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Myosin Gelation Kinetic Study Based on Rheological Measurements†

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Time-dependent shear and mechanical energy loss moduli of chicken breast myosin sols/gels were measured for various protein concentrations and isothermal conditions. Gelation followed second-order kinetics, with rate constants ranging from 0.0024 to 0.88 mL/(mg min). Rate constants changed in a complex manner with respect to temperature. Gels formed at low temperatures (44–56 °C) developed greater shear modulus values and were more elastic than those formed at higher temperatures (58–70 °C). Higher myosin concentration increased the rate of shear modulus development, and final values were higher. Thermal scanning at 1 °C/min produced only $^1/_2$ to $^1/_{15}$ of the potential shear modulus magnitude based on isothermal heating. Overall, gel rheology was strongly dependent on temperature history.

INTRODUCTION

Heat-induced protein gelation has been studied extensively to understand gelation mechanisms and food texture. Ferry (1948) proposed a two-step gelation model: (1) an initiation step involving an unfolding of the protein molecule followed by (2) an aggregation process, resulting in gel formation. Much research has been carried out to determine the effects of heating conditions on protein denaturation/gelation and gel properties (Kinsella, 1976; Laakkonen, 1973; Hamm, 1977; Acton et al., 1981; Asghar et al., 1985; Foegeding et al., 1986).

Ishioroshi et al. (1979) reported that rabbit myosin sols at pH 6 and 0.6 M KCl have two thermal transitions, on the basis of the first derivative of temperature–shear modulus curves. The data also suggested that rabbit myosin thermal transitions begin at temperatures as low as 30 °C and that different heating temperatures make gels of different properties. Wicker et al. (1986), using a linearly increasing temperature of 1 °C/min, determined that both rabbit myosin and chicken myosin had a single transition temperature of 44 °C, on the basis of the change in hydrophobicity, whereas, according to the shear modulus study, the thermal transition temperatures were identified as 48 and 49 °C for rabbit and chicken myosins, respectively.

The force/deformation properties of previously heated gels depend on measuring temperature. Niwa et al. (1988) reported that the contractile force of the elongated kamaboko and expansible force of compressed kamaboko increased with increasing measuring temperature up to 60 °C. This demonstrated the physical effect of the measuring temperature on gel shear modulus.

It appears difficult to determine the contribution of a specific temperature to the shear modulus characteristics solely on the basis of results of thermal scanning experiments without knowing the kinetic and equilibrium information on the gelation processes. The purpose of this study was to investigate the effects of temperature on the gelation process and rheological characteristics. Special emphasis was placed on the rate of development and equilibrium values of rheological properties. Also, an effort was made to relate measuring temperatures and transition temperatures to the rheology parameters.

GENERAL GELATION KINETIC MODEL

At isothermal heating conditions sufficient for gelation to occur, we found the shear modulus–time curves were similar in shape to a typical creep curve (Mohsenin, 1986). The slope of the shear modulus–time curve was relatively large at the beginning but decreased with increasing time and eventually approached zero. We were interested in finding the effects of concentration/temperature on objective parameters, such as rate constants and equilibrium shear moduli. Thus, we desired to establish the relation between the amount of protein in cross-links and shear modulus.

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Nossal (1988) developed a model for the dependence of shear modulus upon the fraction of units (molecular monomer) that are directly incorporated into cross-links in an elastic network structure. Shear modulus is linearly proportional to the difference between the cross-linked fraction α and a coefficient, α_0 (its value is larger than α_c, a critical value of cross-linked units fraction), when the fraction of cross-linked units is relatively large. This model also suggests that shear modulus is zero if the fraction of units involving cross-links is less than the critical value.

Nossal's relation between shear modulus and cross-linked monomers was not used in our gelation kinetic model for chicken myosin due to the unavailability of data related to the gel microstructure. However, the concept that there is no shear modulus when the fraction of cross-linked molecules is less than a critical value is key to our kinetic model.

We assume shear modulus, G, is a function of f, a statistical quantity of cross-linked molecules, and f_c, the critical value of cross-linked molecules

$$G = c(f - f_c) \tag{1}$$

where c is a constant. We further assume f is proportional to the concentration of protein monomers, B, significantly contributing to the shear modulus

$$f = e[\mathrm{B}] \tag{2}$$

where e is a constant. [B] is the concentration of cross-linked monomers. Equation 2 is substituted into eq 1 to give

$$G = g([\mathrm{B}] - [\mathrm{B}]_c) \tag{3}$$

where $g = ce$. If the constructing monomer concentration is used, even after the monomers are involved in cross-linking, the dimensions of g are shear modulus unit per unit concentration.

When protein molecules are heated and denatured, the following overall transition occurs:

$$m\mathrm{A} \xrightarrow{k} (-\mathrm{B}-)_m \tag{4}$$

The transition is irreversible and assumed to be complete. Though the polymerization path is not clear, a combination of all-or-none and open association is likely according to the classification of physical models described by Elias (1972). We will only consider two states, A, un-cross-linked and B, cross-linked. The rate of un-cross-linked protein concentration [A] disappearing should depend on its concentration [A]. The rate law is expressed as

$$-\mathrm{d}[\mathrm{A}]/\mathrm{d}t = k[\mathrm{A}]^n \tag{5}$$

where n is kinetic order of gelation. If the concentration of constructing monomers is used for [B] (even after cross-linking), the stoichiometric coefficient, m, can be avoided. Thus, $[\mathrm{A}] = [\mathrm{A}]_0 - [\mathrm{B}]$ and $\mathrm{d}[\mathrm{A}]/\mathrm{d}t = -\mathrm{d}[\mathrm{B}]/\mathrm{d}t$ can be used to substitute into eq 5 so that

$$\mathrm{d}[\mathrm{B}]/\mathrm{d}t = k([\mathrm{A}]_0 - [\mathrm{B}])^n \tag{6}$$

Integrating eq 6 from $t = 0$ to t and rearranging

$$[\mathrm{B}] = [\mathrm{A}]_0 - \{[\mathrm{A}]_0^{1-n} + (n-1)kt\}^{1/1-n} \tag{7}$$

After time t_c, the shear modulus starts to be detectable. The critical concentration is

$$[\mathrm{B}]_c = [\mathrm{A}]_0 - \{[\mathrm{A}]_0^{1-n} + (n-1)kt_c\}^{1/1-n} \tag{8}$$

[B] and $[\mathrm{B}]_c$ in eq 3 are replaced by eq 7 and 8 and rearranged to

$$G = g\{[\mathrm{A}]_0^{1-n} + (n-1)kt_c\}^{1/1-n} - g\{[\mathrm{A}]_0^{1-n} + (n-1)kt\}^{1/1-n} \tag{9}$$

When n is greater than 1 and t approaches infinity, the second term will be zero; thus, the equilibrium shear modulus G_e is

$$G_e = g\{[\mathrm{A}]_0^{1-n} + (n-1)kt_c\}^{1/1-n}$$

Solving for g

$$g = G_e/\{[\mathrm{A}]_0^{1-n} + (n-1)kt_c\}^{1/1-n} \tag{10}$$

Equation 10 can then be substituted into eq 9 to give

$$G = G_e\{1 - \{[\mathrm{A}]_0^{1-n} + (n-1)kt\}^{1/1-n}/\{[\mathrm{A}]_0^{1-n} + (n-1)kt_c\}^{1/1-n}\} \tag{11}$$

Thus, shear modulus at time t is a function of the rate constant and kinetic order. Critical time, t_c, can be read directly from the shear modulus–time curve (refer to Figure 1 for t_c). On the basis of our assumptions, it can be seen that the shear modulus–time curve is mainly defined by equilibrium shear modulus, kinetic order, and rate constant. Under specified conditions, the equilibrium shear modulus will be temperature dependent. The characteristic of curve slope is determined by the rate constant. If the actual relation between shear modulus and cross-linked molecules concentration is a nonlinear function, this will modify the slope characteristics, affecting the evaluation of the rate constant according to the above equation. However, as long as the initial concentration of protein is in a reasonably small range, the incremental increase in cross-linked protein molecules predominantly determines the shape of the shear modulus–time curve. The contribution to the slope characteristics of the shear modulus–time curve from the nonlinear relation between the shear modulus and cross-linked molecular concentration is probably minor.

MATERIALS AND METHODS

Chicken Myosin Extraction. Chicken muscle myosin was extracted from adult Hubberd-type chickens obtained from New Hope Feed Co. in Goldsboro, NC. The chickens were sacrified by injecting about 2 mL of Ketamine and Xylazine (1:1) into a principal vein under the wing. Breast muscle was taken and immediately placed in ice. The iced muscle was ground with a 10 mM EDTA washed meat grinder (Tinned Chop Rite, MFQ Co., Pottstown, PA). Myosin was extracted according to the method described by Foegeding et al. (1986). Before being subject to the heating treatment, myosin sols in 0.5 M KCl were dialyzed against a 0.5 M NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7) buffer solution (1:10 v/v) for 24 h. The dialyzing solution was renewed every 8 h. The extracted myosin sols were refrigerated at 5 °C. All shear modulus measurement was finished within 4 days after myosin sols were dialyzed.

Myosin Concentration Determination. Myosin concentration was determined by using a spectrophotometer (UV-240, Shimadzu Corp.). The absorbance was measured at 280-nm wavelength, and the myosin concentration (milligrams per milliliter) was calculated by using an extinction coefficient of 0.52 cm^2/mg (Quass and Briskey, 1968). The myosin concentration of the first extraction was 35.7 mg/mL and of the second, 27.3 mg/mL.

Temperature Control and Shear Modulus Measurement. A thermal scanning rheology monitor (TSRM) was employed to measure the change in shear modulus and mechanical energy loss during heating (Montejano et al., 1983; Hamann, 1987). A small sample fixture (cup diameter, 19.1 mm; plunger diameter, 15.7 mm; and plunger length, 48.7 mm) was used, and the fixture was insulated with a thick black foam rubber tube to minimize heat dissipation. The fixture was preheated to a desired temperature by a circulating water bath (EX100DD Neslab

Instrument Inc., Portsmouth, NH) with the temperature controlled by a programmer (MTP-5 Neslab Instrument). The sample fixture (455 g in weight plus hot water circulating around the cup) was a large energy (heat) source relative to the myosin sample size of 7 mL. A plastic syringe was used to inject the myosin sols into the gap (1.7 mm) between the cup and plunger. The sample temperature increased very rapidly, approaching the desired temperature within 20 s. After sample injection, the plunger was immediately aligned in the center of the cup. A few drops of corn oil were used to cover the top of the myosin sols to prevent water evaporation. The shear modulus was monitored at a crosshead speed of 0.5 mm/min (equal to a peak-to-peak displacement of 0.1 mm). The peak-to-peak shear strain was 1.0, and the shear rate was 0.083 s^{-1}. The dynamic shear modulus (pascal) was calculated at 2-min intervals according to the method described by Wu et al. (1985). Mechanical energy loss (percent) was calculated for each shear modulus measurement according to the method described by Hamann (1987). The temperature data were taken from water bath readings corrected by values based on direct measurements of myosin sols, using a thermocouple (871 digital thermometer, Omega Engineering Inc.) that had been calibrated by using both ice and boiling water.

Various isothermal temperatures were selected, varying from room temperature to 90 °C. For each selected isothermal heating temperature, the shear modulus and energy loss were monitored for at least 1 h.

To obtain the measurements for two consecutive isothermal temperatures, myosin sols were subjected to two-stage isothermal heating. After being at 48 °C for 1 h, myosin sols were heated to 68 °C at a temperature rate of 2 °C/min and held at 68 °C for another hour. The shear modulus and energy loss were monitored during the last stage of heating.

To compare the effect of isothermal temperatures with that of a thermal scanning treatment, myosin sols (10 and 25 mg/mL) were heated by thermal scanning from 20 to 80 °C, at a heating rate of 1 °C/min.

Myosin Purity Evaluation. Myosin purity was monitored by using 10% acrylamide sodium dodecyl sulfate (SDS) electrophoresis by the method of Laemmli (1970). We found that myosin sols extracted by using the procedure were quite pure on the basis of the results of SDS electrophoresis.

Data Processing and Parameter Evaluation. Iteration computer programs were written by using THINK C (Symantec, 1989) to determine the parameters (rate constant, equilibrium shear modulus) for all shear modulus–time curves. All experimental runs were repeated.

DATA GENERATION

Kinetic Order Studied by Numerical Least Squares. We did not assume a kinetic order that the gelation process followed. Moreover, actual order is not necessarily integral, and the value should be determined solely by experiment (Laidler, 1987).

Initial myosin concentration $[A]_0$ was known. Critical time t_c was read directly from the shear modulus–time curve. Equilibrium shear modulus was estimated from the shear modulus at the end of 1 h of heating because the un-cross-linked myosin concentration at this time was very low due to a relatively high order and relatively large rate constant. Equilibrium shear modulus could, however, be determined by extrapolating the cross-linked protein concentration (see below for further discussion). Solving eq 11 by using the normal least-squares method is complicated since the normal matrix cannot be easily established from the partial differential equations. However, the model value of shear modulus at time t can be easily calculated if n and k values are assigned. The difference between measured shear modulus G_i and the model modulus $G(t_i)$ should be minimized when the best fit is achieved. For various pairs of n and k selected, the sum of squares, $\sum_i[G_i - G(t_i)]^2$, can be easily calculated. The best fit set of n and k is the one with the smallest sum of squares. If the step values of n and k are infinitesimally small, the best n and k values found should be equivalent to those found by using the usual least-squares method. A computer program written in THINK C was used to estimate the n and k values.

The computer program used did multiple loops of iteration. A range of n values and a range of k values were specified. The program generated values of n (n_1, n_2, n_3, ... n_α) and k (k_1, k_2, k_3, ... k_β). For each n value selected, the computer calculated the sum of the squares between the experimental data and the model value, trying all k values (k_1, k_2, k_3, ... k_β) in sequence. The total number of calculations were $\alpha\beta$ (which was usually larger than 1000). The step values (increment of n or k values) could be freely adjusted to meet the required accuracy. After each calculation, 10 sets of n and k values were output according to ascending magnitude of the sum of squares. From the results, it was found that an order of between 1.6 and 1.8 gave the best fit.

Evaluation of G_e and k. For convenience, all data were analyzed with second-order kinetics imposed (closest integer to the best fit order). Equations 7 and 11 could, thus, be simplified as

$$[B] = [A]_0 - [A]_0/(kt[A]_0 + 1) \quad (12)$$

$$G = G_e\{1 - (kt_c + 1/[A]_0)/(kt + 1/[A]_0)\} \quad (13)$$

To determine the accurate equilibrium shear modulus, we searched for the best G_e and k by an iteration method, similar to that described above.

At a specified time t, the monomer concentration in cross-links is $[B]_f$ with a correspondent shear modulus G_f. Equation 3 can be expressed as g, with [B] replaced by $[B]_f$ and G by G_f

$$g = G_f/([B]_f - [B]_c)$$

Equation 3 can be written

$$G = G_f([B] - [B]_c)/([B]_f - [B]_c) \quad (14)$$

After infinite time, all $[A]_0$ is transformed into [B] and the G will approach G_e, yielding

$$G_e = G_f([A]_0 - [B]_c)/([B]_f - [B]_c) \quad (15)$$

From eq 12, it can be seen that $[B]_c = [A]_0 - [A]_0/(kt_c[A]_0 + 1)$ and $[B]_f = [A]_0 - [A]_0/(t_fk[A]_0 + 1)$. G_e is expressed in terms of k and t_c.

$$G_e = G_f(t_fk[A]_0 + 1)/\{k[A]_0(t_f - t_c)\} \quad \text{or}$$

$$G_e = G_fZ \quad \text{with } Z = t_f/(t_f - t_c) + 1/\{k[A]_0(t_f - t_c)\} \quad (16)$$

This indicates that G_e can be extrapolated from the shear modulus G_f by using the extrapolating factor Z. G_f can be selected at any time as long as a consistent value of t_f (larger than t_c) is used in the extrapolating factor. However, a long time is desired for a good result. We used t_f = 60 min; thus, G_f is the shear modulus at 60 min of heating. Z is always larger than 1. For a specific gelation transition, if k is small, t_c will be large (longer time to reach critical concentration) and Z will be large. If k is fixed, when the original concentration $[A]_0$ is large, t_c will be small (less time to reach critical concentration) and Z will be small.

Before calculation, G_e and k are unknown. Reasonable ranges of G_e and k must be assigned. The computer program then calculates the sum of squares and G_e, using the extrapolating factor Z for each pair of assigned k and G_e. The pair of k and G_e values are selected as model

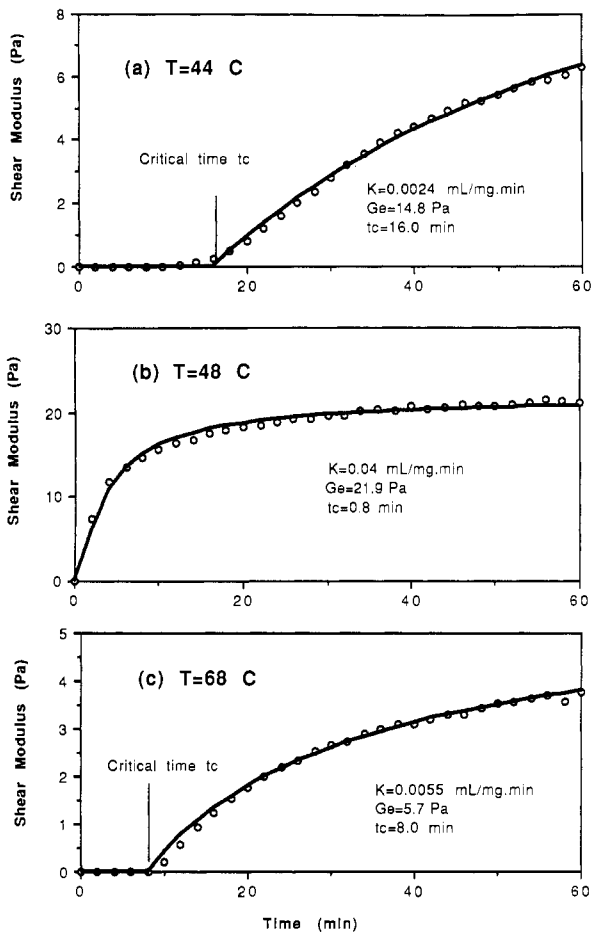

Figure 1. Experimental shear modulus–time data (O) for chicken myosin gelation and kinetic model representation (—) for various isothermal temperatures: (a) 44 °C; (b) 48 °C; (c) 68 °C. Myosin concentration is 10 mg/mL.

constants, which produce the minimum sum of squares and a relative error of less than 3% between the assigned and the calculated G_e. Very small relative errors were always associated with the best fit pair of k and G_e. The gelation process of chicken myosin was well modeled by the second-order kinetic model.

For the data shown in Figure 1a (O), after computer iteration, the best k and G_e were determined to be 0.0024 mL/(mg min) and 14.8 Pa, respectively. The sum of squares of deviation was 0.31, and the relative error was 0.9%. In Figure 1a, the smooth line (—) was generated from the model equation by using k = 0.0024 mL/(mg min) and G_e = 14.8 Pa. It was found that the experimental data were in good agreement with the model, though slight systematic deviation existed due to the approximation in kinetic order.

EXPERIMENTAL RESULTS

Deviation of Chicken Myosin. We found that the shear modulus magnitudes were different among the replicated experiments, even under the same experimental conditions. This deviation was presumably attributed to genetic deviation and seasonal change. Nevertheless, the results were consistent in terms of gelation onset temperature, gelation temperature range, overall temperature–shear modulus/rate constant profiles, and the effect of temperature on gel characteristics. Further, the analyzed data led to the same conclusion.

Effect of Different Isothermal Heating Temperatures. The effects of isothermal temperatures were evident (Table I; Figure 1). Below 44 or above 70 °C, no measurable shear modulus was detected for 10 mg/mL myosin sols. Thus, there were no rate constants for those treatments. At temperatures between 44 and 52 °C, the rate constant increased to a maximum at 52 °C, and thereafter it decreased. The maximum equilibrium shear modulus was found for the gels formed between 48 and 50 °C. At temperatures between 60 and 70 °C, the shear modulus–time curves were totally different from those for lower temperatures. The rate constants were rather small and the shear moduli much smaller. Also, the critical concentration (data not shown) was generally higher compared with low-temperature gelation, indicating an inferior cross-linking at these higher temperatures. The percentage mechanical energy loss generally increased with the temperature, though accurate determination of the energy loss was difficult for the myosin gels formed at high temperatures due to the small force peaks.

Table I. Rate Constant, Equilibrium Shear Modulus, and Mechanical Energy Loss of Myosin Gel Formed at Different Heating Temperatures (Myosin Concentration 10 mg/mL)

temp, °C	critical time, min	rate const, mL/(mg min)	equil shear modulus, Pa	energy loss,[a] %
43[b]				
44	16.0	0.0024	14.8	8.7
46	2.0	0.026	19.0	13.8
48	0.8	0.040	21.9	17.5
50	0.6	0.065	22.3	17.3
52	0.4	0.88	13.2	27.7
54	0.5	0.28	15.1	24.4
56	0.7	0.23	11.7	27.3
58	0.7	0.21	9.9	74.0
60	8.0	0.39	0.7	63.1
62	3.0	0.088	2.5	68.2
66	6.0	0.010	2.9	39.1
68	8.0	0.0055	5.7	19.8
70	12.0	0.0038	4.3	34.5
74[b]				
80[b]				

[a] Energy loss is the average of the readings after shear modulus reached 67% of the equilibrium value for each temperature. [b] No shear modulus was detected for these temperatures.

It appeared (Table I) that there were two extreme types of gelation. The first type was characterized by a high shear modulus and high elasticity (low energy loss) and was produced at 48–50 °C. The second type was less rigid (low shear modulus) and was produced at higher temperatures (above 58 °C). The high energy loss values above 58 °C suggested the equilibrium modulus values are due to viscous resistance to shear rather than elastic. On the basis of the viscosity, we could infer that, at high temperatures, myosin was either polymerized to form large molecular weight clusters or unfolded to increase volume. The low magnitude of the shear modulus also indicated that the protein did not aggregate to form a strong gel structure.

Concentration Effects on the Rheological Properties. The effect of myosin concentration on the shear modulus is shown in Table II. Only one value of rate constant should be expected for the same temperature (48 °C) for various concentrations since, in principle, the reaction rate constant is independent of the initial reactant concentration if the reaction product does not catalyze or inhibit the reaction. We found the mean rate constant was 0.035 mL/(mg min) with a standard deviation of 0.013. Observing the range of rate constants for various temperatures, the values for various concentrations were well centered. Besides the experimental error, other factors might contribute to this deviation. First, the shear

Table II. Effect of Myosin Concentration on the Rate Constant and the Equilibrium Shear Modulus of Myosin Gel Formed at 48 °C Heating Temperature

concn, mg/mL	critical time, min	rate const,[a] mL/(mg min)	equil shear modulus, Pa
5.0	4.0	0.016	2.4
7.5	1.4	0.047	13.9
10.0	0.8	0.040	21.9
15.0	0.28	0.024	36.6
20.0	0.15	0.038	40.4
25.0	0.09	0.048	44.4

[a] The mean of rate constants is 0.035 mL/(mg min) with a standard deviationof 0.013.

modulus may not have been linearly related to concentration when myosin concentrations were high (Figure 3). Second, high-concentration gels were so strong that instrumental pumping was more severely restricted. In this case, thermal expansion of the myosin sols would have raised the plunger, thus retarding shear so the measured values could have been lower than actual values. Also, if the gelation product had an influence on the gelation rate, this effect influenced the apparent kinetic order determined by the integration method. Thus, the apparent order would have changed with changing initial myosin concentration. Unfortunately, the differential method could not be used to determine the accurate order because no shear modulus was detected before the critical time. Finally, in the rate law, we used the un-cross-linked species and assumed previous cross-linking did not affect further cross-linking (eq 5). This assumption may not hold. For those reasons, the second-order rate constant is a statistical average over the whole gelation process. Nevertheless, this macroscopic rate constant appeared to be a good indication of intrinsic gelation speed.

At high concentrations, the shear modulus showed a large increase instantly and then approached the equilibrium value slowly. Most of the magnitude of the shear modulus was obtained in a very short period of time. This type of curve produced a large rate constant. On the contrary, gels formed at low concentrations had slow increases (with time) in shear modulus (see eqs 13 and 16) and a relatively large portion (large Z) to be developed before the equilibrium shear modulus was reached. A small k was found for very low concentration. The small k for very low myosin concentration indicated that these myosin sols required a higher degree of unfolding before overcoming the distance barrier and aggregating into a rigid structure.

By use of critical time and eq 12, the critical concentration could be determined. The mean critical concentration was about 2 mg/mL, with a standard deviation of 0.58, for concentration treatments (48 °C).

Energy loss was large for the gels from low concentrations, and the character was close to a fluid.

Thermal Scanning Treatment and Rheological Properties. Shear moduli of myosin gels formed during a thermal scanning treatment were much lower than those produced by isothermal heating. Myosin gels (25 mg/mL) induced by the thermal scanning treatment (heating rate of 1 °C/min) had a maximum shear modulus of about 18.0 Pa (Figure 2), while the measured shear modulus after 60 min of 48 °C isothermal heating was 44.3 Pa. The thermal scanning shear moduli of a myosin gel of low myosin concentration (10 mg/mL) are shown in Figure 2 (○). During isothermal heating, myosin sols require an extended amount of time to approach an equilibrium shear modulus. In the thermal scanning treatments, the myosin sols did not have sufficient time to develop the equilibrium

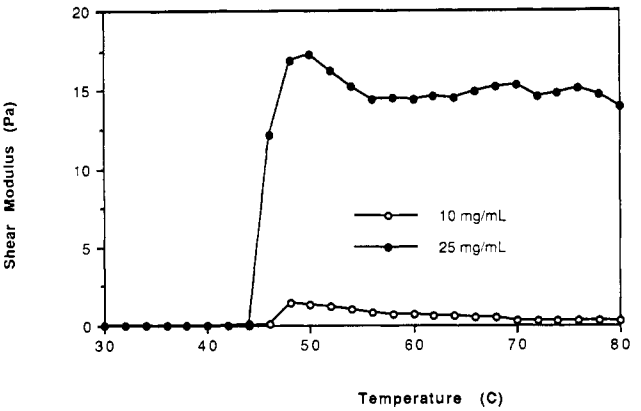

Figure 2. Shear modulus versus temperature for thermal scanning experiments (1 °C/min) with 10 and 25 mg/mL myosin concentrations.

shear modulus, G_e, for a given temperature before the sols reached a higher and less favorable temperature. This kinetic limitation explains why the shear moduli of myosin gels formed by thermal scanning were lower than for gels formed by isothermal heating. The fact that gels obtained from higher heating rates were weaker than those from lower heating rates further supports this explanation. An extreme example was the 80 °C isothermal treatment in Table I. The equivalent heating rate (in average) in the temperature-jumping process was about 180 °C/min. No shear modulus was detected in the whole "holding" hour.

The kinetics explains why the thermal scanning treatment suppressed the potential to fully develop the shear modulus. Furthermore, the gels from low myosin concentrations needed a longer time (small k and large Z) to reach the equilibrium shear modulus than did those from high concentrations. This indicates that, by use of the thermal scanning method, the shear modulus of a gel formed from a lower protein concentration would be more severely suppressed than one from a high concentration. For example, the maximum measured shear modulus for a 10 mg/mL myosin gel was about 1.3 Pa for the thermal scanning treatment, which was $^1/_{15}$ the measured shear modulus of a gel at the same concentration formed by using the isothermal heating method.

The thermal scanning data also indicated that the temperatures between 46 and 50 °C were favorable to shear modulus development since the slope was large and positive. The temperatures higher than 50 °C had a negative effect on the shear modulus. This negative slope could be due to high temperature weakening the intramolecular and cross-linking bonds of myosin gels.

Effect of Two-Stage Heating Treatments on Final Rheological Properties. In the two-stage isothermal heating treatments, the shear modulus increased during the last heating stage (Table III). Mechanical energy loss also increased significantly (Table IV), indicating the increase in equilibrium dynamic shear modulus was due to viscous resistance rather than elastic stiffening. This suggests that even after 1 h at 48 °C, unfolding (increases volume fraction) occurs when the temperature is raised to 68 °C. For a 10 mg/mL myosin gel, the shear modulus increased from 21.2 to 23.5 Pa. When the temperature was increased, chemical bonds became weaker because of an increasing bond vibrational energy (Atkins, 1986). This explains the common phenomenon that most gels will become less rigid upon heating. The increase in the shear modulus during the second-stage 68 °C heating tended to negate the speculation that the different equilibrium moduli in Table I were attributed entirely to the effect of

Table III. Effect of Two-Stage Heat Treatments on the Shear Modulus of Myosin Gel for Various Concentrations

concn, mg/mL	shear modulus,[a] Pa, 1 h at 48 °C	shear modulus,[b] Pa, 48 °C + 68 °C[c]
5.0	1.9	2.7
7.5	13.9	9.1
10.0	21.2	23.5
15.0	36.4	41.4
20.0	39.6	44.4
25.0	44.3	49.5

[a] The shear modulus is the value at the end of heating at 48 °C. [b] The shear modulus values are the average of shear modulus readings during the last half-hour of holding (68 °C). [c] Myosin gel was induced by heating it at 48 °C for 1 h and at 68 °C for another hour.

Table IV. Effect of Different Heating Temperatures on Mechanical Energy Loss of Myosin Gel for Various Concentrations

concn, mg/mL	energy loss,[a] %, 1 h at 48 °C	energy loss,[b] %, 48 °C + 68 °C[c]
5.0	12.6	77.2
7.5	31.6	58.7
10.0	17.5	32.8
15.0	1.1	15.0
20.0	0.0	4.8
25.0	1.7	2.0

[a] Energy loss is the average of the energy loss readings after the shear modulus reached 67% of the equilibrium shear modulus value. [b] Energy loss is the average of the energy loss readings during the last half-hour of holding (68 °C). [c] Myosin gel was induced by heating it at 48 °C for 1 h and at 68 °C for another hour.

measuring temperature on chemical bonds. Rather, the difference in the rheological character was mainly due to the different natures of thermal transitions at different temperatures.

Table IV also shows that for gels of low concentration the mechanical energy loss increased more than for high concentrations.

The first-stage heating treatment mainly determined the final rheological properties, especially shear modulus. After the myosin sols were heated to 60 °C for 1 h and cooled to 48 °C, the measured shear modulus after holding for another hour was 2.0 Pa, which was close to the value for 60 °C single-temperature treatment. On the other hand, the measured shear moduli for two-stage isothermal heating treatments (Table III) were relatively close to the values for their corresponding first-heating treatments. This indicated that the gelation must follow a specific temperature history to get extensive aggregation.

Effect of Measuring Temperature on Rheological Parameters. After a myosin gel (20 mg/mL) formed by two-stage heating was cooled to room temperature, the shear modulus increased from 44.4 to 48.3 Pa, and energy loss decreased from 4.8% to 0.83%. Both of the changes were mainly attributed to an increase in rigidity of chemical bonds (such as hydrogen bonds and covalent bonds) upon cooling. Those effects were minor compared to the effects of the transition-inducing temperatures.

The increase of shear modulus of gels upon cooling as well as the negative slope of the temperature–shear modulus curve for thermal scanning treatments in Figure 2 indicated that high temperatures had small negative effects on the measurement of shear modulus.

DISCUSSION

From the isothermal shear modulus study, the effective gelation temperatures were estimated to be 43–72 °C. This onset gelation temperature is different from that obtained from thermal scanning experiments. For both thermal

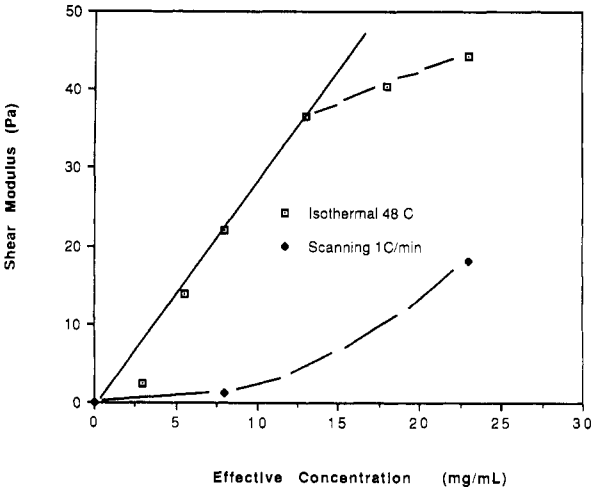

Figure 3. Experimental relation between shear modulus and myosin concentration: (⊡) equilibrium shear modulus versus chicken myosin effective concentration (actual concentration − critical concentration) for isothermal heating at 48 °C; (◆) measured maximum shear modulus versus effective concentration for thermal scanning heating (1 °C/min).

scanning experiments with myosin concentrations of 10 and 25 mg/mL (1 °C/min), no shear modulus was detected until 46 °C (Figure 2). The reason is that slow gelation does not respond quickly enough to catch the increasing temperature. Considering this kinetic lag, we recommended use of the term "apparent onset temperature" for thermal scanning experiments, instead of "onset temperature". The equilibrium shear modulus for any specific concentration was dependent on isothermal heating temperature. The maximum equilibrium shear modulus was obtained at 48–50 °C, and this was about 2–15 times greater than that obtained when a thermal scanning method (1 °C/min) was used. In a DSC study (unpublished data) it was determined that chicken myosin in the same buffer showed three major peaks. The onset temperature of the first peak was 43 °C. The optimum transition temperature (at the maximum rate constant) of the second peak was 58 °C. The optimum temperatures of the first and third peaks were estimated at 50 and 63 °C. The combined temperature range of these three peaks was consistent with the temperature range at which shear modulus was detected. The different physical properties of the myosin gels formed at various temperatures were attributed to those thermal transitions associated with the DSC peaks. It can be inferred that each transition makes a different contribution to the final properties. The first transition produced gels of higher elasticity than did the second and third transitions. When the temperature was close to the onset temperature of the first transition, the rate of shear modulus increase was slow, as indicated by a small rate constant. As the temperature was increased, the rate constant increased. However, when the temperature was higher than 60 °C, the shear modulus appeared not to be caused by the first transition because the critical time was much longer and the measured shear modulus was lower than for low temperatures.

The assumed relation (eq 3) between the shear modulus and the concentration of cross-linked myosin monomers seems valid when the concentration is in a narrow range. Figure 3 shows that for the four lowest concentrations studied, the equilibrium shear modulus (48 °C) was linear with effective myosin monomer concentration (actual concentration subtracted by a critical concentration of 2.0 mg/mL). In this case, when equilibrium was reached, almost all original myosin would have been changed to

aggregated molecules. The aggregated state (in terms of equilibrium shear modulus and elasticity) is temperature dependent. The dashed line in Figure 3 shows the measured maximum shear modulus for thermal scanning results (1 °C/min). The differences can be explained by the different Z values, which are dependent on myosin concentration and rate constant k (eq 16). Physically, low-concentration myosin sols need a longer time to unfold to overcome the distance barrier. Because the transition time for a favorable temperature range is limited in thermal scanning heating, the sols do not reach the potential maximum shear modulus. Further, the lower the concentration, the more suppressed is the shear modulus.

Besides the effects of temperatures on the denatured state, the less rigid and more viscous gels formed at high temperatures would be due to an inferior aggregation because the motions of myosin molecules would be more vigorous at higher temperatures (Atkins, 1986). Myosin unfolding is a process that involves an increase in both enthalpy and entropy because the stabilizing bonds have to be broken and the randomness is increased. Both the reaction enthalpy, ΔH, and the reaction entropy, ΔS, are positive. Thus, unfolding will be favored only at a temperature sufficiently high so that the Gibbs free energy ($\Delta G = \Delta H - T\Delta S$) is negative. On the contrary, an intermolecular reaction is a process that involves a decrease in entropy because the molecules must collide with proper orientation for interaction to occur (Rawn, 1983). Myosin aggregation involves a decrease in entropy (ΔS is negative), resulting in a more "ordered" system. The formation of chemical bonds involves the release of heat (Vollhardt, 1987) and is an exothermic process. The aggregation process of myosin involves the cross-linking of molecules through various bonds and a decrease in enthalpy (ΔH is negative). When the temperature is low and the negative enthalpy change is sufficient to compensate for the decreased entropy, the aggregation will occur. If, however, the temperature is too high, the term $T\Delta S$ is so negative that ΔG is positive and the aggregation will not be favored. There is no temperature at which both the unfolding and aggregation steps are greatly favored.

It has been reported that if aggregation is slow, a finer and more ordered gel network with higher degree of elasticity is produced (Ferry, 1948; Gossett et al., 1984). Gels from rapid heating rates possess greater opacity and lower elasticity (Hermansson, 1979; Schmidt, 1981). It has been generally agreed that a slow process of unfolding also results in a finer gel network. Some of our data appear to reveal an exception to the statements relative to unfolding/aggregation speeds. The myosin gel that is formed rapidly at 48 °C is not only strong but also very elastic. In comparison, the gels formed slowly at temperatures 66–70 °C are less rigid and more viscous. This discrepancy could be explained partially by unfolding states of myosin.

Unfolding rate is usually judged on the basis of heating rate for a thermal scanning experiment. Increasing heating rate does increase the unfolding rate of the protein but does not promote the aggregation since the protein will reach the aggregation-unfavored high temperatures more rapidly, and, thus, shear modulus is low. On the other hand, a slow heating rate slows down the unfolding process but favors the aggregation process. In this case, the overall gelation process is slow due to a slow progress of unfolding. Heating rate does not have a simple relation with the gelation rate. Our isothermal experiments (Table I) show that gelation velocity does not continue to increase with increasing temperature, though unfolding velocity is thought to increase with increasing temperature.

Cross-linking produces the shear modulus and elasticity (Nossal, 1988). Table I shows that for low temperatures the gels are generally stronger and more elastic. This supports the assumption that low temperatures favor cross-linking of myosin molecules.

Slow gelation at high heating temperatures did not produce strong and elastic texture. Instead, the gel was weak and viscous. This demonstrates the importance of temperature (and holding time) rather than gelation rate on the force/deformation properties.

Since low temperatures favor the aggregation process, the sharp onset temperature of gelation can only be explained by the unfolding process, which is driven by molecular kinetic energy. It is likely that proteins are able to aggregate at a temperature below the onset temperature of thermal gelation since slow denaturation of protein is occurring, considering the second law of thermodynamics. Thus, the gelation of protein below the sharp onset temperature is a matter of rate. Connell (1959) showed that cod myosin aggregates at temperatures as low as 0 °C. Fish muscle sols can become transparent and gel rapidly at near 40 °C ("high temperature setting") or more slowly during refrigerated storage (Wu et al., 1985). It can be inferred that proteins of low stability, like fish myosin, have the potential to form a more ordered gel since thermal gelation can occur at a low and aggregation-favored temperature.

When a gel formed at a high temperature is cooled to a low temperature, the aggregation should be more favored, but shear modulus is not increased significantly. It appears that the unfolding and aggregation processes occur simultaneously and cooperatively.

When the myosin concentration was decreased, both the shear modulus and the elasticity of the gels were decreased; a weak and fluidlike texture was obtained which was similar to the texture formed at high temperatures. At low myosin concentrations, the number of myosin molecules in a given volume is limited and a complete and dense cross-linked network is unlikely.

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