Antifreeze Glycoproteins from an Antarctic Fish

QUASI-ELASTIC LIGHT SCATTERING STUDIES OF THE HYDRODYNAMIC CONFORMATIONS OF ANTIFREEZE GLYCOPROTEINS*

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SUMMARY

A quasi-elastic light-scattering technique was used to study the hydrodynamic conformations of antifreeze glycoproteins from an Antarctic fish. Antifreeze glycoprotein is composed of repeating units of Ala-Ala-Thr, with each threonine O-linked to a disaccharide, and it exists as several polymers of different numbers of this repeating unit. Molecular weights of the two major active polymers are 10,500 and 17,500 by such methods as centrifugation and osmotic pressure, but <20 by freezing-point depression. Translational diffusion coefficients at 20°C were 8.35 x 10⁻⁷ cm² s⁻¹ and 6.15 x 10⁻⁷ cm² s⁻¹ for the M₉ 10,500 and 17,500 polymers, respectively. Measurements at -0.2°C in the presence of ice crystals did not indicate any conformational changes of the ice phase, the water phase, and perhaps even a combination of both phases (8, 11). We have used several physical means to examine the glycoprotein structure and properties of the ice phase, the water phase, and perhaps even a combination of both phases (8, 11, 12). We have used several physical means to examine the glycoprotein structure.

The quasi-elastic light scattering technique has been used to probe the hydrodynamic conformations of a group of glycoproteins present in the sera of Antarctic fishes. This group of glycoproteins functions as antifreeze agents (1-6) and structurally consists of repeating units of the glycotripeptides Ala-Ala-Thr, with all threonines glycosidically linked to a galactosyl-2-acetyl-galactosamine disaccharide (7-9). The main active components of these homologous polymeric antifreeze glycotripeptides have molecular weights of 10,500 and 17,500, and contain approximately 80, 50, 23, and 14 amino acid residues, respectively. These substances lower the freezing temperature of water more than NaCl does (>50 times the lowering expected if its function were colligative). Furthermore, they do not affect the melting temperature, whereas substances functioning by colligative action lower both the freezing and melting temperatures (1, 4).

The antifreeze glycoproteins, which account for a major fraction of the protein in the blood serum, comprise a family of at least eight closely related constituents. They have been numbered 1 to 8 on the basis of the relative electrophoretic migrations of their borate complexes. In the sera of two very different Antarctic fishes the total concentration of glycoproteins 1 to 8 is approximately 25 mg/ml. Approximately one-fourth of this amount is in proteins 1 to 5, which all have strong and similar antifreeze activities. Proteins 6, 7, and 8 are termed inactive, although they may have weak activity under some conditions. Each glycoprotein contains two amino acids, alanine and threonine, and two sugars, galactose and N-acetylgalactosamine. Glycoproteins 6, 7, and 8 also contain some proline, which always substitutes for one of the alanines following a threonine. Physically, the glycoproteins have molecular sizes in the reverse order to their numbering, with 1 being the largest and 8 the smallest. Glycoproteins 4, 5, 7, and 8 have molecular weights of 17,500, 10,500, 4,500, and 2,800, and contain approximately 80, 50, 23, and 14 amino acid residues, respectively.

Previously viscosity determinations on protein 5 indicated a relatively expanded or extended molecule and no α helical structure was found by circular dichroism (5). Chemical modifications proved that its function was critically dependent upon the integrity of its carbohydrate side chains (8, 11). Fragments of the glycopeptide were inactive, and kinetic studies of proteolytic cleavages of peptide bonds indicated that hydrolysis of <2 peptide bonds destroyed activity (6). Thus, both the carbohydrate side chains and the polymeric structure are necessary for function (6, 8, 11).

Models for the mechanism by which the antifreeze glycoprotein lowers the freezing point of water can include the structure and properties of the ice phase, the water phase, and perhaps even a combination of both phases (8, 11, 12). We have used several physical means to examine the glycoprotein structure.

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and its interactions with H$_2$O. The present report deals specifically with the use of QELS$^1$ (13-15) to probe possible changes in the conformation of the secondary structure as a result of temperature changes. Since these glycoprotein molecules are small compared to the wavelength of visible light (<10$\mu$m), the conventional light scattering photometer is unable to detect differences in sizes from the angular intensity pattern. QELS, on the other hand, is sensitive to the Brownian motion of the molecules. Since the spectral resolution is very high (>10$^4$), this technique enables us to probe the very slow dynamic Brownian movements of these molecules. In addition, QELS is performed with no external perturbation and with the sample under conditions which are difficult to probe using other techniques.

We are reporting on the effect of temperature on the translational diffusion coefficients and the hydrodynamic conformations of antifreeze glycoproteins. The hydrodynamic conformations also were studied at the temperature and conditions where antifreeze molecules function, i.e. -0.2°C, in the presence of seed ice crystals.

**Experimental Procedure**

**Material**—The glycoprotein mixture (electrophoretically separable components 1 to 8) was separated from the fish blood serum collected from *Trematomus borchgrevinki*, caught in Antarctica as previously described (5-7). The nucleopore filter was obtained from Nucleopore Corporation, Cal. All reagents were of analytical reagent grade.

**Purification of Glycoprotein**—The glycoprotein mixture (2.5 g of mixed numbers 1 to 8) was chromatographed on a DEAE-cellulose column (4.4 X 74 cm) previously equilibrated with an initial buffer (0.0025 M Tris, pH 9.6). Proteins 1 to 5 were separated with the initial buffer, whereas the smaller components, numbers 6 to 8, were eluted with 0.5 M Tris buffer at pH 9.6. The pure samples of components 4, 5, 7, and 8 were desalted on SephadeX G-10, lyophilized, and checked for purity using polyacrylamide gel electrophoresis as previously described (5).

**Determination of Diffusion Coefficient and Radius of Gyration—** Diffusion coefficients were determined using QELS (13, 14). The incident light ($\lambda$ = 5145A) was provided by an argon$^*$ ion laser (coherent radiation, model CRL ion laser). Dust-free protein solutions (5 mg/ml) were prepared and filtered through a filter (0.2 $\mu$m) into dust-free sample tubes (1.1 X 2 cm). The laser beam was focused into the sample tube, which was placed on the axis of a cylindrical thermostated water-alcohol bath. Scattered light from a few coherence areas was imaged onto the surface of a photomultiplier tube (ITTFW130). The alternating current component was suitably amplified by a broad band amplifier prior to being processed by an SA1-42 autocorrelator. The correlation time was 1 $\mu$s per channel for the small molecular weight glycoproteins (7 and 8) and 5 $\mu$s per channel for proteins 4 and 5. Besides studying the diffusion coefficient of protein 4 as a function of temperature above freezing, diffusion coefficients of proteins 4 and 8 were determined also at a temperature above freezing and below melting (−0.2°C) for protein 4 and at 0°C for protein 8 in the presence and absence of ice crystals. The ice crystals were developed in solutions of protein 4 by incubating them at −0.2°C and in solutions of protein 8 by incubating them at 0°C, then seeding by applying a short localized spray of refrigerant. Studies were also conducted on the angular dependence of the spectrum at angles between 30° and 25°. The diffusion coefficient of protein 4 was also determined in the presence of 0.1 M KCl at room temperature using a 45° angle.

**Procedure of Data Analysis—** Scattering of light in a macromolecular solution is caused by fluctuations in the species concentration in the scattering volume. For a monodisperse suspension of spherical macromolecules undergoing only translational motion, the dynamics governing the time-dependent behavior of scattered light is purely translational diffusion. Thus, the measured autocorrelation function, $C(t)$, of the scattered light by intensity fluctuation is given by:

$$C(t) = C_0 e^{-\Gamma t}$$

with $t =$ time; $\Gamma = 2D_0 K^2$; $D_0 =$ translational diffusion coefficient; $K = (4\pi n/\lambda) \sin (\theta/2) =$ magnitude of scattering vector; $n =$ solution refractive index; $\lambda =$ wavelength of laser light in vacuum; $\theta =$ scattering angle. If $D_0$ can be assumed to relate to an effective hydrodynamic radius, $R_h$, via Stokes’ Law:

$$D_0 = \frac{kT}{6\pi n(t)R_h}$$

where $n(T) =$ viscosity of water at temperature $T$; $k =$ Boltzmann’s constant; then a measure of $\Gamma/K^2$ provides direct information on the macromolecular geometry.

If, however, the samples were heterogeneous with respect to molecular weight, and thereby sizes, a noninteracting group of heterogeneous macromolecules would provide a spectrum

$$C(t) = \sum_{i} C_{i} e^{-\Gamma_i t}$$

where $i$ represents the species $i$ present in the mixture. Such a sum leads to a nonsingle exponential resultant correlation spectrum and the interpretation becomes sensitive to data accuracy. In the limiting case where either the $t \rightarrow 0$ slope of $C(t)$ can be measured with accuracy or the sample polydispersity is minimal, one can perform a moments expansion of the correlation function (16):

$$\ln C(t) \approx t \ln C_0 - t^2 \sum \frac{\zeta_i}{\eta_i} \chi_i$$

The first cumulant, $\zeta_1$, divided by $\eta_1$ corresponds to the z-averaged diffusion coefficient and $\eta_1$, the second cumulant, is a measure of the variance of the polydisperse distribution. It has been shown by Pusey (17) that accuracy in the determination of $\eta_1$, poor if the data are rather scattered. Due to instrumental limitation encountered in the present experiments, we decided to simply use a single exponential fit. As a test of how true the correlation spectrum is to a single exponential curve, a fitness parameter, $r$, is defined by Brownlee (18):

$$r = \sqrt{n \sum \left( \frac{\chi_i}{\eta_i} \right)^2 - \left( \sum \frac{\zeta_i}{\eta_i} \right)^2}$$

Here, $n$ is the number of points taken for the analysis. In such an analysis, a truly single exponential curve leads to a value $r \approx 1.00$. Slight polydispersity of the samples causes a deviation from the $r \approx 1.00$ value. We have limited the number of data points taken in the analysis, starting from small time values, so as to keep the value of $r$ as nearly to 1.00 as possible.

**Results**

**Sample Heterogeneity—** In room temperature studies of protein 4 samples, the spectra in $C(t)$ versus $t$ exhibited large departures from linearity. A systematic effort was used to rule out dust or other large nonglycoprotein particles. Besides the careful sample preparations, the inevitable presence of a few dust particles was rendered noncontributory by the use of an electronic advance notice of dust intensity spikes. This is a device which evaluates the alternating current signal. Whenever it senses an anomalously large alternating current contribution, it triggers a stop of the correlator computer until the dust particle has drifted by the field of view. The autoactive resume is controlled by a gating circuit. Such a procedure leaves a very small residual detected contribution due to dust. For very large dust particles which

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$^1$ The abbreviation used is: QELS, quasi-elastic light scattering technique.
escape this procedure, on the &5 µs per channel display, their residual contribution is a flat base-line. We varied the base-line in our fitting procedure to maximize r. However, in no way were we successful in obtaining r ~ -1.00 for an extended record of >20 time points. This indicates that heterogeneity is the basic cause of our non-single exponential behavior, not dust. Fig. 1 shows log [autocorrelation function] versus correlation time taken for differing base-line subtraction of a single experimental run.

To further ascertain if the heterogeneity is a result of interacting glycoprotein molecules in H₂O, 0.1 M KCl was added and the results did not significantly differ from the pure H₂O case. Dependence of D on Scattering Angle—The value of r of Equation 4 was examined over a range of scattering angles from 25–55°. In Fig. 2 it is shown that, for the glycoprotein 4 sample, the $D = F/(2K^2)$ value is not independent of $K^2$ as expected under the procedure outlined in a previous section. This is not the case for either the calibrating Ludos sample or inactive borate complex of glycoprotein 4. Furthermore, a calculation including the contributions of the variation of the scattering structure factor for these small glycoprotein molecules shows that such factors cannot account for observed $D$ versus $K^2$ dependence. In an attempt to explain our $D$ versus $K^2$ results, we recall from the previous section that it was concluded the samples were highly heterogeneous. However, because no reliable $\mu_2$ values were obtainable, we forced our data to fit an effective single exponential curve with the result that the $r$ values of Equation 5 were closer to -0.8 than -1.0, indicating that there is a significant curvature in the ln $C(t)$ versus $t$ display. This procedure results in overweighting the larger fragments of the polydisperse sample even more so than the z-averaging process. Accordingly, at the smaller scattering angles, which are more sensitive to larger spatial fluctuations, the larger fragments of the polydisperse system are overweighted. Hence a lower $D$ was observed as $K$ was decreased.

Since according to Pusey (17) a Gaussian distribution with a ±5% polydispersity is essentially undetectable in curves such as our Fig. 1, we estimate that our apparent sample heterogeneity is in excess of 10%.

Dependence of $D$ on Temperature—In the temperature studies to follow, measurements were made at an appropriately larger angle, $\theta = 45^\circ$, where $r = -0.9$ was maintained. In this series of experiments, the temperature of the samples was varied from room temperature (22°) to the region where the active species were functioning as antifreeze agents. To see if there is a change in species conformation at the temperature range where the antifreeze is functioning, experiments were conducted both with the active protein 4 and the inactive protein 8. The use of Equation 2 was considered valid if $D$ were used in place of $D_0$ and $R_e$ was approximately the z-averaged effective size. After taking into consideration the changing viscosity of the solvent, it was found that the effective particle size, $R_e$, for both species decreased as the temperature was lowered (Fig. 3). The change in $R_e$ was -25% for protein 4 and 30% for protein 8 (Table I). In both cases, the lowest temperature data were taken with and without seed crystals present.

We want to emphasize that the viscosity of H₂O at -0.2° was approximated from supercooled water data, and no attempt was made to account for any more complex change of viscosity of the ice-water mixture in the seeded situation. Dependence of $D$ on Molecular Weight—Since the smaller species of antifreeze glycoprotein are relatively inactive, it was decided to probe the possible difference between species structure as a function of varying molecular weights. At a 45° angle, a study was conducted at room temperature. The results are shown in Fig. 4. Here we see that within the scatter of data, all the samples can fit to a ln(difusion) versus ln(molecular weight) curve with a slope ~ -0.62.
Translational diffusion coefficients (D) of protein 4 and the corresponding calculated effective hydrodynamic radius ($R_e$) as a function of temperature.

**Table I**

<table>
<thead>
<tr>
<th>Antifreeze glycoprotein (number)</th>
<th>Temperature</th>
<th>$D \times 10^3$</th>
<th>$R_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>degrees</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.11</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td>-0.2</td>
<td>0.82</td>
<td>29.7</td>
</tr>
<tr>
<td></td>
<td>-0.2 + ice crystals</td>
<td>3.70</td>
<td>30.1</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>12.1</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>8.4</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>0 + ice crystals</td>
<td>9.2</td>
<td>12.3</td>
</tr>
</tbody>
</table>

**Fig. 4.** Translational diffusion coefficients of protein 4, 5, 7, and 8 plotted against their molecular weights on a log-log graph.

**Discussion**

The present study presents a technique which affords a great opportunity for some detailed studies of antifreezing properties of the noncolligative molecule. It was found that protein 4, which was shown to be a single component using polyacrylamide gel electrophoresis, is polydispersed by QELS and gave molecular sizes varying from $R_e \sim 30 - 42 \, \text{A}$. Determination of diffusion coefficients in the presence and absence of 0.1 M KCl showed that this heterogeneity in sizes is not due to ionic interaction between protein molecules. This does not exclude the possible nonionic intermolecular interactions in protein 4 solution which could lead to aggregation and could be related to the antifreeze mechanism. A more up to date correlation spectrometer is needed for more accurate data to fully assess the extent and meaning of this inhomogeneity of antifreeze glycoprotein.

Our room temperature studies on both the active and inactive proteins indicate that $D \propto M^{-0.46}$. It is known that for the model of a random coil in an average solvent, $D \propto M^{-0.66}$ (19). Our data suggest that there exists a certain amount of excluded volume effect making the coil more extended and somewhat less flexible. Our large $R_e$ values are consistent with such a model.

Comparisons of measurements of diffusion coefficients, at different temperatures and at $-0.2^\circ$ in the presence of ice crystals, did not show that any conformational changes in the proteins occurred under conditions where the protein was functioning, i.e., preventing ice crystal growth at $-0.2^\circ$. Conformational changes or interactions on the surface of the ice crystal, or within the ice crystal, would not be observed by the method employed and therefore are not ruled out by these experiments.

**Acknowledgment**—We greatly appreciate the assistance and suggestions of Dr. Carl W. Schmid in the interpretations of the results.

**REFERENCES**


*The significance of this polydispersity observed by QELS is under further investigation. This extent of polydispersity has not been observed when QELS determinations were made on the borate complexes, or when ultracentrifugal analyses were made with or without borate.*
Antifreeze glycoproteins from an Antarctic fish. Quasi-elastic light scattering studies of the hydrodynamic conformations of antifreeze glycoproteins.
A I Ahmed, R E Feeney, D T Osuga and Y Yeh


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