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THERMODYNAMICAL ASPECT OF G–F TRANSFORMATIONS OF ACTIN

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SUMMARY

1. Thermodynamic analyses were made concerning the polymerization of G-actin to F-actin. The time courses of polymerization were very similar over a wide range of solvent conditions; polymerization curves obtained under varied conditions could be superposed by translation parallel to the logarithmic time axis.

2. The activation enthalpy for polymerization was 20–25 kcal/mole, while the activation enthalpy for depolymerization was about 10 kcal/mole.

3. Based on the fact that polymerization could be regarded as a condensation phenomenon, the apparent enthalpy change associated with the polymerization was estimated from the temperature dependence of the G-actin critical concentration to be about 10 kcal/mole, according to the Clausius–Clapeyron relation. This value, however, could not be directly related to the enthalpy determined by calorimetry, since the polymerization was accompanied by irreversible ATP splitting.

4. A separate calculation of the activation enthalpies in nucleation and growth, of which polymerization consists, was attempted.

5. The activation enthalpy for polymerization did not vary with changes in the ionic conditions, such as ion species, salt concentration and pH.

6. The activation enthalpy for polymerization, which was unaffected by urea, was greatly decreased by ethanol, which also changed its sign.

7. The activation enthalpy for the splitting of ATP at high temperatures and for the exchange reaction of Ca^{2+} and ADP bound to F-actin was about 25 kcal/mole, while that for the splitting of ATP under sonic vibration was about 10 kcal/mole.

INTRODUCTION

As previously reported [1–5], the kinetic and equilibrium analyses of the G–F transformation of actin showed that it is a kind of condensation phenomenon similar to gas-to-liquid condensation. Based on the Clausius–Clapeyron relationship, the apparent enthalpy change associated with this transformation was estimated from the temperature dependence of the critical concentration of G-actin to be in the order of 10 kcal/mole of actin. In the presence of ATP the transformation is accompanied by the irreversible splitting of ATP[3]. However, the kinetic analyses of the ATP splitting of F-actin at high temperatures[6] and on the Ca^{2+} exchange in F-actin[7,8] gave larger values of 20–30 kcal/mole for the activation enthalpy in these processes.

These studies have been extended to thermodynamic analyses of polymeri-

zation and of depolymerization and to various processes in the polymers under varied solvent conditions. In summarizing the results some hypotheses on the nature of the foundation of polymers from the interaction between actin molecules have been put forward.

EXPERIMENTAL

Materials

G-Actin (ATP–G-actin) was prepared according to the method previously described[9–10]. ADP–G-actin was prepared by the method described by MIHASHI[11], based on the method of HAYASHI[12]. The molecular weight of G-actin was assumed to be 57000 (refs. 10, 11), although recent analyses have shown that it is 45000–50000 (refs. 13, 14).

Methods

Viscosity was measured by Ostwald viscosimeters. The flow time of 1 ml water was 30 sec at 20°.

The degree of flow birefringence was measured by a Rao-type home-made apparatus at the shear rate of 10 sec^{-1} (refs. 4, 15). Sonic vibration was performed at 10 kcycles/sec by a sonic vibrator made by Kubota (Japan).

RESULTS

Polymerization

When neutral salts are added to G-actin, the viscosity increases with polymerization. The time courses of the increases in viscosity under different conditions are shown in Fig. 1. In this figure, where the time scale is given in the logarithmic unit, all curves are the same type. The curves at 32.7° in 1 M KCl, at 21.5° in 300 mM KCl and at 21.5° in 50 mM KCl almost overlap, and all other curves can be approximately

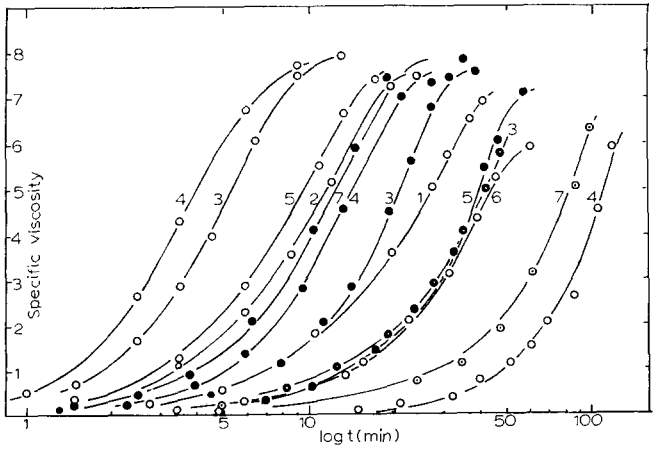


Fig. 1. Polymerization of actin at various KCl concentrations and temperatures. Actin, 1.0 mg/ml; Tris–HCl, 10 mM; ATP, 100 μM; (pH 8.0) and KCl: 20 mM (1), 30 mM (2), 50 mM (3), 100 mM (4), 300 mM (5), 1 M (6) and pH 7.5 and KCl 100 mM (7). O—O, 32.7°; ●—●, 21.5°; ⊙—⊙, 11.0°.

superposed by translation parallel to the abscissa (the log-time axis). When polymerization is almost complete, deviation from the optimal condition for polymerization to a lower temperature, to a lower salt concentration or to a higher salt concentration alters not the overall polymerization process but rather the absolute value of the polymerization rate.

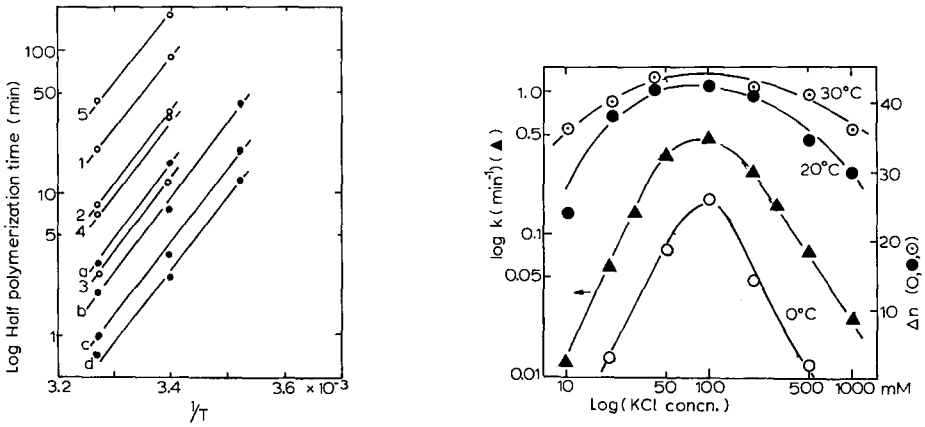


Fig. 2. Arrhenius plots of half-polymerization time ($\tau_{1/2}$) and the reciprocal of temperature ($1/T$). ○—○, actin, 1.0 mg/ml; Tris–HCl (pH 8.0), 10 mM; ATP, 100 μM; and KCl: 20 mM (1), 30 mM (2), 100 mM (3), 300 mM (4), and 1 M (5). ●—●, actin, 1.0 mg/ml; Tris–HCl (pH 8.0), 10 mM; ATP, 100 μM and $MgCl_2$: 0.8 mM (a), 1 mM (b), 1.5 mM (c), 2 mM (d).

Fig. 3. Effect of KCl concentration on the rate and the equilibrium of polymerization. Polymerization was measured by viscosity as in Fig. 1, and the polymerization rate was $k = 0.639/\tau_{1/2}$. The polymerization equilibrium was indicated by the final value of the degree of flow birefringence (Δn). The expected value of birefringence at full polymerization was 45°. Actin, 0.94 mg/ml; Tris–HCl (pH 8.0), 10 mM; and ATP 500 μM. ▲—▲, the rate of polymerization at 31.4°; ○, ●, ⊙, the equilibrium value of the birefringence after 2 days incubation. The temperature is given in the figure.

Shown in Fig. 2 is the temperature dependence of the time required for attaining half of the final viscosity (the half-time for polymerization) obtained from Fig. 1. Arrhenius plots of different solvent conditions are both linearly related and parallel. From the gradient of the relations, the activation enthalpy is calculated to be about 25 kcal/mole of actin. The change in solvent conditions, such as the ion species, the salt concentration and the pH, does not alter the activation enthalpy. A change in the absolute value of the polymerization rate is derived from the differences in the activation entropy.

In Fig. 3 the rate constant (the reciprocal of the half-time for polymerization) of polymerization at 31° is a function of the KCl concentration. In the same figure is given the final equilibrium value of the flow birefringence, which is nearly proportional to the concentration of F-actin. The variations in the rate constant are large in the KCl concentration range where the final equilibrium concentration of F-actin does not greatly change. A similar situation is also found in the effect of $MgCl_2$ (Fig. 4) and of pH.

These results were obtained at a constant protein concentration. It was found, however, that the activation enthalpy has the same value at different protein concen-

trations. As previously reported[5,16], the initial rate of polymerization is approximately proportional to $c_0^{3.5}$, and the half-time for polymerization is proportional to $c_0^{-1.5}$, where c_0 is the total actin concentration. This dependence on the protein concentration is almost independent of the solvent conditions or of the temperature.

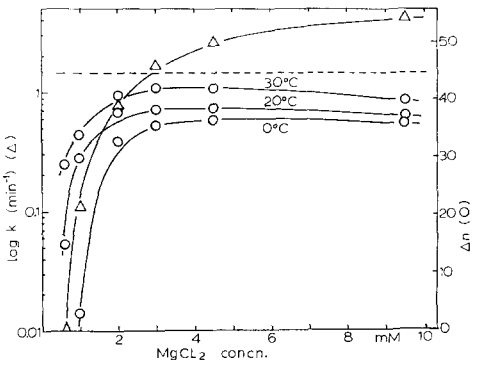


Fig. 4. Effect of $MgCl_2$ concentration on the rate (△) and the equilibrium (○) of polymerization. Methods of measurement are the same as in Fig. 3.

G–F equilibrium

When actin solutions are left standing at constant solvent conditions, an equilibrium between polymerization and depolymerization is established. In the presence of ATP, the polymerization is accompanied by ATP splitting so that the equilibrium is apparent. In this state, it was found that F-actin is stably formed only above a critical actin concentration determined by the solvent condition[1–5]. Above this concentration, the amount of F-actin increases and coexists with a constant concentration of G-actin corresponding to the saturated vapor pressure in the gas-to-liquid condensation. Therefore, according to the Clausius–Clapeyron relationship, the enthalpy change associated with the G–F transformation is calculated from the change in the critical G-actin concentration with the temperature[3].

The method of determining the critical concentration has been previously described[1–5]. By a simplified method, the critical concentration of identical solutions was estimated from the differences in the degree of flow birefringence in actual solutions and from that expected for actin at full polymerization. The degree of flow birefringence at full polymerization is calculated from the total actin concentration and from the birefringence per unit concentration of F-actin which is determined from the increase in the degree of flow birefringence with the actin concentration[2,3,15]. In Figs. 3 and 4 the degree of birefringence expected at full polymerization is about 45°.

The apparent enthalpy change in the G–F transformation obtained by the previous method exists in the range 13–7 kcal/mole. These values are similar to those previously reported[3,4]. The enthalpy does not vary largely with the salt concentration, the pH or the protein concentration. For example, the data in Figs. 3 and 4 give similar enthalpy changes.

Because the experimental conditions in Fig. 1 proximate those for full polymerization, the final viscosity values are almost the same. Nevertheless, a small amount of G-actin coexists with F-actin, and its concentration changes with the solvent condition and with the temperature.

The apparent enthalpy change previously estimated is not necessarily equal to the heat measured directly by calorimetry, if that were possible, because of the accompanying irreversible splitting of ATP.

Depolymerization

According to the former results, the rate and the final state of polymerization show different temperature dependencies. If there is a dynamic equilibrium between G- and F-actin, the rate of depolymerization must also show some temperature dependence.

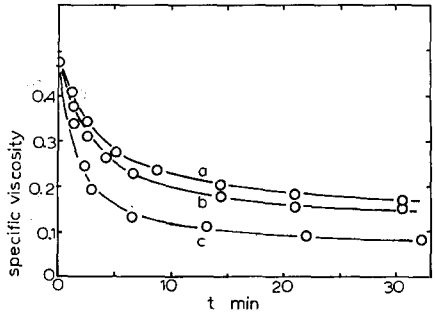
Because it is difficult to instantaneously remove salts from F-actin solutions, the depolymerization was induced by diluting concentrated F-actin solutions. Viscosity was followed immediately after dilution. As shown in Fig. 5, the time course of depolymerization is not expressed by a simple exponential relationship. The initial rapid decrease in viscosity gradually decelerates which may be due to the relative increase in the depolymerized actin of the polymerization reaction.

In Fig. 6, the temperature dependence of the initial depolymerization rate shows the activation enthalpy for depolymerization, which was independent of the final actin concentration, to be about 10 kcal/mole.

ADP–G-actin

When F-actin is depolymerized in the absence of ATP, G-actin containing ADP rather than ATP is obtained. This ADP–G-actin is polymerized by the addition of salts, but the polymerization rate is very slow compared with that of ATP–G-actin[12,17,18]. However, the temperature dependence of the rate was found to be nearly identical with that of ATP–G-actin. Thus, the activation enthalpy is 22–25 kcal/mole in 0.1 M KCl, in 10 mM phosphate buffer at pH 7.5 and in the presence and the absence of 1 mM $MgCl_2$.

As shown later, the rate of depolymerization is almost independent of ATP in the solvent. Therefore, it is expected that the enthalpy changes associated with the G–F transformations of ADP–G-actin in equilibrium and of ATP–G-actin are of the



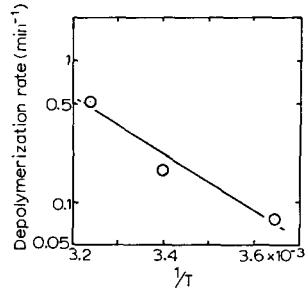


Fig. 5. Time course for depolymerization was followed by the viscosity measurement. A solution of F-actin (5 mg/ml), KCl (20 mM) and Tris–HCl (pH 8.0) (5 mM) at 21.5° was diluted to F-actin (0.5 mg/ml), KCl (0.4 mM), Tris–HCl (pH 8.0) (5 mM) and ATP (500 μM) at different temperatures: 35.4° (a), 21.5° (b) and 1.3° (c).

Fig. 6. Arrhenius plots of the depolymerization rate. Depolymerization rate was calculated from the initial gradient of Fig. 5.

same order. An equilibrium analysis, however, is not easy because ADP–G-actin in the absence of ATP is not stable at high temperatures[12] for long periods.

ATP splitting under sonic vibration

The F-actin solution shows a marked ATPase activity under sonic vibration[19,20]. The rate of ATP splitting was measured at various temperatures. In Fig. 7, the activation enthalpy of this reaction is calculated to be about 10 kcal/mole, although it depends somewhat on solvent conditions.

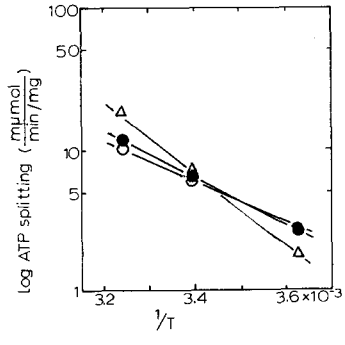
ATP splitting at high temperatures

The F-actin solution shows a marked ATPase activity at 50–60° (ref. 6). From the temperature dependence of this ATPase activity, the activation enthalpy has been estimated to be 25 kcal/mole (ref. 6). On account of this high activation enthalpy, the ATPase becomes negligible at low temperatures. (At 20°, 1 mole of G-actin splits 1 mole of ATP in about 15 h.)

Exchange of Ca^{2+} and ADP in F-actin

As previously reported[7,8], Ca^{2+} and ADP bound to F-actin are slowly exchangeable at room temperature with Ca^{2+}, ADP or ATP in the solvent. The incorporated ATP is split into ADP; this reaction is directly related to the ATPase activity described in previous sections.

The rate of exchange was measured using radioactive Ca^{2+} and ATP (ref. 8). The exchange rate of Ca^{2+} is always more rapid than that of ADP. The activation enthalpies calculated from the temperature dependence of the initial rate of exchange are 20–25 kcal/mole for both Ca^{2+} and ADP. This value is nearly equal to that pre-



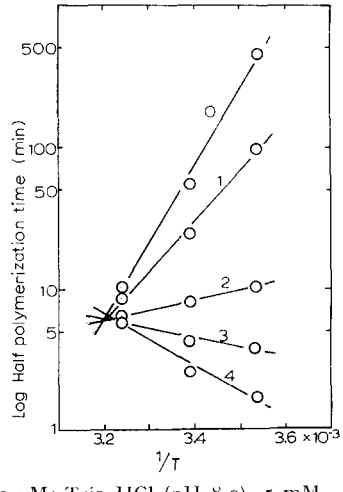


Fig. 7. Arrhenius plots of the sonic ATPase. ATP, 500 μM; Tris–HCl (pH 8.0), 5 mM. △, KCl, 4 mM; actin, 2 mg/ml ($\Delta H^{\ddagger}$ = 13 kcal); ○, KCl, 0.1 M; actin, 2 mg/ml ($\Delta H^{\ddagger}$ = 7 kcal); ●, KCl, 0.1 M; actin, 1 mg/ml ($\Delta H^{\ddagger}$ = 8 kcal).

Fig. 8. Arrhenius plots of the polymerization rate in various concentrations of ethanol. Actin, 0.56 mg/ml; KCl, 67 mM, Tris–HCl (pH 8.0), 6.7 mM; and ATP 100 μM. No ethanol (0) or with ethanol (in weight %): 5.3 (1); 11 (2); 16.6 (3); 20.7 (4).

viously obtained for the ATPase. This suggests that both the exchange and the ATP splitting are due to the same cyclic reaction.

The exchange rates of Ca^{2+} were compared in ADP–F-actin in the presence of ATP and in the absence of ATP and ADP. The rate was nearly equal in two cases. This is remarkable because the rate of polymerization is very different in ATP–G-actin and ADP–G-actin.

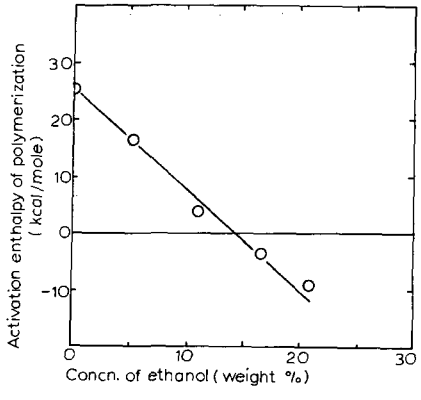
Polymerization in ethanol

Polymerization of actin is accelerated by organic solvents such as ethanol and acetone[4,21]. With ethanol as the solvent, the rate of polymerization, but not the overall shape of the polymerization curve, is changed.

It is interesting that ethanol and not the salt or the pH (Fig. 8) greatly changes the temperature dependence of the polymerization rate. The activation enthalpy of polymerization, obtained from data in Fig. 8, decreases linearly with increasing concentrations of ethanol as shown in Fig. 9. It becomes zero at 15 % ethanol at which point the temperature dependence of polymerization disappears. When the ethanol concentration is higher than 15 %, the enthalpy becomes negative; the polymerization rate is faster at lower temperatures. Data in Fig. 8 also show that at 35–40° the polymerization rate is almost independent of the ethanol concentration.

Polymerization in urea

The polymerization is decelerated by urea, as shown in Fig. 10. As with salts and pH, urea does not change the activation enthalpy of polymerization. The half-time for polymerization is proportional to the concentration of urea. The free energy difference in activation is about 1.1 kcal/mole per mole of urea.



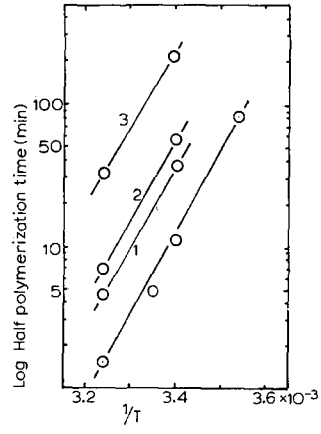


Fig. 9. Dependence of the activation enthalpy on the ethanol concentration. The activation enthalpy ($\Delta H^{\ddagger}$) was obtained from Fig. 8.

Fig. 10. Arrhenius plots of polymerization at various concentrations of urea. Actin, 0.73 mg/ml; Tris–HCl (pH 8.0), 6.7 mM; KCl, 67 mM; and ATP, 100 μM; and urea: no (0); 0.4 M (1); 0.8 M (2); 1.6 M (3).

DISCUSSION

The previous experimental results concerning enthalpy changes are summarized in Table I. A simple mechanism is proposed to explain the data, although the possibility of other mechanisms is not excluded.

TABLE I

THERMODYNAMIC DATA

Reaction	*Rate constant*	*Enthalpy change (kcal/mole)*
(a) G–F equilibrium (condensation)	$G = k_2/k_1$	$\Delta H = 10$
(b) Polymerization	$(k_1 k_n)^{1/2}$	$\Delta H^{\ddagger} = 20$–25
(c) Depolymerization	k_2	$\Delta H^{\ddagger} = 10$
(d) From (a), (b) and (c)		
growth	k_1	$\Delta H^{\ddagger} = 20$
nucleation	k_n	$\Delta H^{\ddagger} = 20$
(e) ATPase at high temperature and exchange of Ca^{2+} or ADP	$(k_1 GF_N, k_2 F_N)$	$\Delta H^{\ddagger} = 20$–25
(f) Sonic ATPase	$(k_1 G, k_2)$	$\Delta H^{\ddagger} = 10$

The following kinetic equation is assumed for the G–F transformation of actin:

$$-dG/dt = k_1 GF_N - k_2 F_N \quad (1)$$

G is the concentration of G-actin, F_N is the number concentration of F-actin; and k_1 and k_2 are kinetic constants of polymerization, respectively[5,18]. With this equation it is demonstrated that polymerization occurs at the ends of F-actin upon collision with G-actin, and the rate is proportional to the product of G and F_N; however, when depolymerization occurs only at the ends of F-actin, the rate is proportional to F_N.

In equilibrium, when $(dG/dt) = 0$, the concentration of G-actin is given by

$$G = k_2/k_1 \quad (2)$$

which is independent of the coexisting concentration of F-actin. Therefore, the expression for the temperature dependence of the critical concentration in this experiment is

$$k_2/k_1 \propto \exp(10/RT) \quad (3)$$

As previously remarked, the value 10 kcal/mole was obtained for the apparent polymerization equilibrium accompanied by the ATP splitting. The enthalpy change for the equilibrium of ADP–G-actin was not directly determined but is expected to be of the same order previously mentioned.

In the depolymerization experiment, the F-actin solution was diluted, and the decrease in the viscosity was followed at different temperatures. In the beginning the F_N of depolymerization did not vary. In Fig. 5 the rate constant of depolymerization is k_2;

$$k_2 \propto \exp(-10/RT) \quad (4)$$

therefore, from Eq. 3 is calculated

$$k_1 \propto \exp(-2o/RT) \tag{5}$$

The simple kinetic Eqn. 1 cannot be applied during the whole process of polymerization because in the initial stage, the formation of polymer nuclei, whose rate of nucleation depends on the solvent condition, is an important process. Therefore, during polymerization under different solvent conditions, the same weight concentration of F-actin, F, is not identical with the same number concentration of F-actin, F_N. Such situations complicate interpretations of the activation enthalpy for the polymerization obtained from Fig. 2.

For the present problem, however, the simple theory on the helical polymerization previously given[5] is useful. Polymerization consists of nucleation and growth. The former presumably occurs in proportion to the p-th power of the monomer concentration, the rate constant being k_n. The occurrence of the latter is expressed by the first term of Eqn. 1 where the rate constant is k_1. When the depolymerization rates were neglected, the integration of kinetic equations related the time t and the degree of polymerization F/c_0 in the following equation:

$$\ln \frac{1 + [1 - (1 - F/c_0)^p]^{1/2}}{1 - [1 - (1 - F/c_0)^p]^{1/2}} = (2p)^{1/2} \cdot (k_1 k_n]^{1/2} \cdot c_0^{p/2} \cdot t \tag{6}$$

This equation shows that the relationships between F/c_0 and log t at different solvent conditions can be superposed by translation parallel to the log-t axis, as observed in Fig. 1.

The polymerization rate (the reciprocal of the half-time for polymerization) is proportional to $(k_1 k_n)^{1/2}$ in the former equation. Therefore, the activation enthalpy for polymerization is related to the enthalpy term in the square root of the product of two rate constants for nucleation and growth. Thus, the present experiment gives:

$$(k_1 k_n)^{1/2} \propto \exp(-2o/RT) \tag{7}$$

Therefore, from Eqn. 5

$$k_n \propto \exp(-2o/RT) \tag{8}$$

Higher temperatures facilitate nucleation. Both rate constants, k_1 and k_n, exhibit nearly the same temperature dependence.

Two mechanisms have been proposed[19,20,22] for the ATP splitting in actin solutions under sonic vibration. One is that the splitting is coupled with the cycle of depolymerization–polymerization at the ends of F-actin, the number of which was increased by sonic vibration[20]. The concentration of G-actin coexisting with F-actin is not changed by sonic vibration, and the average length of F-actin fragments produced by the vibration is assumed to be temperature independent[20]. Therefore, when the solvent conditions are nearly optimal, F_N is independent of temperature. The temperature dependence of the ATPase during the vibration is determined by the dependence of $k_1 G$ or of k_2. The present experiment on the ATPase shows that the activation enthalpy is about 10 kcal/mole; this is consistent with the result (Eqn. 4) obtained from the depolymerization experiment. The second assumption is that

ATPase may occur during intrapolymer structural changes induced by sonic vibration[22] in F-actin such as transformations between partially linear polymers and helical polymers. Such mechanisms can not be excluded.

These two mechanisms probably are applicable to ATPase without sonic vibrations at high temperatures. The activation enthalpy for the ATPase at high temperatures was found to be larger than that under sonic vibration. This difference in activation enthalpies may be due to the temperature dependence of the average length of F-actin or to the difference between intrapolymer structural changes induced mechanically and thermally in F-actin. The experimental data are inadequate to show which mechanism is more probable. However, it is likely that the exchange of Ca^{2+} and ADP bound to F-actin occurs during the ATP splitting. It is remarkable that the exchange rate is not dependent on the presence of ATP or ADP, while the polymerization rate is greatly increased by ATP.

An important finding was that ionic changes have no effect on the activation enthalpy and on the differences in equilibrium enthalpy of the G–F transformation. Although the enthalpy appears not to be derived from ionic interaction between actin molecules, it is not suggested that the ionic property does not contribute to the interaction. Because salts and pH's strongly affect the rate and the equilibrium, it is suggested that the coulomb interaction between charges on actin molecules is of importance. The experimental results show that if this interaction exists, it must be independent of temperature. One explanation for this phenomenon may be that the dielectric constant of the solvent (water) decreases with temperature, so that the product of the dielectric constant and the absolute temperature is insensitive to temperature. However, large enthalpies in polymerization processes may result from nonionic, hydrophobic interactions.

Ethanol was found to change the sign of the activation enthalpy which suggests that the solvent, as a dielectric, participates in the polymerization. The enthalpy varied in proportion to the ethanol concentration, and because the effect of ethanol vanishes at about 35°, the activation entropy can also be expected to be proportional to the ethanol concentration.

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REFERENCES

1 S. ASAKURA, K. HOTTA, N. IMAI, T. OOI AND F. OOSAWA, *Conf. Chem. Muscular Contraction Tokyo, 1957*, Igaku Shoin, Tokyo, 1957, p. 57.
2 F. OOSAWA, S. ASAKURA, K. HOTTA, N. IMAI AND T. OOI, *J. Polymer Sci.*, 37 (1959) 323.
3 S. ASAKURA, M. KASAI AND F. OOSAWA, *J. Polymer Sci.*, 44 (1960) 33.
4 M. KASAI, S. ASAKURA AND F. OOSAWA, *Biochim. Biophys. Acta*, 57 (1962) 13.
5 F. OOSAWA AND M. KASAI, *J. Mol. Biol.*, 4 (1962) 10.
6 H. ASAI AND K. TAWADA, *J. Mol. Biol.*, 20 (1966) 403.
7 M. KASAI AND F. OOSAWA, *Abstr. Conf. Biol. Movement, Tokyo, 1965*, p. 11.
8 M. KASAI AND F. OOSAWA, *Biochim. Biophys. Acta*, 172 (1969) 300.
9 M. KASAI AND F. OOSAWA, *Biochim. Biophys. Acta*, 154 (1968) 520.
10 W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 188 (1951) 559.

11 M. MIHASHI, *Arch. Biochem. Biophys.*, 107 (1964) 441.
12 T. HAYASHI AND R. ROSENBLUTH, *Biol. Bull.*, 119 (1960) 290.
13 M. REES AND M. YOUNG, *J. Biol. Chem.*, 242 (1967) 449.
14 K. TSUBOI, *Biochim. Biophys. Acta*, 160 (1968) 420.
15 M. KASAI, H. KAWASHIMA AND F. OOSAWA, *J. Polymer Sci.*, 44 (1960) 51.
16 M. KASAI, S. ASAKURA AND F. OOSAWA, *Biochim. Biophys. Acta*, 57 (1962) 22.
17 S. HIGASHI AND F. OOSAWA, *J. Mol. Biol.*, 12 (1965) 843.
18 F. OOSAWA AND S. HIGASHI, *Progr. Theoret. Biol.*, 1 (1967) 79.
19 S. ASAKURA, *Biochim. Biophys. Acta*, 52 (1961) 65.
20 Y. NAKAOKA AND M. KASAI, *J. Mol. Biol.*, to be published.
21 K. MIHASHI AND T. OOI, in S. EBASHI, F. OOSAWA, T. SEKINE AND Y. TONOMURA, *Molecular Biology of Muscular Contraction*, BBA Library Vol. 9, Elsevier, Amsterdam and Igaku Shoin, Tokyo, 1965, p. 77.
22 S. ASAKURA, M. TANIGUCHI AND F. OOSAWA, *J. Mol. Biol.*, 7 (1963) 55.