Studies on the Kinetics of Formation and Dissociation of the Actomyosin Complex*

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ABSTRACT: Changes in turbidity measured in a stopped-flow spectrophotometer were used to study the rates of formation and dissociation of the actomyosin complex. The apparent rate constant for complex formation at pH 8 and 20° in 0.5 M KCl–5 $\times 10^{-3}$ M $MgCl_2$ was $1.4 \pm 0.5 \times 10^5$ M^{-1} sec^{-1}. The value is based on the assumptions that myosin molecules are bound independently at sites on F-actin, and that each site consists of two G-actin units. The dissociation of actomyosin by MgATP, MgGTP, MgUTP, MgITP, and magnesium pyrophosphate followed a simple exponential decay and the decay constant was proportional to the concentration of substrate. Dissociation by CaATP did not fit this simple description. The results for magnesium nucleotides and pyrophosphate are consistent with a kinetic scheme in which dissociation is produced by substrate binding by myosin at a site which is distinct from the actin binding site.

A characteristic property of the muscle proteins F-actin and myosin is the formation at high ionic strength of a soluble complex which dissociates on the addition of ATP (Szent-Gyorgyi, 1951; Spicer, 1952; Gergely, 1956). At physiological ionic strength, actomyosin exists as a dispersion, but ATP still appears to cause dissociation, yielding the "clear phase" which is followed by precipitation and superprecipitation as substrate is depleted (Maruyama and Gergely, 1962). It is reasonable to infer that, at least in solution, ATP serves both as substrate for myosin and as regulator of the interaction between actin and myosin. Glycerated muscle fibers or intact muscle in rigor, systems which have in common a depletion of ATP, exhibit a high resistance to stretch (Weber and Portzehl, 1954), presumably due to actin–myosin interaction. Thus ATP may also be supposed to play some part in regulating actin–myosin interaction in muscle and the dissociation observed in solution may be one step in the cyclic process taking place in muscle contraction.

The dissociation of actomyosin has been the subject of a large number of studies, but the kinetics of the process have not been elucidated. In the experiments described here, the formation and dissociation of the actomyosin complex was followed by measuring the change in turbidity in a stopped-flow spectrophotometer. This technique allows the reaction to be examined on a time scale comparable with the contraction cycle in muscle.

The rate of dissociation with a variety of substrates was found to be proportional to substrate concentration over a wide range of concentrations. Rates corresponding to an actomyosin half-life of less than 5 msec were attained (the smallest value that could be measured in the apparatus) without departing from the linear relationship. The kinetic data fit a model in which actin is displaced by binding of substrate to myosin at a site distinct from the actin binding site.

Materials and Methods

Myosin was prepared by a modification of the Holtzer and Lowey (1959) method, as described previously (Finlayson and Taylor, 1969). Actomyosin was obtained by the method of Brynes and Suelter (1965) except that the actomyosin was precipitated three times by dialysis. Acetone powders were prepared by the method of Tonomura and Yoshimura (1962) and F-actin was prepared from the powder by the method of Carsten and Mommaerts (1963). P_i was determined by the Tausky and Shorr (1953) procedure.

Protein concentrations were determined by the difference in optical density at 291 mμ *vs.* 350 mμ in 0.5 M NaOH. The method was calibrated by weight as previously described (Finlayson and Taylor, 1969). Before use myosin, F-actin or actomyosin solutions were clarified by centrifugation at 66,000*g* for 45, 15, and 30 min, respectively.

The operation of the stopped-flow spectrophotometer and the method of data reduction was described in a previous paper (Finlayson and Taylor, 1969).

Results

Turbidity of Actin, Myosin, and Actomyosin Solutions. Viscometric (Szent-Gyorgyi, 1951), sedimentation (Spicer, 1952), and some light-scattering studies (Gergely, 1956) have indicated that the primary effect

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† Present address: Department of Urology, University of Florida, Gainesville, Fla. The recipient of a Special Fellowship from the U. S. Public Health Service.

‡ Supported by U. S. Public Health Service Training Grant GM 780.

§ This study was supported by a U. S. Public Health Service Research Grant, GM 10992. The recipient of a Research Career Development award from the U. S. Public Health Service.

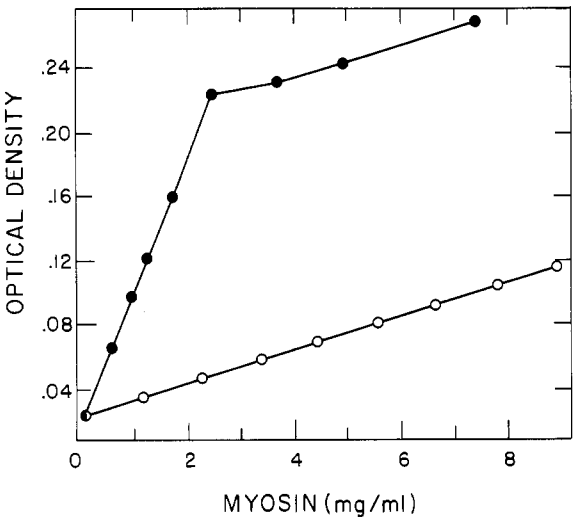


FIGURE 1: Turbidimetric titration of actin. The lower curve is the turbidity of myosin alone (○—○). The upper curve (●—●) is the turbidity of a solution containing 0.72 mg/ml of actin and the amount of myosin indicated by the abscissa. The sharp break in the curve occurs at 3.4 g of myosin/g of actin, 0.5 M KCl, and 0.1 M Tris buffer, 22°, and λ 350 mμ.

of ATP on actomyosin is to cause dissociation. A different view has been expressed by Morales and his collaborators (Gellert *et al.*, 1959; von Hippel *et al.*, 1959) who favor shape changes of large particles as the explanation of changes in the light-scattering envelope while the only components which dissociate are myosin aggregates.

The formation of a complex between actin and myosin and its dissociation by ATP has been described by several investigators in sedimentation experiments, and by using zone centrifugation the products of dissociation can be identified as myosin and F-actin (unpublished observations). Thus it appears reasonable to accept dissociation as the cause of the turbidity change, rather than to enter into a discussion of the interpretation of light-scattering data at low angles in a polydisperse system.

However, even if the two end states of the system, the actomyosin complex *vs.* free F-actin and myosin, can be identified with a difference in turbidity, the use of the change in turbidity with time to measure association–dissociation kinetics requires some justification. In part, the use of turbidity measurements rests on two types of experimental results. First the excess turbidity (the turbidity of a mixture of actin and myosin minus the sum of the turbidities of the components) is proportional to the amount of myosin added. Since the apparent binding constant is large this result implies that excess turbidity is approximately a linear function of the fraction of sites occupied. Second, the change in excess turbidity with time was found to obey simple kinetics $\Delta T = \Delta T_m(1 - e^{-\lambda_d t})$, where ΔT_m is maximum turbidity change. λ_d, the rate constant for dissociation, was proportional to the concentration of the substrate (nucleotide triphosphates or pyrophosphate). This finding would be difficult to explain if turbidity is a complicated function of the fraction of occupied sites.

It could still be argued that the experimental results arise from a series of coincidences and an analysis of the system suggests that this argument is partly correct. An exact treatment of the light-scattering problem is too difficult to undertake, but we have applied the approximate treatment of Gergely and Kohler (1957) to a calculation of the kinetic behavior (Appendix). The results indicate that λ_d is an approximate measure of the intrinsic rate of dissociation per site. Therefore we will first present the experimental evidence and treat the data in terms of binding to independent sites returning to the question of interpretation in the Conclusions section.

Turbidities of solutions of actin or myosin alone are nearly linear functions of concentration for the range used in these experiments. In Figure 1, the lower line shows the turbidity of myosin alone. Addition of increasing amounts of myosin to a fixed concentration of actin gave a linear turbidity increase, as shown by the upper curve with a relatively sharp break at a mass ratio of 3 g of myosin/g of actin. Granting some uncertainties in molecular weights this value corresponds to 3 ± 0.3 actins (as G-actin) per myosin molecule. This figure is higher than the value of 2 actins/myosin calculated from viscosity data (Szent-Gyorgyi, 1951). Estimation of the end point is not very accurate, since as can be seen in the figure, the actomyosin curve is not quite linear or parallel to the myosin turbidity curve in the region just above the break which could lead to an over estimate of the number of actin molecules at the equivalence point.

The rate of formation of the actomyosin complex was measured by mixing actin and myosin in the stopped-flow spectrophotometer and following the turbidity at 350 mμ. The increase in turbidity, which is proportional to voltage change in the range investigated, is shown in Figure 2. The rate equation for a reaction of the type

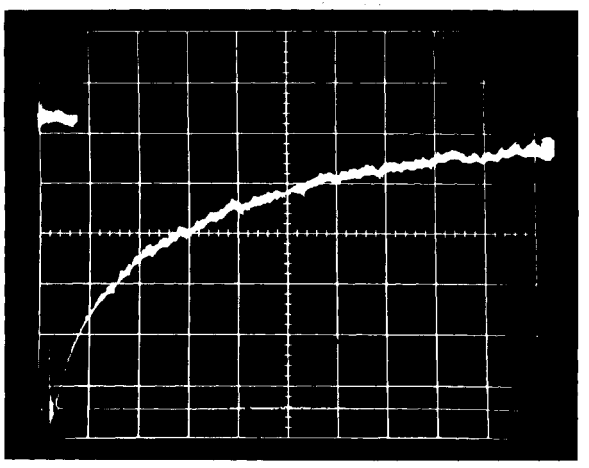
$$A + M \underset{k_d}{\overset{k_r}{\rightleftharpoons}} AM$$

is in general cumbersome to use for evaluation of rate constants. Since the equilibrium constant is large the reverse reaction can be ignored during the early part of the association. For the special case, $[A]_0 = [M]_0$, where square brackets signify molar concentration and the subscript refers to zero time

$$k_r t = \frac{1}{[M]} - \frac{1}{[M]_0}$$

A plot of $1/[M]$ or $1/[AM]$ *vs.* t should be linear, and as discussed by Benson (1960), a linear plot will be obtained even if the initial concentrations are not exactly equal. A difference of a factor of 2 will lead to only a 20% error in k_r.

Experiments were performed at myosin concentrations of 2.2, 4.0, and 5.5 μM, assuming a molecular weight of 5×10^5 for myosin. The G-actin to myosin mole ratio was maintained at 2:1, assuming a molecular



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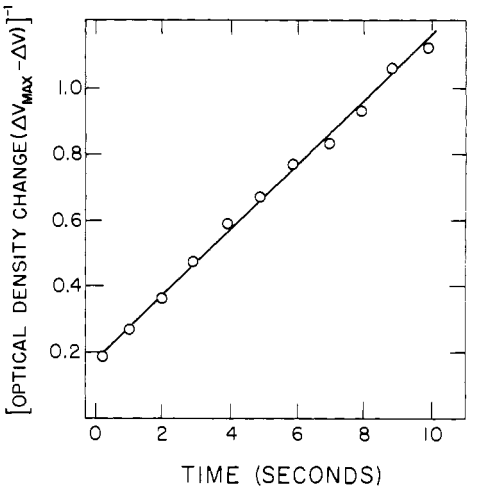

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FIGURE 2: Turbidity change in the reaction of F-actin and myosin. (a) Oscilloscope trace recorded at λ 350 mμ; vertical axis 20 mV/major division; horizontal axis 1.0 sec/major division. Mark at top left indicates the final turbidity reached at long times. (b) Plot of the reciprocal of the voltage difference shown in part a. ΔV_{max} is total voltage change obtained at long times; 5.5 μM myosin, 10.4 μM actin (calculated from molecular weight of G-actin), 0.5 M KCl, 5 mM $MgCl_2$, and 0.09 M Tris buffer, pH 8.0, at 20°.

weight for G-actin of 60,000. Since the reaction goes nearly to completion, the final voltage change ΔV_{max} is proportional to $[M]_0$, and $[M] = [M]_0 - [AM] = \Delta V_{max} - \Delta V$. Thus a plot of $(\Delta V_{max} - \Delta V)^{-1}$ *vs.* t should be linear. ΔV_{max} was obtained by allowing the reaction to proceed until V became constant. Linear plots were obtained at all three concentrations (Figure 2), and the average value of k_r from six determinations was $1.4 \pm 0.5 \times 10^5$ M^{-1} sec^{-1} at pH 8.0 and 20°, in the presence of 0.5 M KCl, 5×10^{-3} M $MgCl_2$, and 0.09 M Tris buffer.

Dissociation of Actomyosin. The turbidity change was measured after mixing actomyosin, natural or synthetic, with a variety of nucleotide triphosphates and pyrophosphate. The two solutions were made up to contain as nearly as possible the same concentrations of KCl and buffer at the same pH. No turbidity transient was detected when actomyosin was mixed with buffered $MgCl_2$ at pH 8. Any transients arising from the twofold dilution or from Mg–protein complex formation are either too slow or too small to be measured. As discussed below, turbidity changes did occur when actomyosin was mixed with $CaCl_2$.

A typical experiment with MgATP is shown in Figure 3. A plot of the data in the form $\log(\Delta V_{max} - \Delta V)$ *vs.* t is linear (Figure 4). (Since the total change in transmission was small, the error arising from equating voltage change to optical density change was less than 2–5%.) In all experiments the data could be represented by $\Delta T = \Delta T_{max}(1 - e^{-\lambda_d t})$, where ΔT is the change in turbidity at time t. This relation is illustrated by the linearity of the semilog plot shown in Figure 4. ΔT_{max} divided by actomyosin concentration was relatively constant from experiment to experiment. It did not depend upon pH in the range from 7.0 to 9.2 and increased about 20% when the temperature was raised from 0 to 15° at pH 8.

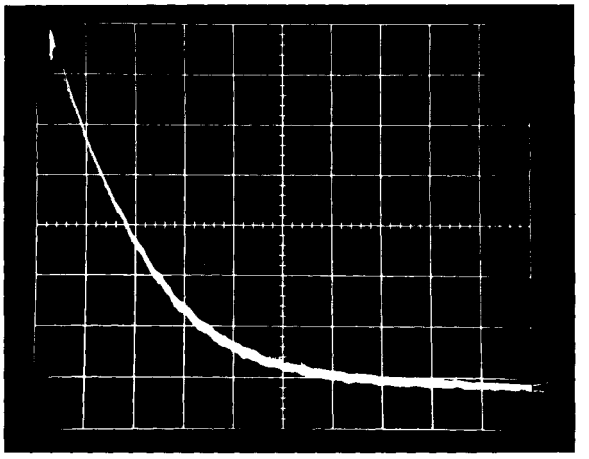

FIGURE 3: Oscilloscope trace obtained in the dissociation of actomyosin by MgATP; vertical axis 50 mV/major division; horizontal axis 0.1 sec/major division. The small hook at the beginning of the trace, at about 10 msec is a noise pulse; 4.5 mg/ml of actomyosin, 0.1 mM ATP, 0.5 M KCl, 10 mM $MgCl_2$, and 0.09 M Tris buffer, pH 8.0, at 0°.

The rate constant, λ_d, depended upon pH, temperature, metal ion, and substrate concentration, and the effects of varying these parameters were investigated in detail. With ATP as substrate the rate constant varied with Mg concentration in a manner which suggested that MgATP was a more effective substrate than ATP itself. As shown in Figure 5, a magnesium concentration of greater than 2×10^{-3} M is necessary to give the maximum rate for a fixed concentration of ATP of 10^{-4} M. The rate remained constant in the range 2×10^{-3} to 10^{-2} M $MgCl_2$, the highest concentration investigated. The curve drawn through

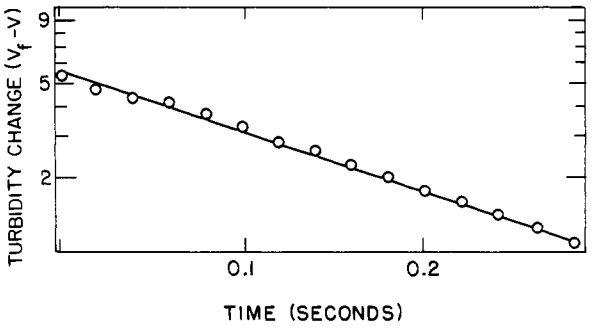

FIGURE 4: A semilog plot of the results shown in Figure 3. $V_f - V$ is the difference between the final voltage and the voltage at time t.

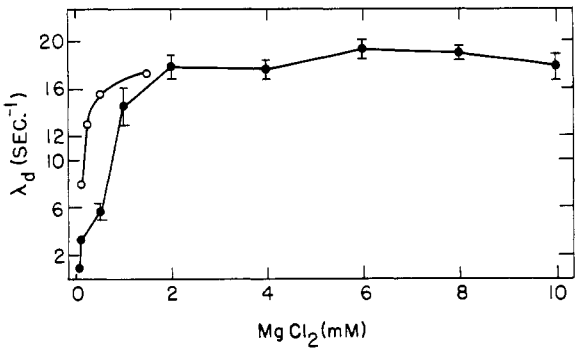

FIGURE 5: Variation of the rate constant for actomyosin dissociation, λ_d, with $MgCl_2$ concentration at a constant ATP concentration of 0.1 mM. Error bars indicate average deviations of triplicate determinations. The curve, O—O, was calculated on the assumption that the rate of dissociation is proportional to MgATP concentration using the stability constant of Khan and Martell; 3.5 mg/ml of actomyosin, 0.1 M Tris buffer, and 0.5 M KCl, pH 7.5, at 15.2°.

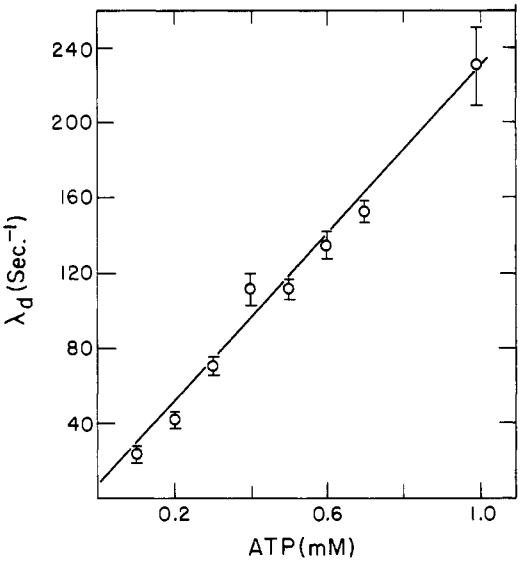

FIGURE 6: Variation of the rate constant for actomyosin dissociation, λ_d, with concentration of ATP; 3.3 mg/ml of actomyosin, 5 mM $MgCl_2$, 0.5 M KCl, and 0.09 M Tris buffer, pH 8.0, at 18.5°. The straight line shown is the least-squares fit.

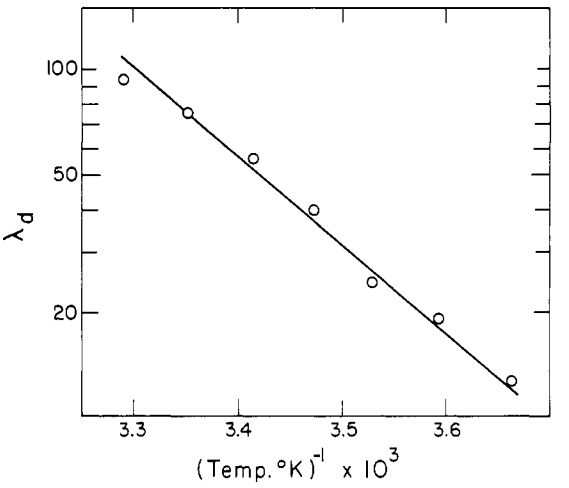

FIGURE 7: Arrhenius plot of λ_d for MgATP dissociation of actomyosin. Buffers were adjusted to pH 8 at the required temperatures. Each point is the average of triplicate or quadruplicate determinations; 2.8 mg/ml of actomyosin, 0.2 mM ATP, 5 mM $MgCl_2$, 0.09 M Tris buffer, and 0.5 M KCl.

the open circles in Figure 5 was calculated from the assumption that the rate is proportional to MgATP concentration, using the value given by Khan and Martell (1966) of 4.18 for log K of MgATP. The shape of the experimental plot corresponds to a value of log k of approximately 3.3. Unfortunately accurate values for the MgATP stability constant do not appear to be available in 0.5 M KCl. Employing the value of 9 for the stability constant of KATP (Botts *et al.*, 1965) gives an apparent value for log K of 3.6–3.7 which provides a much better agreement.

λ_d was proportional to MgATP concentration in the range investigated, which extended from 10^{-4} to 10^{-3} M. In Figure 6, the line is a least-squares fit to the relation $\lambda_d = k[\text{MgATP}] +$ a constant. A similar result was obtained for natural actomyosin, about 15% by weight actin or reconstituted actomyosins, 11 and 35% actin. The slopes of the plots gave values of k of $1.4 \pm 0.3 \times 10^5$ M^{-1} sec^{-1} for two natural actomyosin preparations and $1.8 \pm 0.3 \times 10^5$ M^{-1} sec^{-1} for two synthetic actomyosins, at pH 8 and 18.5°. A fivefold variation in the actomyosin concentration at constant ATP concentration did not produce a significant change in λ_d.

Variation of pH from 7.0 to 9.2 at 10^{-4} M ATP increased λ_d by almost a factor of 2. Variation of temperature from 0 to 30° led to a tenfold increase in λ_d. A plot of log λ_d *vs.* $1/T$ (Figure 7) was essentially linear at pH 8, and the slope corresponded to an activation energy of 11.8 ± 0.1 kcal.

The time course of dissociation was also investigated for MgITP, MgGTP, MgUTP, and MgPP. In all cases the turbidity decrease was accurately fitted by a single exponential and the rate constant, λ, satisfied the relation $\lambda = k[S] +$ constant. The constant did not differ significantly from zero for the various substrates tested except possibly for MgATP, and the value in this case

TABLE I: Rate Constant for Dissociation of Actomyosin at Unit Substrate Concentration.[a]

Ion	Substrate	Temp (°C)	pH	Rate Constant, k (M^{-1} sec^{-1})
Mg	ATP	15	7.5	1.6×10^5
Mg	ATP	0	7.5	5.9×10^4
Mg	ITP	0	8.0	2.6×10^3
Mg	GTP	13.3	8.0	1.25×10^4
Mg	UTP	15.1	8.0	1.98×10^4
Mg	PP	17	8.0	1.1×10^3
Ca	ATP	15	7.5	1.2×10^4
Ca	ATP	0	8.0	4.5×10^3

[a] All values refer to pH 8, 0.5 M KCl, 5×10^{-3} M $MgCl_2$, or 1.5×10^{-2} M $CaCl_2$.

is not much greater than the experimental error. Values for the rate constants sre summarized in Table I.

Effect of CaATP. The dissociation by CaATP did not fit into the simple pattern established for magnesium nucleotides and magnesium pyrophosphate. $CaCl_2$ alone gave a small transient turbidity change which was about 15% of the maximum change obtained with nucleotides. The rapid decrease in turbidity (Figure 8) was fitted by a single exponential with a rate constant of 15 ± 2 sec^{-1} for 1.5×10^{-2} M CaCl at 20°. This decrease was followed by a slow regain of turbidity. The whole process is shown in Figure 9; the rising phase was fitted to two exponentials with rate constants of 1.1 ± 0.1 and 0.35 ± 0.09 sec^{-1}.

As opposed to this complex behavior with Ca alone, CaATP at 15° gave a simple dissociation response which could be represented by a single exponential process with rate constant proportional to concentration, $k = 1.2 \pm 0.4 \times 10^4$ M^{-1} sec^{-1}. Similar values of k were obtained with natural and synthetic actomyosin. The reaction was studied over a range of concentrations from 5×10^{-5} to 10^3 M; consequently, λ_d was much less than 15 sec^{-1} at lower concentrations. Thus the fast decay obtained with Ca alone should have been detectable, if present, by a deviation from linearity in the semilog plot, but no such effect was found.

A comparison of the rate of dissociation at 20° for different calcium concentrations and a constant ATP concentration of 10^{-4} M again gave a dependence upon calcium concentration which could be interpreted as evidence that CaATP is the substrate. Figure 10 shows results obtained from two preparations. The decrease in rate again corresponds to a log K for CaATP which is somewhat lower than the value given by Khan and Martell (1966), but the deviation is small when an approximate correction is made for potassium binding. As opposed to the case with MgATP there appears to be a definite inhibition at high Ca concentrations. The value of 1.5×10^{-2} M $CaCl_2$ used in most dissociation experiments was selected to give the maximum rate.

A further complication arose when dissociation by CaATP was studied at 0°. While all experiments with magnesium nucleotides and magnesium pyrophosphate at 15 or 0° and CaATP at 15° were represented by a single exponential decay, the dissociation by CaATP at 0° showed a lag phase. This feature is illustrated in Figure 11, in which the time scale has been increased to 20 msec/major division to display the plateau at early times. The complete curve is fitted by two exponential terms $y = c_1e^{-\lambda_1 t} + c_2e^{-\lambda_2 t}$, where y is the turbidity change at time t, and the constant c_1 is negative. λ_2 is a linear function of concentration, yielding $k = 4.5 \times 10^3$ M^{-1} sec^{-1}, a value similar to that found for MgITP, at the same temperature. λ_1 is

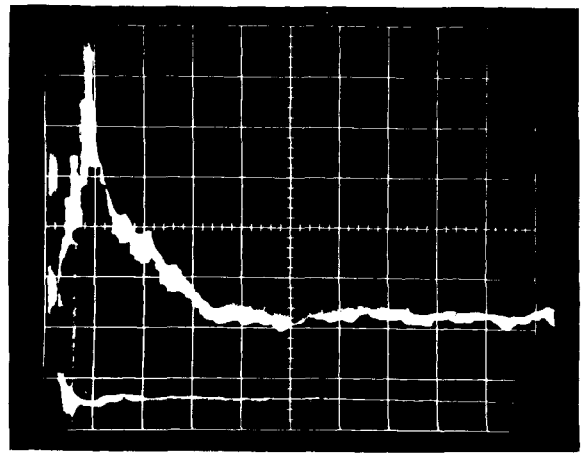

FIGURE 8: The early part of the turbidity transient produced by mixing actomyosin with $CaCl_2$. Upper trace is the photomultiplier signal; the initial rising phase is due to the washout of previous solution. Lower trace shows the end of the piston displacement. Vertical scale: 10 mV/major division; horizontal scale: 50 msec/major division; 3.2 mg/ml of actomyosin, 15 mM $CaCl_2$, 0.5 M KCl, and 0.09 M Tris buffer, pH 8.0, at 20°.

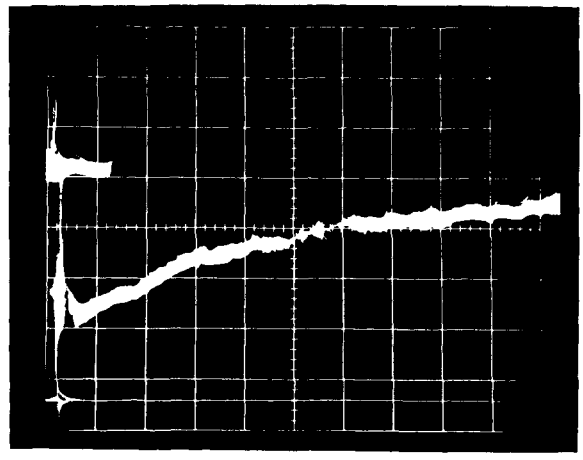

FIGURE 9: Turbidity transient produced by mixing actomyosin with $CaCl_2$. Same system as Figure 8, except that time scale has been increased to 500 msec/major division. The broad mark at the left indicates the final turbidity at long times. The narrow trace near the bottom of the grid is the piston displacement record.

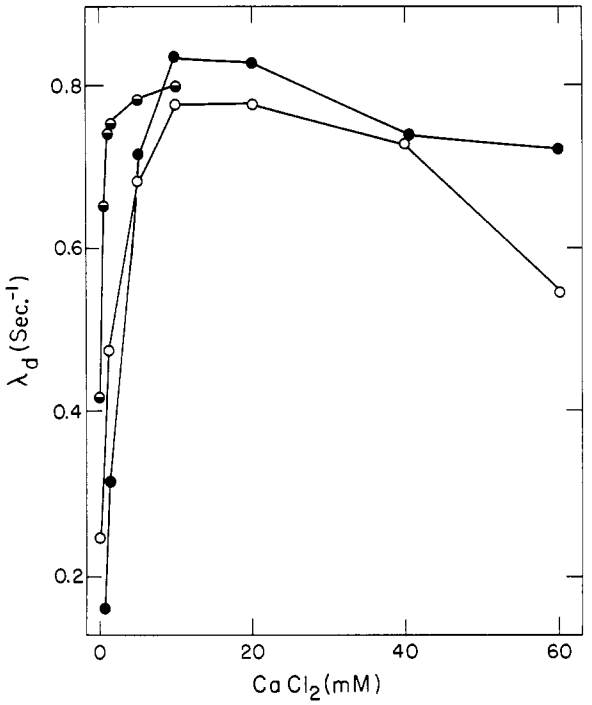

FIGURE 10: Variation of the rate constant for actomyosin dissociation with $CaCl_2$ concentration at a fixed ATP concentration of 0.1 mM. The open and filled circles refer to two different actomyosin preparations: 2.8 mg/ml (●—●) and 3.9 mg/ml (○—○). Each point is the average of duplication or triplicate determinations. The curve, ◑—◑, was calculated from the assumption that the rate of dissociation is proportional to the concentration of CaATP using the stability constant of Khan and Martell (1966); 0.5 M KCl and 0.1 M Tris buffer, pH 7.5, at 15.2°.

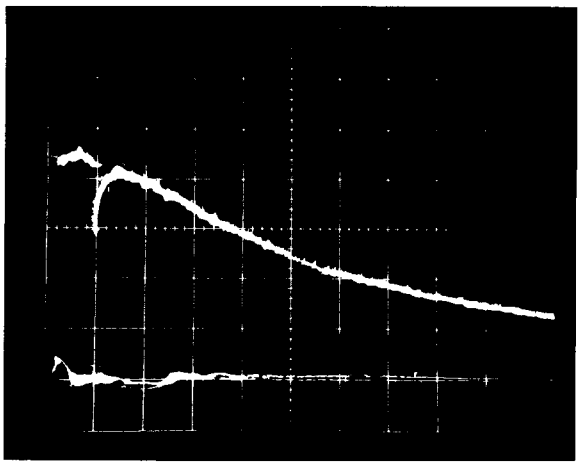

FIGURE 11: Oscilloscope pattern obtained for dissociation of actomyosin by CaATP at 0°. The lower trace is the piston displacement. The upper curve is the photomultiplier signal which shows an early lag phase: vertical axis 50 mV/major division, horizontal axis 20 msec/major division. The sharp drop in signal at about 8 msec after drive stops (or at about 20 msec in the figure) is a noise pulse; 2.4 mg/ml of actomyosin, 2 mM ATP, 21 mM $CaCl_2$, 0.09 M Tris buffer, and 0.5 M KCl, pH 8.0, at 0°.

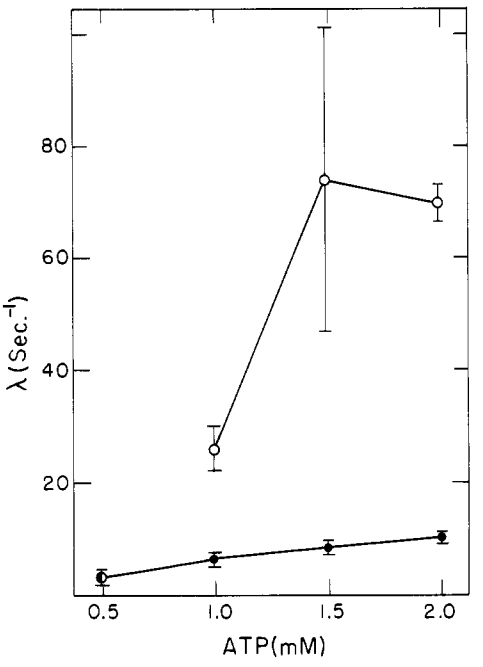

FIGURE 12: Variation of the rate constants with CaATP concentration for the turbidity change associated with the dissociation of actomyosin at 0°, 2.4 mg/ml of actomyosin, 0.5 M KCl, 21 mM $CaCl_2$, and 0.09 M Tris buffer, pH 8.0.

difficult to measure accurately and in view of the errors the apparent concentration dependence (Figure 12) may be an artifact. The value of k, if the data are approximated by a straight line, is the order of 10^5 M^{-1} sec^{-1}.

Recombination of Actomyosin. As the substrate concentration is reduced by hydrolysis the turbidity increases but does not return to its original value. The regain in turbidity averaged 80% of the drop brought about by substrate, which agreed with results obtained by Tonomura and Watanabe (1953). As the substrate concentration is lowered the maximum turbidity decrease is reduced. When the MgATP concentration is approximately equal to the molar concentration of myosin, which was 5–10 μM in these experiments, the decrease in turbidity was slightly less than one-half of the value obtained at high substrate concentrations. It was therefore possible to examine the process of recombination after a stoichiometric substrate pulse.

The regain of turbidity after a MgATP pulse fitted a single exponential process; a typical experiment, at 2.5 μM ATP and 10° is shown in Figure 13. λ_r, the rate constant for recombination, was measured from 0 to 30°, and ranged from 1.4×10^{-3} to 3.5×10^{-2} sec^{-1}. A plot of log λ_r *vs.* $1/T$ was linear, with an activation energy of approximately 19 kcal.

Recombination appears to be a first-order reaction since the constant λ_r did not depend upon actomyosin concentration. At 20° λ_r is 0.01 sec^{-1}, thus the half-life is about 70 sec. Since the system gives an early burst of inorganic phosphate (Imamura *et al.*, 1965), the half-life of free substrate is of the order of a few seconds and the slow recombination occurs in the absence of

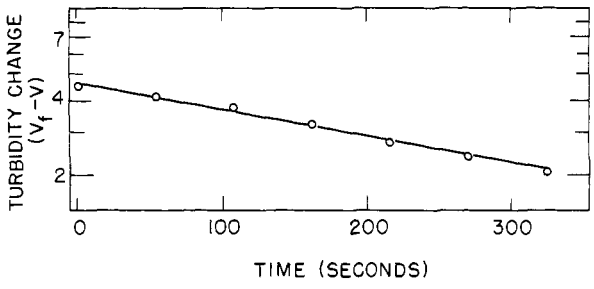

FIGURE 13: Semilog plot of the regain of turbidity during recombination of actin and myosin after a nearly stoichiometric pulse of ATP; 2.81 mg/ml of actomyosin, 2.5 μM ATP, 0.5 M KCl, 5 mM $MgCl_2$, and 0.09 M Tris buffer, pH 8.0, at 10°.

free substrate. The results should be compared with the half-life for formation of actomyosin, when the components are mixed at a total concentration of 3 mg/ml, which is about 1–2 sec for the second-order reaction (Figure 2).

Evaluation of λ_r after CaATP dissociation was difficult to carry out accurately because of the much larger steady-state rate of hydrolysis and the presence of a turbidity transient from calcium alone. At 10^{-5} M CaATP the turbidity change was only 10–15% of the maximum value; consequently, a stoichiometric substrate to myosin ratio could not be used. λ_r was evaluated during the final stage of recombination for various substrate concentrations. It depended slightly upon substrate concentration between 10^{-5} and 5×10^{-5} M but the average value was 0.2 sec^{-1} which is approximately equal to the steady-state rate of phosphate liberation.

Discussion and Conclusions

The change in turbidity which accompanies the formation and dissociation of the actomyosin complex has been used to explore the kinetics of the reaction. With the exception of the effect of CaATP at 0°, the turbidity change accompanying dissociation followed a simple exponential decay process for a variety of substrates and the rate constant was proportional to substrate concentration. The change in turbidity with time during the formation of the actomyosin complex followed the kinetics expected for a second-order reaction. Such simple kinetic behavior suggests that the myosin molecules are bound to independent sites. The simplest kinetic schemes are

$$AM + S \underset{}{\overset{k_1}{\rightleftharpoons}} AM{\cdot}S \xrightarrow{k_2} A + M{\cdot}S \qquad (1)$$

$$AM \underset{k_r}{\overset{k_d}{\rightleftharpoons}} A + M, \quad M + S \underset{}{\overset{k_1}{\rightleftharpoons}} M{\cdot}S \qquad (2)$$

The symbols A and AM refer to a free or occupied site on F-actin. In mechanism 1 substrate binds to myosin at a site different from the actin binding region and the substrate-myosin-complex has a low affinity for actin. The mechanism will lead to exponential decay if $k_2 \gg k_1[S]$. A second possibility is illustrated by mechanism 2. Substrate and actin compete for the same site but free myosin is trapped in one or more enzyme–substrate or enzyme–product complexes which block actin binding. Mechanism 2 could lead to exponential decay with rate constant proportional to substrate concentration if the rate-limiting step is substrate binding to free myosin.

The experimental data satisfied the equation $\Delta T = \Delta T_m(1 - e^{-\lambda_d t})$, where $\lambda_d = k[S]$ while either of the mechanisms considered would lead to $[AM] = [AM]_0 e^{-\lambda t}$. In order to identify the decay constant, λ_d, obtained from turbidity measurements with the constant λ, which refers to a single, independent site, it is necessary to solve the kinetic problem explicitly, taking into account that the contribution of a given molecule to the turbidity depends upon the number of sites occupied. An approximate treatment of the problem (Appendix) showed that dissociation of independent sites with rate constant λ would yield a decrease in turbidity which could be fitted approximately by a single exponential. However, the complete result includes the algebraic sum of three exponential terms with decay constants λ, 2λ, and 3λ. Consequently λ_d can be treated as an approximate measure of the rate constant for dissociation but the magnitude of all the constants presented in Table I may be in error by a factor of 2 (see Appendix). The condition which must be satisfied in order for mechanism 2 to yield a rate of decay proportional to substrate concentration is $k_d[AM]_0 = k_r[A]_0[M]_0 \gg k_1[S][M]_0$, where subscript zero refers to $t = 0$. $k_r[A]_0$ must be considerably greater than 100–200 sec^{-1} to account for the rate of dissociation by MgATP at high concentrations. The value of k_r was measured as 2×10^5 M^{-1} sec^{-1}, while the free actin concentration was certainly not greater than 10^{-5} M. Therefore $k_r[A]_0$ is less than 2 sec^{-1} and the condition cannot be satisfied.

Thus the kinetic evidence supports a mechanism for dissociation in which the affinity of myosin for actin is reduced by substrate binding at a site distinct from the actin site. A distinction between the substrate and actin sites was made by Barany (1959) based on the differential effect of sulfhydryl titration on binding and ATPase activity. Eisenberg and Moos (1968) have recently concluded that the affinity of the actin–heavy meromyosin complex is reduced by substrate binding, possibly at the hydrolytic site.

In all cases the rate constant for dissociation was proportional to substrate concentration, *i.e.*, $\lambda_d = k[S]$, where k has the dimensions of a second-order rate constant. The rate of dissociation did not appear to depend upon the structure of the purine or pyrimidine base, since similar values were obtained with CaATP, MgITP, MgGTP, and MgUTP (Table I). Data on ADP were not obtainable because of adenylate kinase contamination but the kinetic behavior of MgPP was similar to the nucleotide triphosphates although the rate was ten times smaller, and agreed with the value reported by Morita and Tonomura (1960) for the rate of turbidity change measured by light scattering.

MgATP differed from all the other nucleotide triphosphates in that the rate was ten times larger at the same concentration and temperature. With this substrate the rate exceeded 250 sec^{-1} at high concentrations and became too fast to measure in the apparatus.

The scheme shown in mechanism 1 is presumably an oversimplified description of the steps involved in dissociation. The simplest interpretation of the mechanism requires k to be the rate constant for substrate binding to myosin. However, the values given in Table I are in the range from 10^3 to 10^5 M^{-1} sec^{-1} which are decidedly low values for rates of formation of an enzyme–substrate complex. With MgATP as substrate, the finding of a fast proton-liberating step with a maximum rate of 100–200 sec^{-1} at a substrate concentration of 10^{-4} M requires a rate constant greater than 10^6 M^{-1} sec^{-1} for a site on the enzyme (Finlayson and Taylor, 1969). It could be argued that substrate is bound to a "dissociating site" rather than the hydrolytic site as postulated by Weber and Portzehl (1954) and Eisenberg and Moos (1965).

The low values could also be explained by introducing a two-state model for the actomyosin complex corresponding to the two states of myosin which may be necessary to explain the enzyme kinetics. It is worth noting that with MgATP a second apparent rate constant was found in the proton studies which is equal to the apparent rate constant for dissociation, *i.e.*, 2.5×10^5 M^{-1} sec^{-1} at 18.5° and 4×10^4 M^{-1} sec^{-1} at 0° compared with values of 1.6×10^5 and 6×10^4 M^{-1} sec^{-1}, respectively, from kinetics of dissociation. This agreement, if not fortuitous, may indicate that similar intermediate steps are common to both processes and the interpretation of the apparent rate constant would depend upon the kinetic model. The available evidence does not allow a choice to be made among possible kinetic schemes.

The recombination of actin and myosin after a stoichiometric pulse of MgATP was a first-order reaction with an apparent rate constant of the same magnitude as the rate constant for ATP hydrolysis by myosin. A reasonable interpretation is that recombination and ATP hydrolysis are both rate limited by the same first-order reaction, presumably the decay of an enzyme–phosphate or enzyme–ADP intermediate.

Appendix

Kinetics of Actomyosin Turbidity Change. Gergely and Kohler (1957) have presented an approximate treatment of the scattering of actomyosin solutions which accounts satisfactorily for the dependence of 90° scattering upon myosin content. We will employ the same approximations to treat the kinetic problem. The Rayleigh ratio is given by

$$R_\vartheta = K \sum_{j=0}^{n} P_\vartheta^{\,j} M_j^{\,2} X_j$$

M_j is the molecular weight of species j; $M_j = M_A + jM_m$, where M_A and M_m are the molecular weights of F-actin and myosin, respectively. $P_\vartheta^{\,j}$ is the particle scattering factor. It is assumed that all F-actin molecules have n binding sites and that $P_\vartheta^{\,j}$ decreases linearly with j, *i.e.*, $P_\vartheta^{\,j} = P_\vartheta^{\,A}(1 - bj)$, where $P_\vartheta^{\,A}$ is the scattering factor for F-actin. The molecular weight of actin is taken as 2.5×10^6, and the molecular weight per binding site as 10^5; consequently, $n = 25$. The molecular weight of myosin is 5×10^5. Thus R_ϑ can be expressed as

$$R_\vartheta = K_1 \sum_{j=0}^{n} (1 - bj)(1 + 0.2j)^2 X_j \qquad (1)$$

Gergely and Kohler assumed no interaction between binding sites thus X_j, the molar concentration of the species with j sites occupied, is given by a binomial distribution. With these assumptions the authors were able to predict the value of R_{90} *vs.* myosin content employing measured values for the scattering factors of actin and actomyosin.

To determine the change in R_ϑ with time it is necessary to evaluate the sums ΣX_j, ΣjX_j, Σj^2X_j, and Σj^3X_j as functions of time for a particular kinetic scheme. We considered two models for independent binding sites which reduced to an equation of the form

$$AM \xrightarrow{\lambda} A + M$$

where $\lambda = k[S]$ is the rate constant for dissociation at a single site. The equation for the rate of change of the species with $n - j$ sites occupied is $\dot{X}_{n-j} = -(n - j)\lambda X_{n-j} + (n - j + 1)\lambda X_{n-j+1}$. Beginning with the equation $\dot{X}_n = -n\lambda X_n$, the equations the X_n, X_{n-1}, etc., can be solved successively and for solution for the general term can be inferred.

$$X_j = (-1)^q \sum_{q=0}^{n-j} \left(\sum_{k=q}^{n-j} \frac{(j + k)!}{j!(k - q)!q!} \times X_{j+k}^0 \exp -(j + q)\lambda t \right)$$

where X_j^0 is the concentration of the j'th species at time zero. To evaluate

$$\sum_{j=0}^{n} X_j$$

we take the sums of the coefficients of exp(0), exp $-\lambda t$, exp $-2\lambda t$, etc., and obtain

$$\sum_{0}^{n} X_j = \sum_{0}^{n} X_j^0$$

as expected since this result expresses the conservation of F-actin. By the same procedure we obtain

$$\sum_{0}^{n} jX_j = \left(\sum_{0}^{n} jX_j^0 \right) \exp -\lambda t$$

$$\sum_{0}^{n} j^2 X_j = \left(\sum_{0}^{n} jX_j^0 \right) \exp -\lambda t + \left(\sum_{0}^{n} j(j - 1)X_j^0 \right) \exp -2\lambda t$$

$$\sum_0^n j^3 X_j = \left(\sum_0^n j X_j^0\right) \exp -\lambda t + \left(\sum_0^n 3j(j-1) X_j^0\right) \exp -2\lambda t + \left(\sum_0^n j(j-1)(j-2) X_j^0\right) \exp -3\lambda t$$

It is evident that substitution of these results into 1 will give for the time dependence of R_ϑ a sum of three exponential terms. In order to estimate the contributions of the three terms we evaluate the time dependence of the 90° scattering, ΔR_{90}, using the values for the scattering factors given by Gergely and Kohler. If the binding sites at $t = 0$ are saturated by myosin the sums $\Sigma j X_j^0$, $\Sigma j(j-1) X_j^0$, and $\Sigma j(j-1)(j-2) X_j^0$ are approximately $1:24:24 \times 23$.

$$1 - \frac{\Delta R_{90}}{\Delta R_{90\ \max}} = \frac{C_1 \exp -\lambda t + C_2 \exp -2\lambda t - C_3 \exp -3\lambda t}{C_1 + C_2 - C_3}$$

The coefficients are approximately $C_1 = \frac{1}{2}$, $C_2 = 1$, and $C_3 = \frac{1}{2}$. A plot of the logarithm of the $L{\cdot}H{\cdot}S$ *vs.* t gives a nearly linear relation with apparent rate constant of 1.2λ. The negative term, which arises because of the increase in P_ϑ as dissociation proceeds, tends to make the value of the apparent decay constant approach λ more closely. For example, omission of this term raises the apparent decay constant to 1.5λ.

To obtain the turbidity, eq 1 must be integrated over φ. The resulting equation will have the same form but a different value for the coefficient b. Thus the parameter λ_d determined from the experimental relation $\Delta T = \Delta T_{max}(1 - \exp \lambda_d t)$ will be an average value lying between the limits λ and 2λ. The scattering due to free myosin was not included in the equations but it is evident that it contributes a term in $\exp -\lambda t$.

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