresis. Acrylamide and bis(acrylamide) were purchased from Bio-Rad Laboratories, Richmond, CA. Tris, glycine, and alcian blue dye were purchased from Sigma. Oligomers of hyaluronate were electrophoresed by using a mini-slab gel apparatus (Idea Scientific) with 10 cm × 15 cm microscope slides and 0.8-mm spacers. Wells were 4 mm wide. The procedure used was essentially that due to Ornstein (Williams & Reisfeld, 1964).

Spectroscopy. Samples of hyaluronate oligomer were dissolved in water and then dialyzed 4 times against a 250–500-fold excess of buffered mixed solvent either at 4 °C or at room temperature. All HA oligomer samples in acidic mixed solvent were buffered, containing 7.5 mM H_3PO_4 and 12.5 mM NaH_2PO_4. Concentrations are in terms of the fundamental disaccharide unit.

Circular dichroism spectra were recorded at a minimum of four different temperatures between 15 and 30 °C. Between sets of CD spectra, the sample concentration was assessed by optical density measurement using a Cary 15 or Cary 219 spectrophotometer with nitrogen purge. Sample concentrations were determined by using an extinction coefficient of 9500 M^{-1} cm^{-1} at 188 nm for the disaccharide of HA. After a set of CD spectra had been recorded, the sample material was recovered from the optical cell with dilution by an aliquot of the buffered mixed solvent and was then redialyzed in preparation for the next series of measurements. This process was continued until measurements had been made for each oligomer at 10–15 different concentrations in the range of 0.2–20.0 mM disaccharide. Following such a series of measurements, the sample was recovered, dialyzed against distilled water, and lyophilized. This recovered material was then compared by electrophoresis to a portion of the original oligomer sample stock. Other spectroscopic details are given in the preceding paper (Staskus & Johnson, 1988).

Data Analysis. Models were fit to the data with a simplex-directed search technique (Nelder & Mead, 1965; Deming & Morgan, 1973). Briefly, the n unknown parameters (or factors) in the model for the transition (in this case, the transition enthalpies, entropies, and CD of the associated form) are considered to define a factor space of dimension n. The simplex is a geometric figure defined by $n + 1$ vertices in the factor space. Thus, a simplex in two dimensions is a triangle. After reasonable boundaries for the factor space are established, the simplex is placed at random within the boundaries to be searched. It then moves through the space according to strict rules governing which vertex should be discarded and how its replacement will be generated relative to the remaining hypersurface. The simplex functions by moving away from a vertex with a poor response. To judge a vertex, we calculated the CD predicted by the model for each experimental condition of temperature, oligomer concentration, and oligomer length utilizing the known experimental conditions and the factor values corresponding to the vertex coordinates. The predicted CD was compared to the observed value for each condition, and the sum of the squares or the absolute values of the differences comprised the vertex response. This allowed least-squares fitting of a rather complicated nonlinear function of several variables. The simplex search was typically repeated in excess of 50 times to help determine the uniqueness of the solution and to discriminate secondary local minima from global minima.

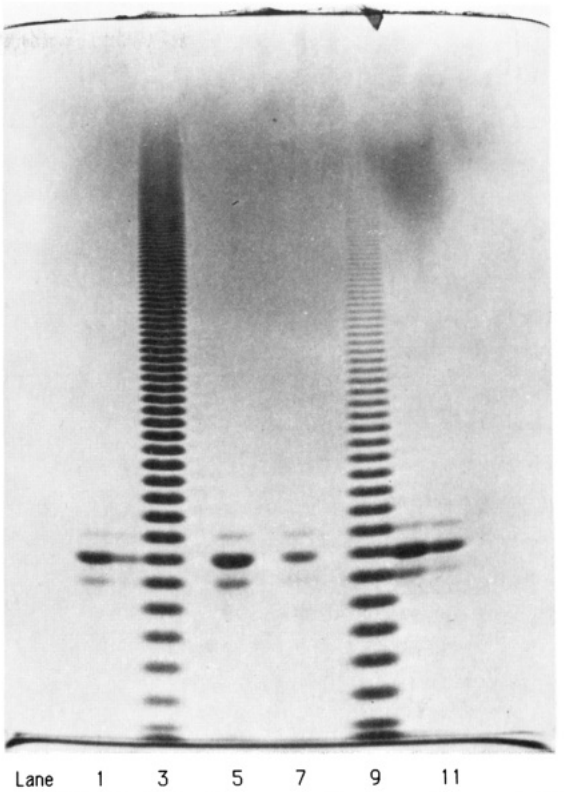

FIGURE 1: PAGE of HA oligomer samples comparing material from before (lanes 1, 5, and 10) with that recovered after a series of CD measurements (lanes 2, 7, and 11). With any end effect on staining intensity neglected, soft laser densitometer scans of the gel show 70–80% of the staining material to be 12 disaccharides in length, with 10–15% contamination by each of the 11- and 13-disaccharide species. Similar results were obtained with other oligomers. Lanes 3 and 9 contained unfractionated digests of HA cleaved with testicular hyaluronidase.

RESULTS

Gel Electrophoresis of Oligomers. Figure 1 presents a comparison of oligomeric HA sample material recovered after CD spectroscopy (lanes 2, 7, and 11) with an aliquot reserved from the original sample (lanes 1, 5, and 10). The original sample was obtained by pooling fractions in a cut from a chromatographic separation of HA total digest. Lanes 3 and 9 in the figure contain total enzymatic digests of HA, with a range of oligomer sizes from 6 disaccharides (near the tracking dye front) to greater than 50 being resolved on the polyacrylamide gel. The assignment of an oligomer length to an electrophoresed band was made by comparing the electrophoretic profile of a chromatography fraction to its position in a plot of HA concentration versus fraction number. That

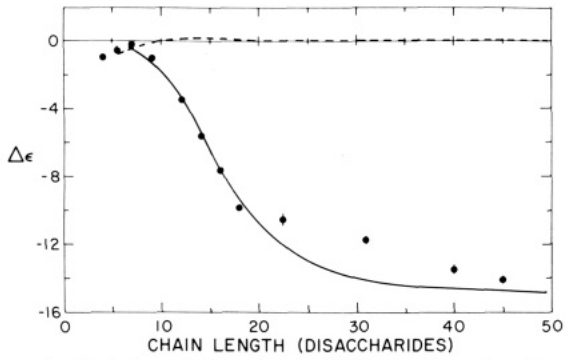

FIGURE 2: Chain length dependence of CD at 190 nm for oligomers of HA (45% by volume ethanol, 12.5 mM monobasic sodium phosphate, and 7.5 mM phosphoric acid, 25 °C). Sample chain length was determined by electrophoresis, and values are population averages. CD's for 9-, 12-, 14-, 16-, and 18-disaccharide species were interpolated at 5 mM concentration from plots of CD versus concentration for each oligomer. CD was relatively insensitive to concentration for the other species plotted. The solid line represents the CD calculated from the best fit assuming a cooperative zipper transition with two associating strands which are allowed to slide relative to one another in the duplexes. Error bars represent noise levels of the CD measurements.

plot presented well-resolved peaks of concentration for oligomers shorter than 10 disaccharides. The band assignments were confirmed by electrophoresis of fractions using a protocol which resolves HA oligomers as small as seven disaccharides (Turner & Cowman, 1985).

For the sample in Figure 1, 70–80% of the oligomeric material stained by alcian blue is 12 disaccharides long, with 10–15% contamination by each of the 11- and 13-disaccharide species. There is relatively less of the 11-disaccharide contaminant present in material recovered after spectroscopy, due to the extensive dialysis of the sample performed between CD measurements (compare lanes 5 and 7 in Figure 1). However, integration of densitometer scans of the gel shows that the difference is not enough to affect the average HA oligomer length within the sample population.

The clarity of the oligomer ladders observed in lanes 3 and 9 confirms the purity of the HA used as substrate in the digestion. The presence of contaminating chondroitin sulfate would be expected to result in the creation of hybrid oligomers due to transglycosylation events during digestion of an HA and chondroitin sulfate mixture. Chondroitin sulfate has a higher linear charge density than HA. Since charge density contributes to electrophoretic mobility, a hybrid oligomer would be expected to have a higher mobility than HA of the same chain length, giving a second set of bands. In preliminary experiments involving digestion of mammalian HA and PAGE of exclusion column fractions, two sets of stained bands were observed within the gel lanes. The set of bands with the higher mobility migrated out of phase with the bands of an unfractionated HA digest ladder used as a reference. It is believed that the set of higher mobility bands is due to hybrid oligomers in the sample resulting from chondroitin sulfate contamination.

Chain Length Dependence of CD. The chain length dependence for the CD of HA in acidic mixed solvent is shown in Figure 2, which presents the $\Delta\epsilon_{190}$ for oligomers at 25 °C and approximately 5 mM concentration of disaccharide. The solid line in the figure is the CD predicted by a cooperative zipper duplex model. We will discuss this fit later. The dashed line in the figure represents the CD at 190 nm for various oligomers of HA in acidic aqueous solution at 25 °C. This CD is practically zero over most of the range of oligomer lengths investigated. The rise above base line for oligomers from 12 to 16 disaccharides long does not exceed the noise level

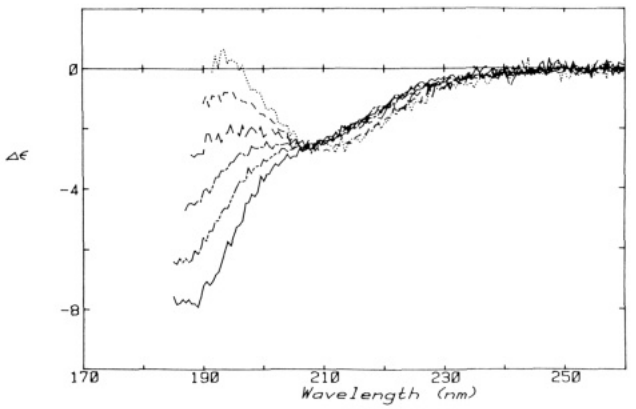

FIGURE 3: Concentration dependence of CD (per disaccharide) for the 16-disaccharide oligomer of HA was measured in acidic ethanol–water solvent at 30 °C. Sample concentrations in millimolar of disaccharide are 0.14 (···), 0.28 (- - -), 0.80 (– –), 2.31 (–·–), 5.24 (–··–), and 8.67 (—).

of the measurements. However, the negative CD for the smallest oligomers studied is clear and reproducible, approaching a value of -1 M^{-1} cm^{-1} for the five-disaccharide species. A similar dependence of HA CD on chain length has been noted previously in neutral aqueous solution by Cowman et al. (1981, 1983).

For oligomers in acidic mixed solvent and shorter than five disaccharides, the 190-nm CD at 25 °C is small and negative, becoming less negative with increasing chain length until a minimum magnitude is reached at seven or eight disaccharides. With further increase in size of the oligomer, the negative CD at 190 nm increases dramatically, the rate of increase with chain length being maximal between 12 and 18 disaccharides. The negative CD observed in oligomers five disaccharides and shorter is likely due to end residue contributions. The differences in CD between acidic aqueous and acidic mixed solvent in this size range are small but reproducible. They correspond to the type of perturbations observed in HA monomer spectra with introduction of organic solvent shown in the preceding paper (Staskus & Johnson, 1988). Thus, the CD of the seven-disaccharide species of HA appears to represent an (disordered oligomer) end point in the chiroptical transition of the molecule as a function of chain length at 25 °C and 5 mM concentration of the repeating disaccharide. The large increase in negative magnitude for $\Delta\epsilon_{190}$ with increasing chain length is representative of the dependence on chain length expected for a conformational transition requiring cooperation between adjacent residues in the polymer.

The limit of $\Delta\epsilon_{190}$ for HA at infinite chain length is not clearly indicated in Figure 2. Measurements for oligomers averaging 40 and 45 disaccharides in length are -13.6 and -14.2 M^{-1} cm^{-1}, respectively. Measurements of HA polymer gave -12 M^{-1} cm^{-1} when dissolved in unbuffered mixed solvent and -15 M^{-1} cm^{-1} as a gel in the buffered mixed solvent. Since this end point is difficult to estimate, we will make it one of the unknown model parameters to be provided for us by the data fitting procedure.

Concentration Dependence of Oligomer CD. We wish to determine whether or not the conformational change in HA reflected by the chiroptical transition in mixed solvent involves strand association. Examination of oligomers can reveal concentration effects not observable in longer molecules, because the polymer effect establishes a lower limit to the local residue concentration as the molecule hairpins or loops upon itself. In the size range of 10–16 disaccharides, oligomer CD spectra are quite sensitive to sample concentration under our experimental conditions (Figure 3). Analyses of sets of spectra

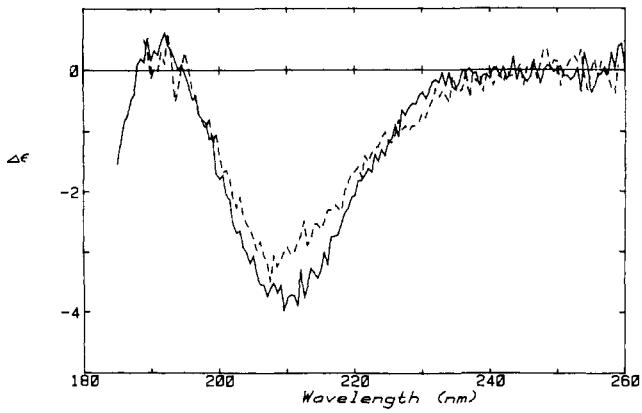

FIGURE 4: CD spectrum per disaccharide for the 12-disaccharide oligomer of HA in water (—) and in acidic ethanol–water solvent (---) at 15 °C and 0.214 mM concentration of disaccharide.

for orthogonal components, as described in the preceding paper (Staskus & Johnson, 1988), detect only two components above the noise level of the measurements, demonstrating that there are only two environments for the chromophore. The spectral series as a function of oligomer concentration in Figure 3 is reminiscent of the series for HA polymer as a function of solvent composition and presumably reflects the same conformational change.

In order to analyze transition data for HA oligomers in terms of transition models, it would be useful to know the two end-point spectra for the transition. The CD for a disordered structure of a particular oligomer is presumably that observed at infinite dilution, but this is not practically attainable. We approximate it as the acidic aqueous solution CD. Indeed, the CD spectra of the 12-disaccharide HA in acidic aqueous solution and acidic mixed solvent at low oligomer concentration appear identical (Figure 4). The CD for the totally ordered HA oligomer is more difficult to estimate than the infinite dilution end point, and we would have less confidence in any value that we chose. Therefore, we will let the CD for ordered HA remain as an unknown to be determined by the data fitting procedure.

Concentration Dependence as a Function of Temperature. A common and simple method of analyzing cooperative polymer transitions for strand association involves measurements of melting curves for oligomers of the molecule at several different concentrations. The initiation and growth energies can be determined from the slopes of the melting curves at the transition midpoints and the shifts in temperatures for melting midpoints as the oligomer concentrations are changed. Measurement of complete melting curves was not possible for our system. In our case, difficulties with sample aggregation at very low temperatures required to attain an end point in the transition, as well as instability of the solvent at very high temperatures, limited the temperature range. We compensate for this by expanding the concentration domain of our measurements (Figure 5).

Use of a moderate temperature range has the advantage of reducing the demand on the buffer during the course of the transition. The importance of carboxyl group protonation and its effect on the transition have been noted by Park and Chakrabarti (1978), who used titration of HA solutions to show that the conformational transition of HA promotes protonation of its carboxylate groups. Thus, melting or diluting out the ordered structure may result in proton absorption from or release into the medium, and a resulting pH change could affect the further course of the transition. All of our HA oligomer solutions are buffered with phosphate. The effect

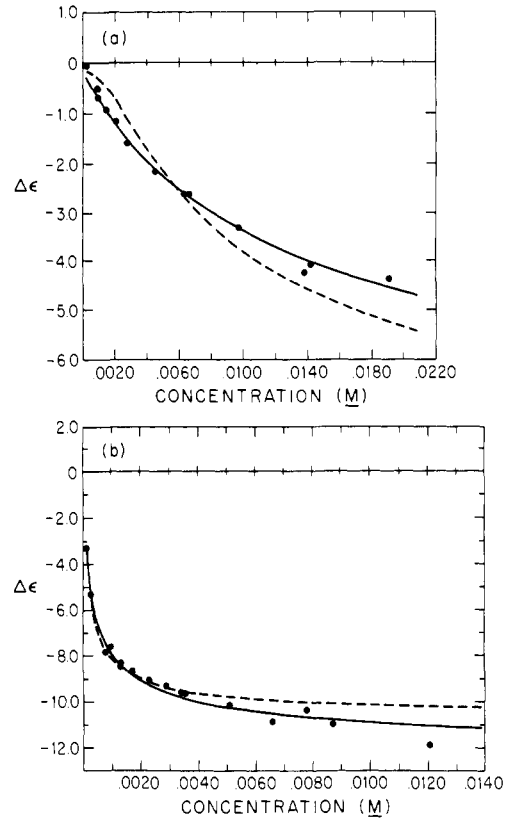

FIGURE 5: Graph of CD at 190 nm versus concentration for HA oligomers. The solid line is the least-squares fit of a cooperative transition model with two associating parallel and in-register strands. The dashed line is the corresponding fit for three associating strands. Panel a is a plot of 12-disaccharide oligomer data at 30 °C, and panel b is that for the 16-disaccharide oligomer at 15 °C. Inspection reveals that the three-strand model fits were poorest for the shorter oligomers at higher temperatures (a) and the longer oligomers at lower temperatures (b). No such trend could be detected for the two-strand model fits.

of sample dilution on system pH is removed by the use of dialysis between sets of CD measurements. Upon repeating a series of measurements, our conditions give no detectable inconsistency in HA oligomer spectra recorded as a function of concentration at a given temperature, whether the sample was previously dialyzed at 4 °C or at room temperature (20 °C). This is consistent with the presence of adequate buffering of the system.

Analyzing the Results with Transition Models. Our results for the HA oligomers were analyzed as an intramolecular transition in the noncooperative case and with the zipper model (Cantor & Schimmel, 1980). Furthermore, the following models for intermolecular transitions were investigated: two-state duplex (two-strand association); finite cooperative (zipper) duplex with parallel or antiparallel strands both in register and with the in-register condition relaxed (sliding); two-state triplex (three-strand association); zipper triplex both in register and with the in-register condition relaxed (Applequist & Damle, 1963, 1965; Staskus, 1987). While the mathematical details of these models and the computerized search procedure are presented elsewhere (Staskus, 1987), the results of simplex fitting our data with various intermolecular transition models are presented in Table I. Note that the units are per mole of duplex (or triplex) of the fundamental disaccharide residue. The solution listed for a given model corresponds to that with the smallest fitting error found in excess of 50 simplex trials, using the sum of either the least-squares (L2) or least absolute value (L1) of the residuals to judge the fit. The energy parameters with subscript ind

Table I: Results for Various Transition Models

	energy terms					
	length independent/initiation		per residue/growth		ordered	av fitting
	ΔH_{ind} (cal mol^{-1})	ΔS_{ind} (eu mol^{-1})	ΔH_{res} (cal mol^{-1})	ΔS_{res} (eu mol^{-1})	CD_{com} (M^{-1} cm^{-1})	error/point (M^{-1} cm^{-1})
two-state models						
duplex	−9160	−32.8	−1590	−4.1	−12.2	0.09 (L2)
	−7290	−26.3	−1670	−4.4	−12.1	0.23 (L1)
	0.0	−1.82	−2249	−6.34	−12.1	0.10 (L2)
	0.0	0.0	−2162	−6.18	−12.4	0.12 (L2)
triplex	−580	−0.8	−4100	−11.5	−10.4	0.30 (L2)
cooperative zipper models						
duplexes						
parallel in-register	−14700	−53.5	−1320	−3.3	−14.5	0.09 (L2)
	−16300	−58.7	−1160	−2.8	−14.4	0.22 (L1)
antiparallel in-register	−14180	−51.5	−1345	−3.36	−14.3	0.09 (L2)
sliding	−18370	−66.8	−1088	−2.56	−16.4	0.09 (L2)
	0.0	−4.87	−2560	−7.50	−15.7	0.11 (L2)
	−19700	−71.3	−950	−2.1	−16.4	0.22 (L1)
triplexes						
parallel in-register	8335	28.9	−4936	−14.4	−11.0	0.27 (L2)
parallel sliding	8411	29.1	−5078	−15.0	−12.0	0.23 (L2)
	0.0	0.72	−4430	−12.8	−12.1	0.23 (L2)

are for the chain length independent or end effect contribution to the transition energy in two-state models and the initiation step in cooperative models. The parameters with subscript res are the per residue contributions in two-state models and growth step values in cooperative models. The $\Delta H_{ind} - T\Delta S_{ind}$ in the cooperative models corresponds to $RT \ln \alpha$ of Applequist and Damle (1963), and $\Delta H_{res} - T\Delta S_{res}$ corresponds to $RT \ln s$. Here, s is the equilibrium constant for transition of a residue in a polymer when at the interface between the two conformations, and α is a constant that corrects an s value to the equilibrium constant not at an interface. Since the equilibrium constant for strand association in the zipper model is αs, the enthalpy and entropy for this step are actually the sums of the listed initiation and growth values.

When we examine our results for fitting experimental data with various models, we can make the following observations:

(1) Direct visual comparison of the fits for theoretical models with the raw data (Figure 5) shows the three-strand models to be poor at the limits of the transition. The CD intensity for the ordered structure predicted by these models is too small, being only −10 to −11 M^{-1} cm^{-1}. Furthermore, in plots of $\Delta\epsilon_{190}$ versus concentration for shorter HA oligomers, the three-strand models predict a region where the second derivative is negative, which is not displayed by the data. Thus, we chose two-strand models as the best description of the data.

(2) Two-state models (infinite cooperativity) provide smaller estimates of CD intensity for ordered structure than the models with finite cooperativity, so finite cooperativity agrees better with observations.

(3) The fitting error is higher in two-state models than in those with finite cooperativity and is higher in three-strand models than in two-strand models. Thus, fitting error also favors duplex models with finite cooperativity.

(4) Fitting error displays a clear minimum as a function of the magnitude of the CD for the ordered structure when predicted by the simplex trials. For example, the fits with smallest error for the in-register duplex models are those with the ordered CD fixed near −14.5 M^{-1} cm^{-1}.

(5) In cooperative two-strand models, the effect of allowing chains to slide within the complex involves a sharpening of the initiation step of the transition with temperature as the initiation enthalpy and entropy both increase. There is a concomitant broadening of the growth step as its enthalpy and entropy decrease. Fits are similar, so the sliding model can be preferred only because of its generality.

(6) Attempts to simplify models by removing any contribution of the initiation step to the transition enthalpy (i.e., $\Delta H_{ind} = 0.0$) consistently result in a larger fitting error as the total transition enthalpy is absorbed by the growth step.

All in all, the cooperative zipper model for duplexes with sliding chains seems to be the best choice. When we consider all but the most unreasonable simplex solutions for this model as a group, we find that the parameters in terms of a duplex of disaccharide units are −20 ± 3 kcal mol^{-1} for ΔH_{ind}, −1.0 ± 0.3 kcal mol^{-1} for ΔH_{res}, −71 ± 15 eu mol^{-1} for ΔS_{ind}, −2.3 ± 1.3 eu mol^{-1} for ΔS_{res}, and −16.4 ± 0.5 M^{-1} cm^{-1} for CD_{com}.

Discussion

Extrapolation to Polymeric HA. A comparison of the chain length dependence for HA CD at 190 nm with the CD predicted by using the sliding duplex zipper model with thermodynamic parameters from Table I shows that the model has difficulty in predicting the CD of oligomers longer than approximately 20 disaccharides (Figure 2). Potential reasons for the discrepancy include applicability of the zipper model and existence of associated species higher than duplexes.

The zipper model as presented by Applequist and Damle disallows regions of unordered residues surrounded by ordered regions in the polymer. As chain length increases in an oligomeric series, the likelihood of such a stretch of unordered residues will increase until in the infinite polymer such regions will alternate with ordered ones, the average lengths depending upon the equilibrium constants for initiation and growth of ordered regions. Therefore, we also analyzed the data set using a matrix-generated partition function of the type originally due to Zimm and Bragg (1959) and used to analyze transition data for collagen model peptides (Shaw & Schurr, 1975). This model allows "bubbles" of unordered residues within ordered regions.

When we fit our experimental data using this matrix-generated partition function, we find that the thermodynamic parameters for growth and initiation of ordered regions are not very different from the zipper model. The fit of the bubble model to HA oligomer data as a function of chain length as in Figure 2 is virtually identicial with that of the zipper model. Furthermore, the CD predicted for longer oligomers is larger than that experimentally observed. Thus, it does not appear that the applicability of the zipper model is the cause of the

discrepancy between the observed CD in long oligomers and that predicted by model fits of the shorter oligomer CD.

A second potential explanation for the discrepancy is the possible existence of HA oligomer duplexes that overlap so as to utilize more than two strands. Once we have estimated the energy parameters for the transition with the zipper model, we can use expressions similar to that in eq 24 of Applequist and Damle (1965) to estimate the equilibrium constants for formation of multistranded species. When we do this, we find, for example, that in excess of 30% of our sample molecules may participate in three-strand duplexes under our experimental conditions, as an oligomer forms a double-stranded region with more than one partner. The analysis becomes complex, and predicting the CD associated with such structures is difficult. It is clear that the possibility exists for less complete pairing of HA strands because of this circumstance, especially for longer oligomers, with a concomitant decrease in the contribution of ordered structure CD to the total signal. It would be preferable to avoid this difficulty by investigating the transition in that region of sample concentration and temperature where it behaves in an all-or-none fashion (Applequist & Damle, 1965). However, the two-state limit region for the present system is inaccessible, due to the extremely low sample concentrations and temperatures required (Staskus, 1987).

Structure for HA in Organic–Aqueous Solvent. Only one fiber diffraction pattern is presently proposed to represent a double-helical structure in HA. The fiber from which it was obtained was drawn at low pH in the presence of various monovalent cations (Sheehan et al., 1977). A recent reanalysis of the data suggests that the cations lie between double helices in the fiber and that carboxyl groups are protonated to the extent that they can provide hydrogen-bonding links between paired strands within double helices (Arnott et al., 1983). Our results are consistent with this model. Cations are not required for the appearance of ordered structure CD. If they are present, HA polymer will form a soft gel in the aqueous–organic solvent. This may be due to higher order association of double-helical domains to create a network, as suggested by Rees and co-workers (Robinson et al., 1980; Rees & Welsh, 1977) to explain features of the self-interaction in carrageenan solutions.

Rheological studies demonstrating network formation in HA solutions are also consistent with our results. Welsh et al. (1980) showed that the network character of HA which is accentuated by lowering the pH or reducing the water content of the solution can be specifically inhibited by oligomers of the same copolymeric sequence. Oligomers 2–4 disaccharides long did not affect network formation, while those 60 disaccharides in length inhibited or abolished it. This was presumably due to the long oligomers competing with and breaking polymer–polymer junction zones, which small oligomers could not do. We would predict from our CD results that the critical size for competition of oligomer with polymer in these junctions (formed in our solution conditions) is the size that can first form ordered structure (in the range of 11–15 disaccharides). This length may be increased at lower alcohol content of the solvent.

The ability of HA to self-associate, as well as interact with other glycosaminoglycans (Turley & Roth, 1980) and proteins (Hascall & Heinegard, 1974; Turley, 1982; Turley et al., 1985), may be critical to its physiological function in processes such as cell–substrate adhesion, cell motility, and tissue matrix organization. The existence of double-stranded HA under physiological conditions is speculative at present. The small fraction of total polymer residues necessary to create a transient network through junction zones may escape optical detection at neutral pH in aqueous solution. However, rheological evidence of its existence is observed (Morris et al., 1980a,b).

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REFERENCES

Applequist, J., & Damle, V. (1963) *J. Chem. Phys. 33*, 2719–2721.

Applequist, J., & Damle, V. (1965) *J. Am. Chem. Soc. 87*, 1450–1458.

Arnott, S., Mitra, A. K., & Raghunathan, S. (1983) *J. Mol. Biol. 169*, 861–872.

Bitter, T., & Muir, H. M. (1962) *Anal. Biochem. 4*, 330–334.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, pp 1056–1060, W. H. Freeman, San Francisco, CA.

Cowman, M. K., Balazs, E. A., Bergmann, C. W., & Meyer, K. (1981) *Biochemistry 20*, 1379–1385.

Cowman, M. K., Bush, C. A., & Balazs, E. A. (1983) *Biopolymers 22*, 1319–1334.

Deming, S. N., & Morgan, S. L. (1973) *Anal. Chem. 45*, 278A–283A.

Dische, Z. (1947) *J. Biol. Chem. 167*, 189–198.

Hascall, V. C., & Heinegard, D. (1974a) *J. Biol. Chem. 249*, 4242–4249.

Hascall, V. C., & Heinegard, D. (1974b) *J. Biol. Chem. 249*, 4232–4241.

Martin, F. H., Uhlenbeck, O. C., & Doty, P. (1971) *J. Mol. Biol. 57*, 201–215.

Morris, E. R., Rees, D. A., & Welsh, E. J. (1980a) *J. Mol. Biol. 138*, 383–400.

Morris, E. R., Rees, D. A., Robinson, G., & Young, G. A. (1980b) *J. Mol. Biol. 138*, 363–374.

Nakamoto, K., Suga, H., Seki, S., Teramoto, A., Norisuye, T., & Fujita, H. (1974) *Macromolecules 7*, 784–789.

Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308–313.

Nelson, J. W., Martin, F. H., & Tinoco, I., Jr. (1981) *Biopolymers 20*, 2509–2531.

Park, J. W., & Chakrabarti, B. (1978) *Biochim. Biophys. Acta 541*, 263–269.

Rees, D. A., & Welsh, E. J. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 214–224.

Robinson, G., Morris, E. R., & Rees, D. A. (1980) *J. Chem. Soc., Chem. Commun.*, 152–153.

Shaw, B. R., & Schurr, J. M. (1975) *Biopolymers 14*, 1951–1985.

Sheehan, J. K., Gardner, K. H., & Atkins, E. D. T. (1977) *J. Mol. Biol. 117*, 113–135.

Staskus, P. W. (1987) Ph.D. Thesis, Oregon State University.

Staskus, P. W., & Johnson, W. C., Jr. (1988) *Biochemistry* (preceding paper in this issue).

Turley, E. A. (1982) *Biochem. Biophys. Res. Commun. 108*, 1016–1024.

Turley, E. A., & Roth, S. (1980) *Nature (London) 283*, 268–271.

Turley, E. A., Bowman, P., & Kytryk, M. A. (1985) *J. Cell Sci. 78*, 133–145.

Turner, R. E., & Cowman, M. K. (1985) *Arch. Biochem. Biophys. 237*, 253–260.

Welsh, E. J., Rees, D. A., Morris, E. R., & Madden, J. K. (1980) *J. Mol. Biol. 138*, 375-382.
Williams, D. E., & Reisfeld, R. A. (1964) *Ann. N.Y. Acad. Sci. 121*, 373-381.
Zimm, B. H., & Bragg, J. K. (1959) *J. Chem. Phys. 31*, 526-535.
Zimm, B. H., Doty, P., & Iso, K. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1601-1607.

Structure Elucidation of Glycosphingolipids and Gangliosides Using High-Performance Tandem Mass Spectrometry†

Bruno Domon and Catherine E. Costello*
Mass Spectrometry Facility, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: Glycosphingolipids and gangliosides have been investigated by using fast atom bombardment high-performance tandem mass spectrometry (FABMS/MS). Homologous compounds have been investigated in order to ascertain the fragmentation. Collision-induced dissociation spectra of the molecular species in the positive ion mode mainly afford information on the ceramide constitution (aglycon as a whole, *N*-acyl residue, and long-chain base), whereas negative ion spectra show fragments informative of the sugar sequence and the degree of branching of the carbohydrate. In the case of gangliosides carrying a complex oligosaccharide moiety, collision spectra of fragment ions have been performed in order to gain additional structural data. The advantage of tandem mass spectrometry over conventional fast atom bombardment mass spectrometry (FABMS) consists in the fact that collision spectra of the individual components from mixtures, as usually encountered with these kinds of samples, can be recorded. Furthermore, the exclusion of most of the interfering signals from the matrix allows the identification of pertinent fragments at low mass.

The growing interest in glycosphingolipids (GSLs)[1] and gangliosides, involved in many vital functions on cell membranes, such as cell surface antigens, cell–cell recognition sites, specific receptor for signal molecules, and signal transductor through the membrane (Hakomori, 1981; Ledeen & Yu, 1982; Karlsson, 1986), has stimulated the investigation of new techniques for their structural analysis. Mass spectrometry, a highly sensitive method, has been a key tool for the determination of of the primary structure of derivatives of these compounds (Sweeley & Nunez, 1985). Soft ionization techniques, such as field desorption (FD) (Costello et al., 1980; Kushi & Handa, 1982) and more recently fast atom bombardment (FAB) (Arita et al., 1983a,b; Hemling et al., 1983; Sonnino et al., 1986; Egge & Peter-Katalinic, 1987), have now made possible the analysis of underivatized glycosphingolipids. The latter method yields pertinent fragment ions as well as molecular weight information, but the identification of these signals, especially at low mass, is often hindered by the matrix background. On the other hand, heterogeneity in the ceramide as well as in the oligosaccharide moiety reflects the complexity of these structures and can complicate the interpretation of spectra.

Tandem mass spectrometry (MS/MS) (McLafferty, 1983) overcomes some of these limitations and thus increases the structural information. The recent availability of commercial high-performance tandem mass spectrometers has added a new dimension in the analysis of biomolecules (Biemann, 1986). In MS/MS, the ion associated with the molecular weight ($[M + H]^+$, $[M - H]^-$) or a fragment ion is selected in the first mass spectrometer MS-1 at a resolution of 1 mass unit. These ions collide with an inert gas such as helium in a collision cell located in the field-free region between MS-1 and MS-2. Finally, the product ions are analyzed, also at unit resolution, in the second mass spectrometer MS-2. The major advantage of this two-step process is that mixtures can be analyzed in a manner that yields structural information related solely to the ions selected by MS-1. This technique has already been used successfully for the analysis of polypeptides (Biemann, 1986; Biemann et al., 1986) and the determination of the structure of small peptides (Johnson & Biemann, 1987). The present work describes the application of this approach for the characterization of glycosphingolipids and gangliosides.

Experimental Procedures

Materials. Semisynthetic *N*-palmitoyl- (**1**), *N*-stearoyl- (**2**), and *N*-lignoceroyl- (**3**) dihydrogalactocerebrosides, *N*-palmitoyl- (**4**), *N*-stearoyl- (**5**), and *N*-lignoceroyl- (**6**) dihydrolactocerebrosides, and *N*-palmitoyl- (**7**), *N*-stearoyl- (**8**), and *N*-oleoyl- (**9**) galactocerebrosides were obtained from Sigma Chemical Co., St. Louis, MO (see Chart I). Glycosphingolipid asialo-GM_1 (**10**) and gangliosides GM_1 (**11**) and GD_{1a} (**12**) were obtained from Supleco Inc., Bellefonte, PA. Compound GD_{1b} (**13**) was supplied by Bachem Inc., Torrance, CA. Samples were dissolved in DMSO (5 $\mu g/\mu L$), and this solution

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* Address correspondence to this author.

[1] Abbreviations: FABMS, fast atom bombardment mass spectrometry; FABMS/MS, fast atom bombardment tandem mass spectrometry; CID, collision-induced dissociation; FD, field desorption; $[M + H]^+$, protonated molecular ion; $[M - H]^-$, deprotonated molecular ion; E, electric sector; B, magnetic field; MS-1, first of two high-resolution mass spectrometers in tandem; MS-2, second of two high-resolution mass spectrometers in tandem; GSL, glycosphingolipid; LCB, long-chain base; HPLC, high-performance liquid chromatography; *m/z*, mass to charge ratio; Hex, hexosyl or hexose; Sia, sialyl or sialic acid; Cer, ceramide.

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