Effect of Aspect Ratio on Protein Diffusion in Hydrogels

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To elucidate the effect of the aspect ratio of protein on the diffusion in gels, the diffusion of a rodlike protein, tropomyosin, in two kinds of polysaccharide gels, agarose and carrageenan, has been investigated by electronic speckle pattern interferometry method and compared with that of a globular protein, myoglobin. In an agarose gel that has a comparatively large pore size, the diffusion coefficient D of the tropomyosin (aspect ratio $R = 26$) shows a behavior similar to that of the globular myoglobin ($R = 1.6$), decreasing with the fiber volume fraction ϕ with a scaling exponent $\alpha = -0.3$ to -0.4, which is close to the Rouse model of $\alpha = -0.5$. Whereas in the λ-carrageenan gel that has a smaller pore size, the diffusion of tropomyosin is quite different from that of myoglobin and consists of two regions: a region at a low fiber volume fraction $D \approx \phi^{-0.3}$, close to the Rouse model and a region at a high fiber volume fraction $D \approx \phi^{-1.8}$, close to the reptation model of $\alpha = -1.75$. As far as the authors know, this is the first reptationlike behavior observed for the diffusion of rodlike protein in a hydrogel.

Introduction

Diffusion coefficients of protein through gels have been widely measured by many methods[1−6] including dynamic light scattering,[3] pulsed field-gradient nuclear magnetic resonance (PFGNMR),[4] holographic interferometry (HI),[5] and electronic speckle pattern interferometry (ESPI).[6] However, relatively little information is available on the parameters, such as the surface charge of the protein, the shape and the aspect ratio of the protein, etc., of the diffusion in gels.

To investigate the effect of charge on protein diffusion, we have studied the diffusion of myoglobin in two kinds of polysaccharide gels, neutral agarose gel and anionic carrageenan gel in a wide range of pH and ionic strengths by using the electronic speckle pattern interferometry (ESPI) method.[7] As reported in the preceding paper,[7] in the uncharged agarose gel diffusion of myoglobin was not effected by the change in pH and the ionic strength over a wide range, indicating no electrostatic interaction between the gel and the myoglobin. Whereas in the negatively charged λ-carrageenan gel, the diffusion of myoglobin was accelerated by the electrostatic attraction when the pH was lower than the isoelectric point (pI) of the protein, and it was extensively hindered by the electrostatic repulsion when pH > pI. The charge effect was weakened with the increase in the ionic strength and effectively screened at a salt concentration of 0.5 M.

Another important factor affecting the diffusion process is the shape of the diffusing molecules, in particular, the aspect ratio of the molecules. The purpose of this study is to elucidate the effect of the aspect ratio of proteins on the diffusion by investigating the diffusion coefficients of the rodlike protein tropomyosin (aspect ratio $R = 26$)[8] in agarose and carrageenan gels and comparing the diffusion coefficients with those of the globular protein myoglobin ($R = 1.6$).[8] As a result, we have found that for the tropomyosin diffusion in λ-carrageenan gel, the diffusion coefficient decreases with the increase in the fiber volume fraction of the gel in a power law, and a transition in the scaling exponent of the power law occurs at a certain fiber volume fraction. This is explained in terms of a transition from Rouse-like to the reptation-like diffusions. The pore size of gels is estimated from the diffusion of the globular protein myoglobin, and the relationship between the filament length of the tropomyosin and the pore size of the gel at which the transition occurs is discussed.

Experiments

Materials. Agarose (Agarose S, no. 312-01193) and κ-carrageenan (no. 033-09292) were obtained from Wako Co. Ltd. and λ-carrageenan (no. 24203-1610) was obtained from Junsei Chemical Co., Ltd. and used without further purification. The gelation temperature of agarose and κ-, λ-carrageenan was approximately 42 °C. To prepare the gel phases for diffusion measurements, the polysaccharide solution at a prescribed weight percentage (0.5−4 wt %) was prepared by dissolving the polysaccharide powder in deionized water or in 40 mM buffer solution (Tris-malate at pH = 6.8 and pH = 9.0) and slowly heating to the solution boiling temperature. The KCl salt concentration was changed from 0.01 to 0.5 M. The solution was kept at this temperature until the polysaccharide was completely dissolved. The solution was then cooled to approximately 80 °C, being stirred until it appeared homogeneous. After that, it was transferred to a glass spectrophotometric cuvette using a syringe. The cuvette was then cooled to room temperature for at least an hour to ensure a complete gelation. A piece of rectangular plastic was inserted into the cuvette to keep the surface of the gel flat. The gel length in the cuvette was about 1.8 cm.

Myoglobin from horse muscle (no. M-0630) was obtained from SIGMA (St. Louis, MO) and used without further purification. The myoglobin aqueous solution was prepared by dissolving the myoglobin (5 wt %) in deionized water or 40 mM buffer solution (Tris-malate at pH = 6.8, Tris-HCl at pH = 9.0). The KCl concentration was changed from 0.01 to 0.5 M. The molecular weight of the myoglobin is $M = 17\,600$ and its molecular size is 4.5 nm × 3.5 nm × 2.5 nm.[8] The isoelectric

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point of the myoglobin is pI = 6.73.[8] The self-diffusion coefficient D_0 of myoglobin at pH = 6.8 is 9.06×10^{-7} cm^2/s [7] as determined by NMR measurement, which gives out a hydrodynamic radius of 2.09 nm using the Stokes−Einstein equation.

Tropomyosin was prepared from scallops by the method of Ebashi et al.[9,10] The striated adductor muscles (200 g) of scallops were homogenized with 600 mL of 40 mM KCl, 10 mM potassium phosphate (pH = 7.0), 0.2 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3, 1 mM $MgCl_2$, and 5 mM 2-mercaptethanol in a Waring blender for 15 s; homogenization was repeated four times at 1-min intervals. The homogenate was then mixed thoroughly with 1400 mL of the same solution and then centrifuged at 8000*g* for 15 min, removing the supernatant. The washing was repeated three times. Muscle residues thus obtained were suspended into 800 mL of 0.4 M LiCl (pH = 5.0) and extracted for 1 h by gently stirring at 4 °C, maintaining the pH at 5.0 with 0.1 N HCl. The extract was obtained by centrifugation at 10000*g* for 20 min, and the supernatant was adjusted to pH = 7.0 with 0.1 N NaOH, followed by fractionation with ammonium sulfate. The fraction precipitated at 40 and 60% saturation with ammonium sulfate was dissolved and dialyzed against 0.4 M LiCl, 1 mM sodium bicarbonate, and 5 mM 2-mercaptethanol. The solution containing tropomyosin was then clarified by centrifugation at 100000*g* for 40 min. After adjusting the protein concentration was adjusted to 15−20 mg/mL, the solution was brought to pH = 4.5 with 0.1 N HCl at 0 °C and centrifuged at 10000*g* for 10 min. The precipitate was dissolved in and dialyzed against 40 mM buffer solution (Tris-malate at pH = 7.0, Tris-HCl at pH = 9.0). The KCl concentration was changed from 0.01 to 0.5 M. The purity of prepared tropomyosin was confirmed by SDS−gel electrophoresis. The molecular weight of one chain of the tropomyosin is M = 35 000. The isoelectric point of tropomyosin is pI = 5.1.

According to the literature, tropomyosin is a rodlike protein that consists of two similar helical chains with a coiled-coil structure. The native tropomyosin molecule is a two-chain, α-helical coiled-coil. It comprises two essentially completely α-helical polypeptide chains arranged side-by-side, in parallel and in register, and given a slight supertwist.[9−12] The molecular size of the tropomyosin is 38.5 nm (length) × 1.5 nm (width). The self-diffusion coefficient of tropomyosin is $(2.22-2.43) \times 10^{-7}$ cm^2/s[8]. Tropomyosin plays a central role in the calcium dependent regulation of vertebrate-striated muscle contraction in association with the troponin complex.

Measurements. The mutual diffusion coefficients of the proteins in the gels were measured by the ESPI method. The principle of the experimental setup has been reported in previous papers.[6,7] A continuous wave He−Ne laser emitting coherent light at 632.8 nm is used as the light source. The laser beam is divided into a reference beam and an object beam by a beam splitter. Each beam is focused through a pinhole spatial filter by a 25× microscope objective, and then the beams pass through collimating lenses. The object beam traverses the diffusion cell and, the reference beam is reflected in the same way as the object beam. The two beams impinge on the CCD array. The interference fringes are recorded with a cooled CCD camera. The total image area contains 1280 × 1024 pixels. The diffusion cell is a 1.0 cm × 4.5 cm spectrophotometric cuvette with a 5 mm light path.

The protein solution is put into the diffusion cell, and the dual-illumination images are sequentially taken and stored on the hard disk with an interval time of 15 min for 15 h. The background noise is subtracted from all the images taken in the image acquisition step.

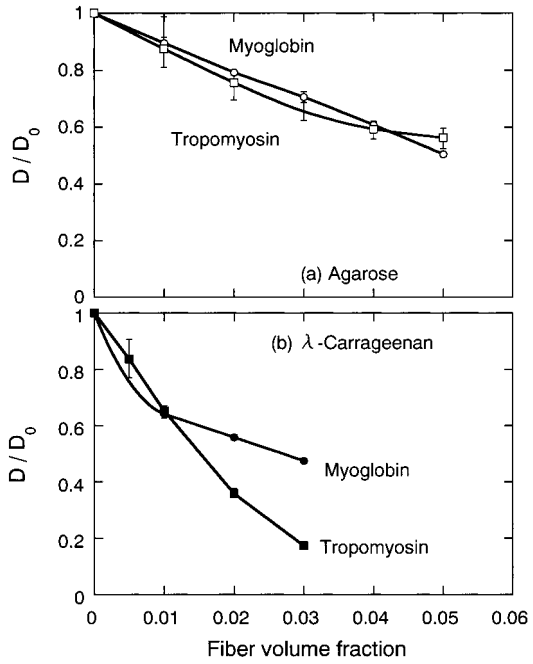

Figure 1. Effect of fiber volume fraction of the polysaccharide gels on the diffusion coefficient of proteins at 20 °C. The pH and ionic strength of solution are 6.8 and 0.5 M KCl, respectively: (a) in agarose gel (opened marks); (b) in λ-carrageenan gel (closed marks); (○, ●) myoglobin; (□, ■) tropomyosin.

The diffusion constant was evaluated using the method described in the previous report.[6,7] The positions of the three next neighboring fringes were determined to estimate the diffusion constant.[7] Measurements were made in at least duplicate at 20 °C. The main source of the errors comes from the coarseness of the fringe peak in the digital image processing. The diffusion coefficient was the average value estimated from 30 images. The maximum value and the minimum value are shown as the range of error bars in the figures. A detailed discussion on the errors of ESPI was given in one of our previous papers.[6]

Results and Discussions

Figure 1 shows the diffusion coefficients of myoglobin and tropomyosin in agarose gels (Figure 1a) and λ-carrageenan gel (Figure 1b), respectively. The experiment was carried out at pH = 6.8 in the presence of 0.5 M KCl. Since the isoelectric points (pI) of myoglobin and tropomyosin are 6.8 and 5.1, respectively, the net surface charge is zero for myoglobin and negative for tropomyosin at this pH. As shown in Figure 1a, the diffusion coefficients of the proteins are only modestly hindered by the increase in the fiber volume fraction of the agarose gel and the differences in diffusion behavior between myoglobin and tropomyosin are not substantial. However, in λ-carrageenan gel, the diffusion coefficients of the proteins are strongly hindered by the increase in the fiber volume fraction. In addition, the differences in the diffusion behaviors between myoglobin and tropomyosin are prominent and the diffusion coefficient of tropomyosin substantially decreases with the fiber volume fraction.

One can easily see that the diffusion coefficient in the λ-carrageenan gel is smaller than that in agarose gel under the same gel fiber volume fraction ϕ for both myoglobin and tropomyosin. According to our previous study, a difference in

TABLE 1: Gel Pore Size, *R*, Estimated from the Clague−Philips Model for Agarose Gel and from the Tsai−Strieder Model for λ-Carrageenan Gel[a]

	R (nm)	
ϕ	agarose	λ-carrageenan
0.005	96.6	14.8
0.01	67.6	10.4
0.02	46.8	7.2
0.03	37.4	5.9
0.04	31.7	5.0
0.05	27.7	4.5

[a] The pore size of a gel at a certain fiber volume fraction, ϕ, is defined as the hydrodynamic radius of a globular protein, which has a mobility equal to $^1/_{10}$ of that in the free solution.

the gel fiber size brought about a pronounced effect on the diffusion process in the gel network.[7] The different behavior in agarose gel and in λ-carrageenan gel should, first of all, be attributed to the difference in fiber size of the gels. Agarose fiber has an average radius of 1.9 nm, whereas λ-carrageenan fiber ranges from 0.2 to 0.5 nm, depending on the concentration.[13] Therefore, the average pore size of agarose gel is much larger than that of λ-carrageenan gel at a same fiber volume fraction. The strong suppression of the diffusion with an increase in the fiber volume fraction in the λ-carrageenan gel is attributed to the smaller pore size.

Slater et al.[14] used the Ogston theory[15] to evaluate the average pore radius, *R*, of an agarose gel of a certain concentration as the radius of gyration of the DNA molecular size, which has a mobility equal to half the free solution mobility at that gel concentration. As has been clarified in our previous paper,[7] the diffusion coefficient of the globular myoglobin in agarose gel obtained by ESPI measurement can be well expressed by the Clague−Philips model,[16]

$$\frac{D}{D_0} = \left(1 + \frac{2}{3}\alpha\right)^{-1} \exp[-\pi\phi^{0.174\ln(59.6(R_h/R_f))}] \qquad (1)$$

while that in λ-carrageenan gel can be expressed by the Tsai−Strieder model,[17]

$$\frac{D}{D_0} = \left(1 + \frac{2}{3}\alpha\right)^{-1} \qquad (2)$$

without using any arbitrary parameters. Here

$$\alpha = \phi\left(\frac{R_f + R_h}{R_f}\right)^2 \qquad (3)$$

where R_h is the hydrodynamic radius of the diffuser, R_f is the fiber radius of the gel, and ϕ is the volume fraction of the fiber. Therefore, we can use these two models to estimate the pore size of the agarose gel and λ-carrageenan gel at various fiber volume fractions by employing the same approach as that of Slater et al. We define the pore size as the hydrodynamic radius of a globular protein which has a mobility equal to $^1/_{10}$ of that in the free solution, that is, to estimate the value of R_h at $D/D_0 = 0.1$. Table 1 shows the pore sizes of the agarose and the λ-carrageenan gels at various fiber volume fractions estimated from eq 1 (Clague−Philips model) for agarose gel and from eq 2 (Tsai−Strieder model) for λ-carrageenan gel using R_f(agarose) = 1.9 nm and R_f(λ-carrageenan) = 0.29 nm.[7] As shown in the table, the pore sizes of agarose thus obtained are smaller than those obtained from DNA diffusion by Slater et al., though we set the criteria at a diffusion coefficient being suppressed to 10% of that in the free solution instead of 50%, which is the case of Slater et al. We consider that the pore size thus obtained is closer to the real pore size of the gel than that obtained from DNA diffusion, since Equation 1 and 2 were found to be in good agreement with the experimental data of myoglobin, which is a compact globular protein, and its diffusional behavior should be more Stokes-like. The pore size obtained from DNA diffusion might be overestimated due to the contribution from reptation mode.

As shown in Table 1, the pore size of the λ-carrageenan gel is only $^1/_6$ of that of agarose gel at a similar fiber volume fraction. The molecular size of myoglobin is smaller than the pore size even at the highest λ-carrageenan gel concentration. However, the length of the tropomyosin filament (38.5 nm) becomes larger than the pore size when the agarose concentration is higher than 4 wt %. On the other hand, the pore size of the λ-carrageenan gel is much smaller than the length of the tropomyosin filament even at the lowest fiber volume fraction in this study.

In polymer theory, the diffusion of polymer chains can be divided separately into two regions based on the chain length relative to pore size *R*.[18,19] The first region for a Gaussian chain is observed when the gyration radius of the chain $R_g < R/2$, where the diffusion is described by the Rouse model. For the charged macromolecule,[20]

$$D \approx R^2/\tau_{Rouse} \sim \frac{1}{N}\left(1 + \frac{2AC_s}{C}\right)^{1/2} \qquad (4)$$

where τ_{Rouse} is the relaxation time at Rouse region, *C* is the gel concentration (which is proportional to the polymer volume fraction ϕ), C_s is added simple salt concentration, *A* is the average number of monomers between charges in the gel, and *N* is the diffuser segment length. In the low-salt limit $C \gg 2AC_s$, the diffusion coefficient is fiber volume fraction independent, $D \sim C^0 \sim \phi^0$. In the high-salt region $C \ll 2AC_s$, the diffusion coefficient decreases as the square root of the fiber volume fraction of the gel decreases, $D \sim C^{-1/2} \sim \phi^{-1/2}$.

The second behavioral region is observed when $R_g > R/2$, where the reptation theory, first proposed by de Gennes,[21] describes the movement of an unattached chain by Brownian motion in a many-chain or gel system. Obstacles of the polymer network limit the lateral movement of the chain. In the reptation region, the diffusion coefficient for charged macromolecules is displayed by[20]

$$D \approx R^2/\tau_{Rep} \sim \frac{1}{N^2C^{1/2}}\left(1 + \frac{2AC_s}{C}\right)^{5/4} \qquad (5)$$

In the low-salt regime ($C \gg 2AC_s$), $D \sim C^{-1/2} \sim \phi^{-1/2}$, while in high-salt regime ($C \ll 2AC_s$), $D \sim C^{-7/4} \sim \phi^{-7/4}$.

Since the Debye length at 0.5 M KCl is 0.43 nm, which is much smaller than the estimated pore size of the gel, we can approximately neglect the electrostatic interaction and apply the scaling relations for neutral polymers, $D \sim \phi^{-0.5}$ in the Rouse region and $D \sim \phi^{-1.75}$ in the reptation region.

To observe the scaling relation, a logarithmic plot from Figure 1 is shown in Figure 2. The diffusion behaviors of myoglobin in the two kinds of polysaccharide gels are quite similar; they show a slight curvature which might suggest different diffusion regimes. However, neither the electrostatic effect nor the pore size change can explain this curvature since agarose gel is neutral and its pore size is 6 times larger than that of λ-carragenan gel. An average scaling exponent of −0.26 can be obtained from Figure 2a, which is close to the prediction of the Rouse model. In contrast, the behavior of tropomyosin in

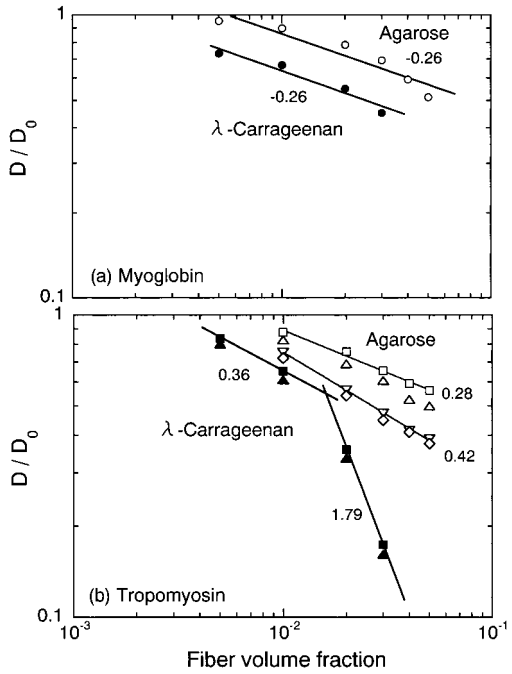

Figure 2. Logarithmic plot between the fiber volume fraction of gels and the diffusion coefficients of myoglobin (a) and tropomyosin (b) in polysaccharide gels. The diffusion of myoglobin in agarose gel (○) and in λ-carrageenan gel (●) were carried out at pH = 6.8 and 0.5 M KCl. Diffusion of tropomyosin in agarose gel (open marks): (□) pH = 6.8, 0.5 M KCl; (△) pH = 9, 0.5 M KCl; (▽) pH = 6.8, 0.01 M KCl; (◇) pH = 9, 0.01 M KCl. Diffusion of tropomyosin in λ-carrageenan gel (closed marks): (■) pH = 6.8, 0.5 M KCl; (▲) pH = 9, 0.5 M KCl. The straight lines with slopes shown by the numbers in the figure are the fits for the experimental data at pH = 6.8. The scaling exponents of the other experimental data of tropomyosin are summarized in Table 2.

the two kinds of gels is quite different (Figure 2b). In agarose gel, tropomyosin showed a nice single scaling exponent of −0.28. In λ-carrageenan gel the diffusion coefficients of tropomyosin reflect two behavioral regions. When $\phi < 0.01$, it has a scaling exponent of −0.36, which is in the Rouse region. However, it seems to fall in the reptation region when $\phi > 0.01$. The actual fit of the data gives a scaling exponent of −1.79, which is close to the theoretical value −1.75 (Table 2).

The diffusion of tropomyosin at pH = 9 and 0.5 M KCl is shown in Figure 3. As shown in Figure 3, diffusions of tropomyosin in polysaccharide gels were slightly hindered with increasing pH, even in the neutral agarose gel, which might be attributed to an increase in the molecular size at a higher pH. However, as in pH = 6.8, we observed a very similar behavior in the decrease of D with the fiber volume fraction.

To investigate the effect of electrostatic interaction between the tropomyosin and the gel, the diffusion of the tropomyosin in 0.01 M KCl is further investigated. However, the experiment only succeeded in agarose gel since λ-carrageenan could not form a consistent gel at such a low KCl concentration to perform the diffusion experiment. As shown in Figure 3, the diffusion is extensively hindered when the ionic concentration is low, but the difference between diffusion at pH = 6.8 and pH = 9 is not distinct. The hindered diffusion at the low ionic concentration should be attributed to the formation of double filament aggregation.[22−25]

The log−log plots of the experimental data for tropomyosin at pH = 9 and 0.01 M KCl are also shown in Figure 2. The scaling exponents for various conditions are summarized in Table 2. In agarose gel, only the Rouse region is observed even

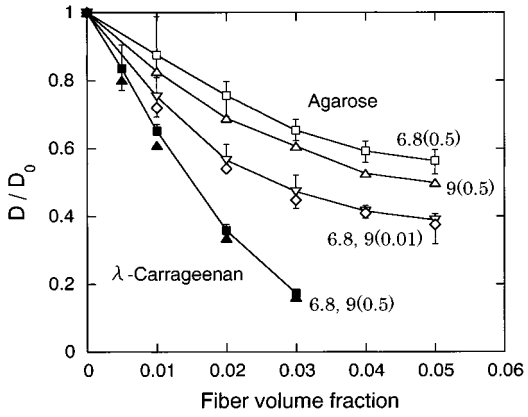

Figure 3. Effect of fiber volume fraction of the polysaccharide gel on the diffusion coefficient of tropomyosin with various pH and KCl salt concentrations at 20 °C. Open marks, in agarose gel: (□) pH = 6.8, 0.5 M KCl; (△) pH = 9, 0.5 M KCl; (▽) pH = 6.8, 0.01 M KCl; (◇) pH = 9, 0.01 M KCl. Closed marks, in λ-carrageenan gel: (■) pH = 6.8, 0.5 M KCl; (▲) pH = 9, 0.5 M KCl. Numbers in the figure are pH values, and the numbers in the parentheses are KCl concentrations in M. For clarity, only the error bars for pH = 6.8 are shown.

TABLE 2: Scaling Exponent α Obtained from $D/D_0 \sim \phi^\alpha$ for the Diffusion of the Rodlike Protein, Tropomyosin, in Gels[a]

	agarose		λ-carrageenan			
	pH = 6.8	pH = 9	pH = 6.8		pH = 9	
α (0.5 M KCl)	−0.28	−0.32	−0.36	−1.79	−0.40	−1.81
α (0.01 M KCl)	−0.42	−0.41				

[a] The scaling exponents −0.28 to −0.42 are close to the Rouse model of α = −0.5, and the scaling exponents −1.79 to −1.81 are close to the reptation model of α = −1.75.

at pH = 9 and 0.01 M KCl. The data at low salt concentration (0.01 M KCl) show a slightly stronger scaling exponent with the fiber volume fraction than do the high salt concentration results (0.5 M). Since the agarose gel is electrically neutral, the formation of the double filament aggregation of tropomyosin at low salt concentration might account for the difference.[22−25] In λ-carrageenan gel, tropomyosin shows a transition around $\phi \approx 0.01$ at pH = 9 and 0.5 M KCl, the same as that at pH = 6.8, with a scaling exponent of −0.40 in the Rouse region and −1.81 in the reptation region. The effect of electrostatic interaction on the diffusion of the tropomysin in the charged λ-carrageenan gel could not be studied in the present study since the experiment in the λ-carrageenan gel can only be carried out at a high KCl concentration of 0.5 M.

The abrupt transition in the diffusion behavior of tropomyosin in λ-carrageenan gel occurs at $\phi = 0.01-0.02$, corresponding to a pore size range of 10−7.2 nm. This critical pore size for the transition of the diffusion from Rouse-like to reptation-like is much smaller than the contour length of tropomyosin (38.5 nm). Two factors should be taken into consideration. One is that the pore size might be underestimated, though the estimation gives us the relative size change of the two gels. The other might originate from the semiflexible nature of the coiled-coil of the tropomyosin. Due to the lack of the information about the rigidity, we compare the experimental diffusion data in solution with the theoretical value for stiff rodlike molecules given by the following:[19]

$$D_R = \frac{D_{\parallel} + 2D_{\perp}}{3} = \frac{kT \ln(L/b)}{3\pi\eta_s L} \qquad (6)$$

where L is the contour length, b is the diameter of the filament,

k is the Boltzmann constant, T is the temperature, and η_s is the viscosity of the buffer, which could be approximated by the viscosity of water. Using $L = 38.5$ nm, $b = 1.5$ nm, $T = 293$ K, and $\eta_s = 10^{-3}$ N s/m^2, we have $D_R = 3.6 \times 10^{-7}$cm^2/s. The experimental data ($D = 2.4 \times 10^{-7}$ cm^2/s) and the theoretical value are in the same order of magnitude, and the experimental data are lower by a factor of 1.5, which indicates a good agreement and that the rigid rodlike model can express the diffusion of the tropomyosin well.

We could only find one reference regarding the reptation of semiflexible molecules, which described the direct imaging of reptation of F-actin filaments in the filaments' semidilute solution.[26] In that work, the filament self-diffusion coefficient was found to decrease approximately linearly as the filament length increased, in agreement with the reptation model. However, no filament concentration dependence was observed within experimental error.

Thus, reptationlike diffusion of the rodlike protein, tropomyosin, is first observed in λ-carrageenan gel in the present study.

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