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THERMAL AND SOLVENT STABILITY OF PROTEOGLYCAN AGGREGATES BY QUASIELASTIC LASER LIGHT-SCATTERING

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ABSTRACT

The dissociation behavior of several species of proteoglycan aggregates (PGA) has been studied quantitatively by monitoring the changes in particle size by dynamic laser light-scattering. Firstly, studies of the thermal dissociation of reconstituted PGA from bovine nasal septum and bovine fetal epiphysus are described. The effect of link protein in stabilizing reconstituted PGA against changes in temperature has been demonstrated. It was also confirmed that, upon prolonged heating at 70°, the dissociation of link-containing, reconstituted PGA is effectively irreversible. Secondly, the dissociation characteristics of native PGA isolated from chick chondrocyte and rat chondrosarcoma cell-cultures were investigated in aqueous solvents containing increasing concentrations of guanidine hydrochloride. It was found that the more densely packed, native, aggregate structure has a higher susceptibility to thermal disruption than the reconstituted, link-containing material. Considerable loss of subunits from the native aggregates occurs at temperature where the link protein retains its activity. The dissociation of native PGA is irreversible, in the sense that reconstituted PGA always exhibits smaller sizes than the native PGA, reflecting a smaller degree of aggregation of the subunits.

INTRODUCTION

Proteoglycans constitute the major carbohydrate-containing, macromolecular component of the ground substance of the intercellular matrix[1]. The native struc-

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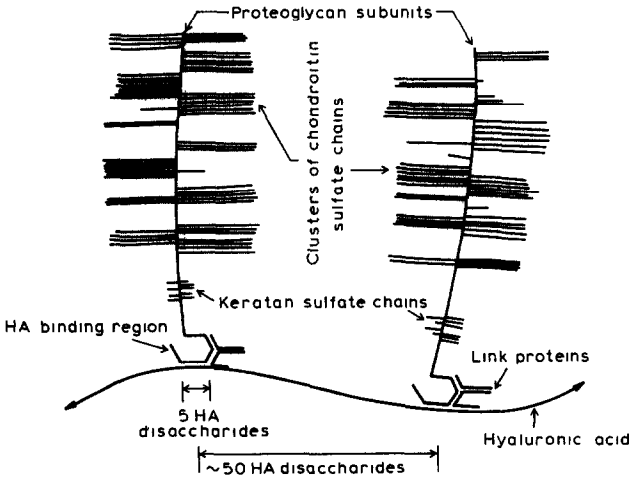

Fig. 1 Schematic of cartilage proteoglycan aggregate structure [Two distinct monomeric forms of cartilage link protein have been identified for several tissues However, the precise nature of the link protein binding complex has not been established]

ture of cartilage proteoglycan (PG), shown in Fig. 1, consists of an aggregate (PGA) composed of a number of proteoglycan subunits (PGS; M_r = 1–4 × 10^6) which are non-covalently attached to a central, hyaluronic acid chain (HA, M_r = 0.5–2 × 10^6). Cartilage PGS consists of a linear, protein core (M_r = 2–3 × 10^5) with side chains of keratan sulfate ($M_w \simeq$ 12,000) and chondroitin sulfate ($M_r \simeq$ 20,000) bound covalently to L-serine and L-threonine residues. The PGS–HA interaction involves one end of the core protein that is largely free from carbohydrate chains and is stabilized by a small protein known as link protein (LP).

Proteoglycan is usually extracted from homogenized cartilage in the form of its dissociated components: typically, the extraction is done with 4M guanidine hydrochloride (Gdn·HCl) under conditions that dissociate the aggregate. PGA is then reconstituted by dialysis to associative conditions (0.4M Gdn·HCl or 0.1M NaCl). Recently, a "native" aggregate has been isolated by using solvents which do not disrupt the PGA structure. This can be done (in high yields) from cultures of cartilage cells by using 0.5M Gdn·HCl. Also, it has been demonstrated[2] by electron microscopy that the native aggregates (PGA-aA1) isolated from chick chondrocyte cultures differ from reconstituted PGA (PGA-raA1) in having a higher density of subunits attached to HA.

Light-scattering studies of PGS have been reported by several laboratories. It is important to note that PGS and PGA are polydisperse species, and hence it is necessary to specify precisely the history of the preparation of the sample. Pasternak *et al.*[3] and Kitchen and Cleland[4] carried out total intensity studies on PGS preparations from bovine nasal septum isolated *via* CsCl density centrifugation under associative and then dissociative conditions (labelled A1D1). These furnished a weight-average molecular weight, M_w = 1.6–2.3 × 10^6, and a *z*-average radius of

gyration, $R_{g,z}$ = 410–570 Å. Reihanian *et al.*[5] reported a dynamic light-scattering study of a PGS preparation from bovine nasal septum subjected to a second CsCl density centrifugation (A1D1D1). This study determined the hydrodynamic (Stokes) radius (R_h) to be 735 Å in several solvent systems, and indicated a higher molecular weight, $M_w = 3.97 \times 10^6$ (which was to be expected, as the A1D1D1 sample is a high-density fraction than that of A1D1). An interesting observation was made, in that the subunit exhibits, in aqueous NaCl, a reversible self-association which is inhibited[5,6] by addition of 0.4M Gdn·HCl. More recently, Harper *et al.*[7] showed that this effect is due to the reversible formation of a small concentration of very large aggregates which dominate the light-scattering characteristics.

Light-scattering studies of PGS and PGA fractions from chick chondrocyte cultures have been reported by Shogren *et al.*[8] and Ohno *et al.*[9]. The specimens were PGS fractions isolated by chromatography on Sepharose 2B in 4M Gdn·HCl, and also a reconstituted[10] PGA isolated from the void volume of 2B columns in 0.4M Gdn·HCl. Shogren *et al.*[8] determined $M_w = 1.42 \times 10^6$, $R_{g,z}$ = 565 Å, and R_h = 376 Å, for PGS, and $M_w = 45 \times 10^6$, $R_{g,z}$ = 2170 Å, and R_h = 1380 Å for PGA[10]. Maeda and Oike[11] obtained similar results by static light-scattering studies of chick chondrocyte PGS in 0.4M Gdn·HCl, and also showed that, in 0.5M NaCl, these PGS preparations undergo self-association. Lyon *et al.*[12] reported both static and dynamic light-scattering studies of bovine, femoral articular PGS in 4M Gdn·HCl, and reported $M_w = 1.2 \times 10^6$, $R_{g,z}$ = 420 Å, and R_h = 307 Å. These authors further demonstrated that this PGS also self-associated in NaCl. Ohno *et al.*[9] compared static and dynamic light-scattering data for native and reconstituted PGA, and observed that the molecular weights of the native PGA are 5–10 times those for reconstituted PGA, indicating a larger aggregation number, and confirming earlier work based on electron microscopy[2].

The role of the interaction of proteoglycan subunit with link protein in stabilizing the proteoglycan aggregate structure has been established[13,14]. In the absence of link protein, proteoglycans bind reversibly to oligosaccharides[13]. In contrast, link-containing aggregates do not show competitive binding with oligosaccharides[13]. The link protein effectively locks proteoglycan onto the hyaluronate chain. The stabilizing effect of link protein persists[13] on heating to 50° in 0.5M Gdn·HCl, and even to 60° in 0.15M NaCl at pH 7.4. Also, the link-stable aggregate is dissociated[14] by high concentrations of Gdn·HCl, $CaCl_2$, $MgCl_2$, and sodium dodecyl sulfate, but is not dissociated by 4M urea.

We now describe, for the first time, light-scattering studies of the dissociation of PGA when it is subjected to changes in temperature and solvent conditions. Our first experiments[15] compared the stability of two specimens of reconstituted bovine fetal epiphyseal PGA: a link-free preparation formed by mixing HA and PGS-A1D1D1, and link-containing PGA prepared by mixing PGS, HA, and soluble link-protein[16]. We also describe a study of the dissociation of native PGA preparations isolated from chick chondrocyte and rat chondrosarcoma cultures.

EXPERIMENTAL

Sample preparation — (*a*) *Reconstituted proteoglycan aggregates from bovine nasal septum and bovine fetal epiphysus.* Three species of reconstituted proteoglycan aggregates were investigated. Two preparations were made in 0.15M NaCl, 0.01M 2-(4-morpholino)ethanesulfonic acid (MES), pH 7.0, according to the methodology of Tang *et al.*[16]. One of these contained[17] 3 mg of bovine fetal epiphyseal PGS/mL, and 0.03 mg of rooster comb HA/mL, but no link protein. The specimen of HA was generously supplied by Dr. D. Swann, Shriner Burns Institute, Boston, MA. The second solution contained 3 mg of proteoglycan subunit/mL, 0.03 mg of HA/mL, and 0.21 of a soluble form of link protein/mL. Aliquots of each solution were dialyzed against 0.15M NaCl, 0.01M MES, at pH 7 and at pH 5. The solutions for light-scattering analysis were then diluted to 0.25 mg of PGS/mL by additions of weighed amounts of solvent. A third sample was bovine nasal septum PGA-A1, isolated by standard methods[18], and purified as the void volume on Sepharose CL-2B.

(*b*) *Chick chondrocyte proteoglycans.* Native PGA (PGA-aA1) was isolated from day-8, chick limb bud chondrocyte cell-cultures using 0.4M Gdn·HCl containing proteolytic inhibitors and purified from the densest (A1) fraction in a CsCl density-gradient centrifugation as the void volume on[18] Sepharose CL-2B.

(*c*) *Rat chondrosarcoma proteoglycans.* Native PGA-aA1 was obtained from the laboratory of Dr. V. C. Hascall, NIDR. These samples were isolated under associative conditions from day-8 rat chondrosarcoma cultures as described[19]. The PGA used for light-scattering analysis was again obtained as the void volume on Sepharose CL-2B.

Light-scattering. — Static and dynamic light-scattering were performed by using two light-scattering instruments: a custom-built system described elsewhere[26] and a commercial unit from Brookhaven Instruments Corporation, Amherst, MA, comprising a BI 2020 correlator and a BI 240 photogoniometer.

Light-scattering from PGA solutions was analyzed by conventional methods[21]. Intensity data were analyzed by using Zimm plots of the following relationship:

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_w}\left(1 + \frac{q^2 R_{g,z}^2}{3}\right) + 2A_2 c, \qquad (1)$$

from which were obtained the weight-average molecular weight, M_w, the z-average radius of gyration $R_{g,z}$, and the second osmotic virial coefficient A_2. Here, $q = (4\pi\bar{n}/\lambda)\sin\theta/2$ is the scattering vector, θ is the scattering angle, $\lambda = 6328$ Å is the wavelength of the laser light, and $\bar{n} = 1.333$ is the refractive index of the solvent.

The time correlation function $g^{(1)}(\tau)$, from dynamic light-scattering was interpreted by the method of cumulants[22,23] according to the relationship:

$$\ln g^{(1)}(\tau) = -\bar{\Gamma}\tau + \frac{1}{2!}\frac{\mu_2}{\bar{\Gamma}^2}(\bar{\Gamma}\tau)^2 - \ldots \quad (2)$$

From the first cumulant, $\bar{\Gamma} = D_{t,z}q^2$, we determined the z-average translational coefficient, and the normalized second cumulant $\mu_2/\bar{\Gamma}^2 = (\bar{\Gamma}^2 - \bar{\Gamma}^2)/\bar{\Gamma}^2$ provided information about the distribution[21,22] of D_t.

It is important to note that, in order to monitor the thermal denaturation of PGA, we measured $D_{t,z}$ at comparatively large scattering angles (40° or 50°). The correlation function for mixtures of subunits and aggregates is approximately bi-exponential:

$$g^{(1)}(\tau) = A_1\exp(-\Gamma_1\tau) + A_2\exp(-\Gamma_2\tau). \quad (3)$$

At $\theta = 40°$, an appropriate value for Γ for PGS is 550 s^{-1}, with $\Gamma_2 = 100$ s^{-1} for[6] PGA. The scattering amplitudes A_i (A_1 or A_2) can be approximated, by using the Guinier relation for the interference function, as:

$$A_i = c_iM_i\exp(-q^2R_{gi}^2/3), \quad (4)$$

Where c_i = weight concentration of species i of molecular weight M_i and radius of gyration R_{gi}. For our present purposes, it is convenient to set the molecular parameters equal to those reported for bovine nasal septum proteoglycan[6], *viz.*, M_w(PGS) = 3.2 × 10^6 and M_w(PGA) = 2.5 × 10^6, R_g(PGS) = 1050 Å, and R_g(PGA) = 3940 Å. Because of the high value of the ratio $M_{PGA}/M_{PGS} = 78$, it is advantageous to choose a comparatively large scattering angle where the interference function ratio

$$P_A/P_S = \exp(-q^2R_{gPGA}^2/3)\exp(-q^2R_{gPGS}^2/3) \quad (5)$$

is relatively small, so that the unaggregated PGS molecules make a significant contribution to the total scattering. At $\theta = 40°$ and $\lambda = 6328$ Å, $q = 90{,}536.8$ cm^{-1} and the ratio $P_A/P_S = 55.5$, and hence, in Eq. 3, $A_{PGS}/A_{PGA} = 0.713$ (C_M/C_A). The net result is that the scattering amplitudes of the PGS and PGA are approximately equal at equal weight concentrations when $\theta = 40$–$50°$.

We limit ourselves herein to a discussion of the behavior of $D_{t,z}$ and the Stokes hydrodynamic radius, R_h, calculated from $D_{t,z}^0$ by the Stokes–Einstein equation

$$D_{t,z}^0 = kT/6\pi\eta_sR_h, \quad (6)$$

where k is the Boltzman constant, T is the absolute temperature, and η_s is the solvent viscosity.

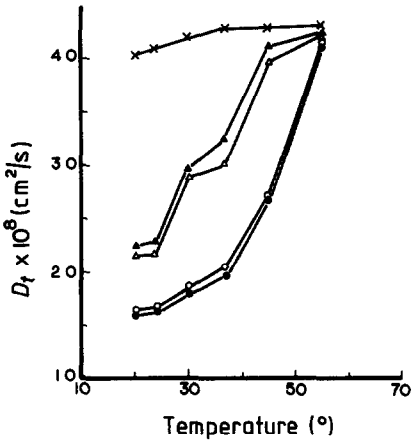

Fig. 2 Temperature-dependence of *z*-average translational diffusion coefficient of link-free bovine epiphysial PGA [Key: ●, pH 7 0, ○, pH 5.0, △, pH 4.0; ▲, pH 3 0; ×, pH 2 0]

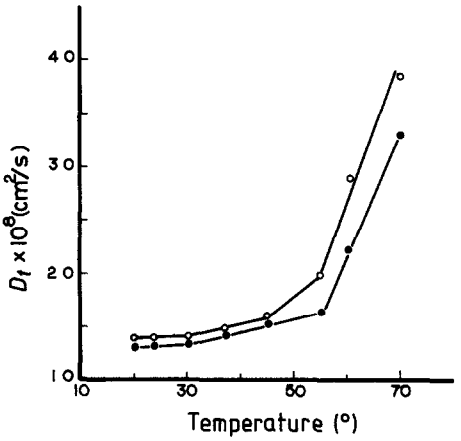

Fig. 3. Temperature-dependence of *z*-average translational diffusion coefficient of link-containing bovine epiphysial PGA. [Key. ●, pH 7 0; ○, pH 5 0]

RESULTS

(*a*) *Reconstituted proteoglycans.* — The thermal dissociation of link-free and link-containing reconstituted PGA, prepared by mixing an A1D1 PGS from bovine fetal epiphysus with soluble link protein and rooster comb hyaluronate as already described, was monitored in aqueous 0.15M NaCl/0.01 MES as a function of pH by observing the temperature dependence of the photon correlation function of the scattered light. Figs. 2 and 3 compare the temperature-dependence of the *z*-average diffusion coefficients of link-free and link-containing PGA at several pH values. The data have been normalized to the viscosity of water at 25°. In both systems, the

diffusion coefficient increases with temperature, corresponding to a decrease in particle size as the aggregates dissociate into their component macromolecules. Fig. 2 indicates that, for link-free PGA, decrease in pH at a given temperature destabilizes the aggregate. Also, comparing Fig. 2 *versus* Fig. 3 at pH 5 and 7, the presence of link protein stabilizes the aggregate structure, because the average particle-size at a given temperature is always larger for the link-containing preparations.

A feature of our photon correlation functions is an increase in non-exponentiality as the temperature increases, reflected by an increase in the second moment, $\mu_2/\bar{\Gamma}^2$. As already noted, an advantage of photon correlation spectroscopy for studies of this kind is that it is possible to analyze separately the light-scattering contributions from free subunit and aggregate structures.

It is important to realize that the dissociation of these reconstituted PGAs does not occur instantaneously. Fig. 4 shows the time dependence of the apparent z-average diffusion coefficient D_t^{app} of bovine nasal septum PGA (A1) in 0.15M NaCl, pH 7.4, at $T = 70°$, measured at a scattering angle of $\theta = 50°$. There is an exponential variation of $D_{t,z}$ from an initial value characteristic of the aggregate to a final value corresponding to the free subunit. The time dependence of the apparent diffusion coefficient can be fitted by an expression of the form:

$$D_t^{app}(t) \times 10^8 = 3.01 - 1.31\exp(-0.013t). \tag{7}$$

The half-life of the dissociation process is 53 min.

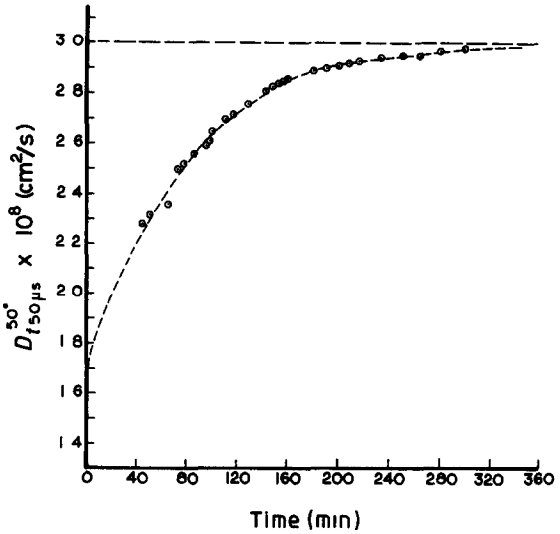

Fig. 4. The kinetics of thermal dissociation of reconstituted bovine nasal septum PGA at $T = 70°$, monitored by observing the time evolution of the apparent translational diffusion coefficient measured at scattering angle $\theta = 50°$ and correlation time increment 50 μs.

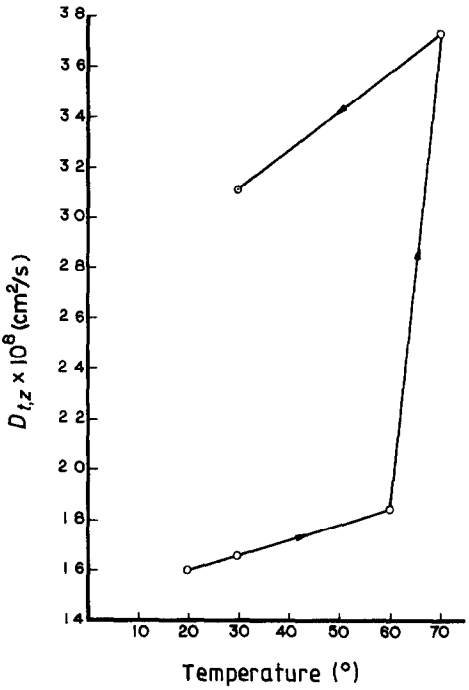

Fig 5 Temperature-dependence of the *z*-average translational diffusion coefficient of reconstituted bovine nasal septum PGA [After maintaining the solution for 5 h at 70°, $D_{t,z}$ decreases only slightly on cooling, and remains at this value for several weeks, indicating that irreversible denaturation of the PGA had occurred]

In Fig. 5, we show results of a thermal denaturation experiment in which the degree of reassociation of the link-containing, reconstituted bovine nasal septum PGA was studied in 0.15M NaCl after maintaining the temperature of the solution for 5 h at 70°. As the diffusion coefficient evidently remains at a value characteristic of the proteoglycan subunit, even after a period of weeks has elapsed, we concluded that irreversible denaturation of the link protein, or the hook region, or both, occurs.

(*b*) *Native chick chondrocyte proteoglycans.* — The dissociation of native PGA from chick limb bud chondrocytes was examined by monitoring the change in $D^0_{t,z}$ (the *z*-average diffusion coefficient extrapolated to zero PGA concentration) as a function of molar concentration of Gdn·HCl in the solvent. Typical data are presented in Fig. 6. Above 1.0M Gdn·HCl, there is a sudden increase in $D^0_{t,z}$, corresponding to a decrease in the average Stokes radius (R_h) from 192 to 39 nm. Upon dilution back to associative conditions, *i.e.*, from 4M to 0.4M Gdn·HCl, the aggregate structure is partially reconstituted. Fig. 7 shows the time dependence of the apparent diffusion coefficient, $D_{t,z}$, measured at a single scattering-angle (θ = 50°) following dilution to 0.4M Gdn·HCl. After 70 h, $D^{app}_{t,z}$ has decreased to a value

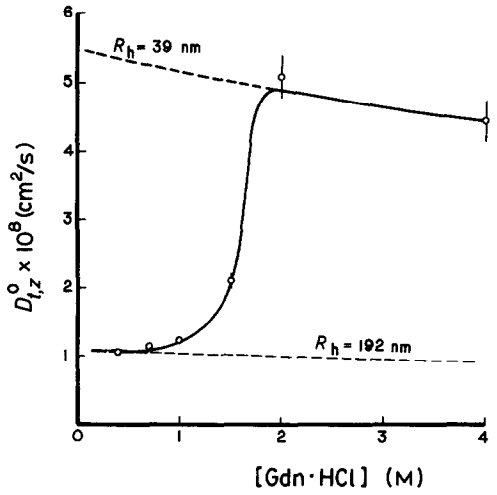

Fig 6 Dissociation of native chick limb bud PGA by Gdn · HCl is examined by monitoring the variation in *z*-average translational diffusion coefficient normalized to the viscosity of water at 20°

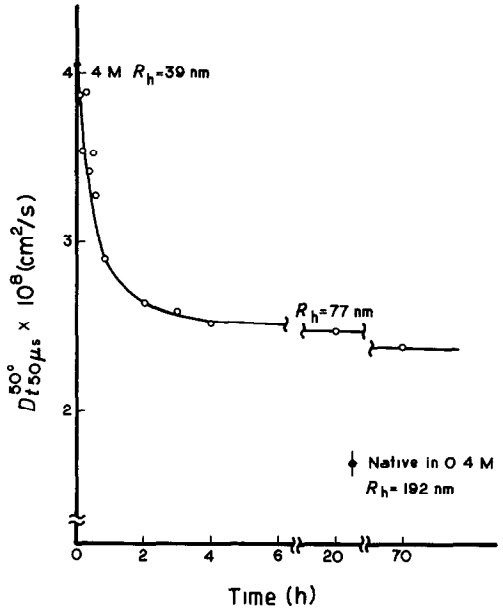

Fig 7 Reconstitution of chick limb bud PGA is examined by monitoring the time-dependence of $D^{50°}_{t50\mu s}$ The value of the native aggregate is shown for comparison.

which, on appropriate extrapolation, corresponds to a Stokes radius of 770 Å. However, the initial value of the native PGA (1920 Å) is never regained. These observations are entirely consistent with those of our previous light-scattering study[9], in which we found that the sizes of PGA-raA1 are always smaller than those of PGA-aA1.

Thermal dissociation of native PGA has also been investigated under associative conditions, *viz.*, in 0.4M Gdn·HCl, by monitoring the temperature-dependence of $D^0_{t,z}$. The results are shown in Fig. 8, together with the calculated Stokes radii. Also presented are the Stokes radii measured when solutions are cooled to room temperature after heating to 50° and to 60°. In each case, the aggregate fails to recover its initial size. In fact, the value obtained on cooling from 60° remains unchanged. Evidently, the densely packed, native PGA structure cannot re-form after thermal dissociation.

(*c*) *Rat chondrosarcoma proteoglycans.* — Experiments similar to those performed for the chick chondrocyte PGA were conducted for native PGA extracted from rat chondrosarcoma cultures. As shown in Fig. 9, dissociation of aggregates occurs when the concentration of Gdn·HCl is increased above 1.0M, as evidenced by an increase in $D^0_{t,z}$ corresponding to a decrease in Stokes radius from 135 to 38.5 nm, which is the value for pure PGS. Evidently, Gdn·HCl has comparable effectiveness in dissociating both the rat chondrosarcoma and the chick chondrocyte PGA

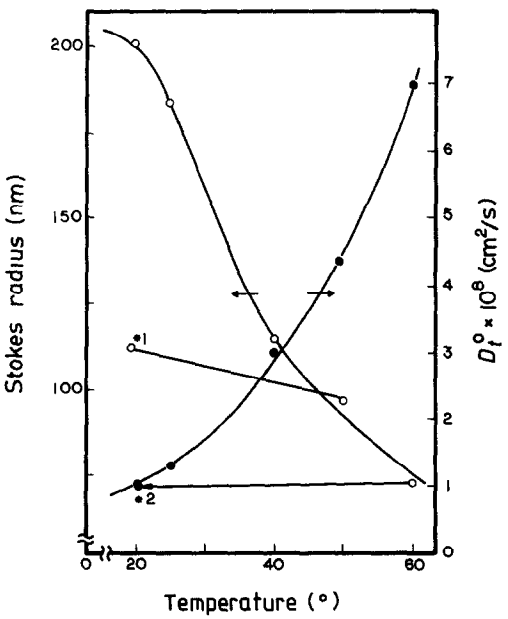

Fig 8 Temperature-dependence of the z-average translational diffusion coefficient of native chick limb bud PGA, and the respective Stokes Radii. Also shown are values of Stokes radii on cooling from 50° (1) and 60° (2)

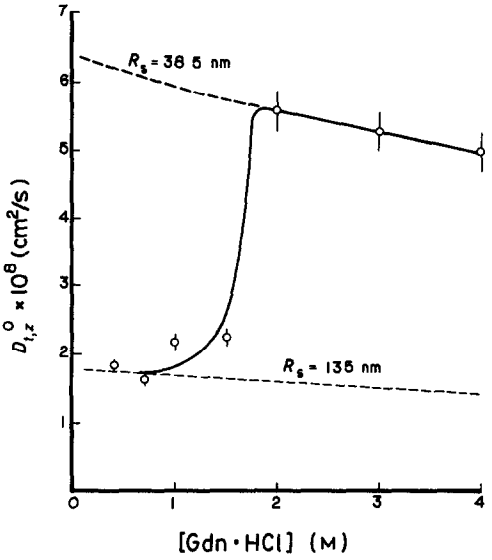

Fig 9 Dissociation of native rat chondrosarcoma PGA by Gdn·HCl is examined by monitoring the variation in z-average translational diffusion coefficient corrected to the viscosity of water at 20°

DISCUSSION

The diffusion data in Figs. 2 and 3 illustrate the role of link protein in stabilizing the proteoglycan aggregate structure against pH and thermal dissociation. It is interesting to compare our results from light-scattering measurements with those of previous studies of the stability of reconstituted PGA determined with the aid of viscometry[13], velocity sedimentation[13,16,17], and electron microscopy[17] methods. Consistent with Figs. 2 and 3, Hardingham[13] and Tang *et al.*[16,17] found that, at room temperature, both the link protein-containing and the link protein-free reconstituted PGA resist dissociation at pH values >5, but that the link protein-containing PGA has a larger degree of aggregation in this pH range than has link protein-free PGA. The viscometric data of Hardingham[13] indicated that link protein-free PGA is substantially dissociated in 0.5M Gdn·HCl at pH 4.0 and room temperature, in agreement with our light-scattering results (see Fig. 2), which indicated essentially complete dissociation of link protein-free aggregates in 0.15M NaCl at 20° and pH 4.0. Also based on viscometric data, Hardingham[13] found that, on heating in 0.15M NaCl at pH 7.4, link protein-containing PGA retains a substantial degree of aggregation up to 60°, whereas link protein-free PGA is completely dissociated under the same conditions. Again, these observations are in agreement with our light-scattering data at pH 5 and 7 in 0.15M NaCl, as presented in Figs. 2 and 3. On prolonged heating of reconstituted, link protein-containing PGA in 0.15M NaCl at 70°, irreversible thermal denaturation of the HA-binding region, or link protein, or both, occurs (see Figs. 4 and 5). Hardingham[13] found the thermal denaturation of

link protein-containing PGA to be reversible up to 60° in 0.15M NaCl at pH 7.4, and up to 50° in 0.05M Gdn·HCl–0.05M NaOAc at pH 5.8.

Kimata *et al.*[24] investigated the stability to dissociation by pH and Gdn·HCl of native PGA isolated from rat chondrosarcoma cultures. These authors found[24] that native PGA is stable up to 1.5M Gdn·HCl and is partially dissociated by 2M Gdn·HCl over a period of 24 h. Our experiments (see Figs. 6 and 9) indicated the presence of small amounts of dissociation at 1.5M Gdn·HCl, and complete dissociation of native PGA from both rat chondrosarcoma and chick chondrocyte cultures at 2M Gdn·HCl. The slight discrepancy may reflect solvation effects (the work of Kimata *et al.*[24] involved Cs_2SO_4 density centrifugation) or small differences in sample preparation.

Kimata *et al.*[24] also studied the kinetics of reassociation of PGA after pH dissociation. They observed substantial reaggregation of rat chondrosarcoma PGA after equilibration for 4 h under associative conditions This is quite consistent with our kinetic data (see Fig 7) for reconstitution of chick chondrocyte PGA after Gdn·HCl dissociation. We note, however, that Kimata *et al.*[24] gave evidence suggesting that, under pH dissociation, the link protein–monomer complexes dissociate as bimolecular units from hyaluronic acid, whereas, in 4M Gdn·HCl, the link protein–monomer complex is completely dissociated. In spite of this, the kinetics of reassociation appear to be similar. Our experiments showed further (see Fig. 7) that reaggregation produces a PGA of size smaller than that of the native species This result is consistent with previous studies[2,9] which showed that reconstituted PGAs have lower molecular weights than the native species. Thus, the more densely packed, native PGA structure cannot re-form after denaturation[2,9] *in vitro*.

Finally, with regard to thermal dissociation of native PGA (see Fig. 8), it is evident that these species suffer considerable dissociation at temperatures where the reconstituted form is stable (see Fig. 3). However, the hydrodynamic radius of the thermally dissociated, native PGA at 60° is the same (see Fig. 8) as that of the reconstituted form of this species. Also, from earlier studies[13,14] and Fig. 3, it seems that the link protein and hyaluronic acid-binding region retain their native form at temperatures up to 60°. Thus, we conclude that, at each temperature, there exists an equilibrium mixture of native and reconstituted (disordered) PGA structures which arises because of thermal stress on the more densely packed monomers of the native PGA. This equilibrium shifts toward the disordered form with increase of temperature. At present, we cannot determine whether partially disordered PGA species occur, or whether the transition from native to disordered PGA is all-or-nothing in character. Thus, analysis of aggregate stability by light-scattering agrees well with that by other methods, and allows direct examination of the sizes and kinetic evolution of species produced during dissociation and reassociation of proteoglycan aggregates

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