THE EFFECT UPON ACTOMYOSIN OF STOICHIOMETRIC AMOUNTS OF ADENOSINETRIPHOSPHATE REGENERATED IN A COUPLED ENZYME SYSTEM*

By W. F. H. M. MOMMAERTS‡ AND JEAN HANSON§

(From the Department of Biochemistry, Western Reserve University School of Medicine, Cleveland)

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In this paper, we are reporting our first efforts to study coupled enzyme systems in which the common substrate, ATP,[1] is regenerated as it is used. One partner of the enzyme system is actomyosin, which hydrolyzes ATP to ADP and phosphate, and which undergoes a reversible physical change (here detected with the light-scattering method), to the extent that it is transitorily combined with the substrate. The other partner is pyruvate kinase, which regenerates ATP by phosphate transfer from phosphoenolpyruvate (PEP),[1] which serves as the reservoir substrate. At this preliminary stage, we have not accomplished a formal kinetic study of this multiple enzyme system. We restrict ourselves to a descriptive presentation of the main qualitative features.

Observations of this nature assume additional interest in view of the coupling effects that lead to the relaxation of contracted muscle models (3, 9, 11, 12), which differ from the present system in that the actomyosin compound occurs as a structurally organized entity, allowing specialized manifestations of activity.

Methods

Light-Scattering Measurements.—Originally, the physical condition of the actomyosin was followed through the course of an experiment by repeated determina-

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[1] The following abbreviations have been used in this paper: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; PEP, phosphoenolpyruvate.

tion of the apparent turbidity, τ' according to the method of Brice *et al.* (7).[2] Since the completion of the dual beam recording light-scattering photometer (Mommaerts (16)) all work has been done with that instrument. Its advantage is that it provides a continuous record linearly reflecting all changes to which τ' is subjected, without distortions due to the conservative absorption of light in the solution (16). The rectangular scattering cuvet, with an internal horizontal cross-section of 2 × 2 cm., contains 12 ml. of actomyosin solution to which small, measured amounts of ATP are added with a rapid mixing device (16). A cooling coil (painted dull black) has been attached to the inner endfaces of the photometer compartment facing the lamp housing and the power supplies; by means of circulating tapwater, the considerable heating otherwise occurring during prolonged experiments is effectively avoided, but no accurate constancy of temperature is claimed. A constant temperature mantle surrounding the cuvet provides accurate regulation of the temperature of the solution, but most experiments reported herein were done before this improvement was added.

Actomyosin.—This protein was prepared from rabbit muscle as in the preceding investigation (16), with a method aiming at the complete removal of myosin as well as of contaminating enzymes such as adenylate kinase. The protein, which was first clarified by centrifugation for 2 to 3 hours at 40,000 *g*, was added to a solvent consisting of 0.4 M KCl, 0.05 mixed buffer (succinate and tris-(hydroxymethyl) aminomethane, pH 6.9 or 7.4) to a final concentration of the order of 0.5 mg. per ml. The solvent was clarified by filtration through UF grade fritted glass filters immediately before use. Other solutions, employed in small volume only, were clarified by centrifugation or added as such.

Pyruvate Kinase.—This enzyme was initially prepared according to Kornberg and Pricer (10), but was later crystallized from rabbit muscle according to Beisenherz *et al.* (2), its solution being kept in the frozen condition for several months. Its activity is assayed according to Kornberg and Pricer (10) by means of PEP, ADP, reduced DPN, and lactic dehydrogenase (crystallized from rabbit muscle according to Beisenherz *et al.* (2), or from beef heart according to Meister (13)). The assay of the preparation used in the experiments here described was carried out according to Kornberg and Pricer (10)[3] giving the potency of the stock solution as 20 units per ml.

Adenosine triphosphate and other nucleotides are commercial preparations, further purified by paper electrophoresis in a citrate buffer of pH 4.0, and eluted from the paper with water. From the aqueous eluate, the nucleotide is adsorbed at weakly acid reaction with charcoal (acid-washed Darco, tested to have no hydrolyzing effects upon triphosphates) and eluted from the adsorbent with 50 per cent ethanol containing 0.001 M $KHCO_3$. The concentration of the final solution after evaporation of the alcohol *in vacuo* is determined by ultraviolet spectrophotometry after suitable dilution. The phosphoenol pyruvate used is a synthetic preparation of the

[2] The apparent turbidity τ is the turbidity value derived from measurement of the light scattered at 90°, without corrections for dissymmetry or depolarization (Mommaerts (15)).

[3] These determinations were performed by Mrs. Grace Huff.

Ba-Ag salt. This was converted into the Ba salt by decomposition with a small excess of HCl, filtration through a cation exchange resin in the K cycle, and reprecipitation as Ba salt. This was stored as such, and converted into the K salt in the same fashion. A stock solution of 10 mM concentration was kept in the frozen condition to serve a series of experiments.

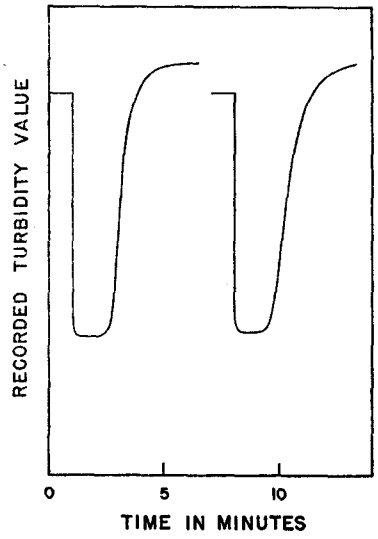

FIG. 1

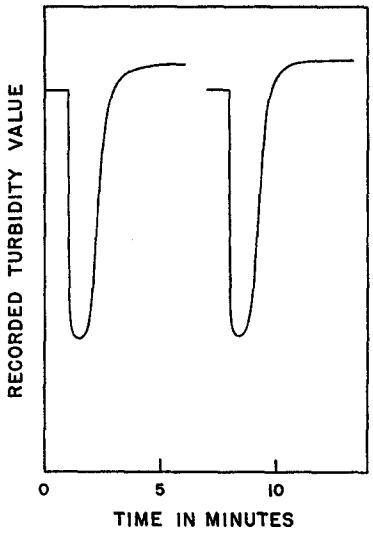

FIG. 2

FIG. 1. Turbidimetric response of actomyosin to ATP as such (first curve) and in the presence of pyruvate kinase (second curve). Actomyosin concentration 0.53 mg. protein per ml. in KCl-buffer medium, pH 6.9, with 0.002 M $MgCl_2$, with or without 0.2 ml. stock solution of pyruvate kinase. At zero time, addition of 0.048 μM of ATP.

FIG. 2. Turbidimetric response of actomyosin to ATP as such (first curve) and in the presence of 2 μM of PEP (second curve). Details of the experiment as in Fig. 1 except that it was performed at a slightly higher temperature.

RESULTS

Effect of ATP.—The response following the addition of ATP has been described in the preceding publication. Experiments described in Figs. 1 and 2 show that the effect of ATP in the presence of PEP does not differ from that in its absence, showing that actomyosin has no activity with respect to PEP or is not inhibited by it, and is not contaminated with pyruvate kinase; and that the reaction is likewise unaffected by pyruvate kinase.

Turbidity Response in Coupled System.—The effect of a trace amount of ATP is small and transient, the rapid splitting of the substrate causing a quick reversal. The changes are more persistent in the presence of Mg^{++} than in its

absence or, especially, than in the presence of Ca^{++}. In a system with Mg^{++} containing also PEP and pyruvate kinase, the response becomes persistent (Fig. 3). Its final reversal is due to exhaustion of the reservoir substrate, as can be demonstrated by the reestablishment of the full response upon renewed addition of PEP. Fairly large amounts of pyruvate kinase are necessary since, especially at pH 6.9, the enzyme works under suboptimal conditions. Correspondingly, no sustained effect is obtained in the absence of Mg^{++}, but appears promptly after adding this ion (Fig. 4). Under favorable conditions of pH and enzyme quantity, when a small response is maintained without

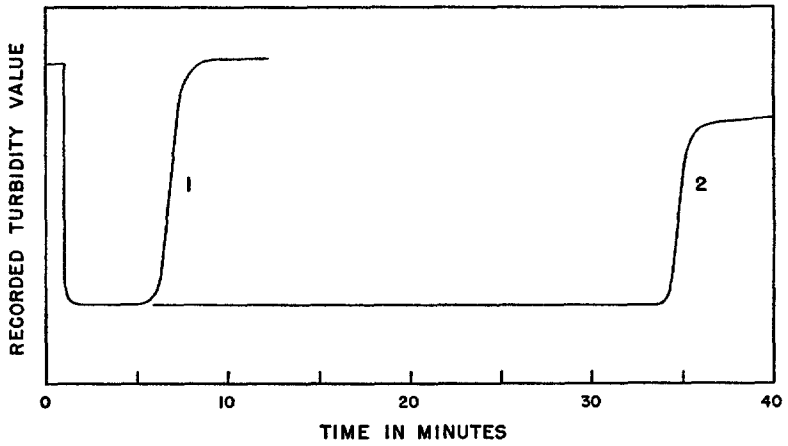

FIG. 3. Turbidimetric response of actomyosin to 0.096 μM of ATP as such (curve 1) and in the presence of 2 μM of PEP and 0.2 ml. pyruvate kinase (curve 2). Composition of medium as in Fig. 1.

Mg^{++}, addition of Ca^{++} abolishes this due to its activation of the ATPase (1, 17) and inhibition of the kinase (5, 6).

Recovery of Original Turbidity.—It depends on the conditions of the experiment whether, after the depletion of the reservoir substrate and the subsequent hydrolysis of ATP, the initial turbidity of the actomyosin becomes fully restored. Although the ATP levels are low, the times during which they can exert a depolymerizing effect upon the liberated F-actin are long, and Mg^{++} has already been described as promoting incomplete recovery (16). It is found that at pH 7.4, after maintaining the actomyosin in the dissociated condition for about 30 minutes, the turbidity is only about halfway reestablished. At pH 6.9, where the depolymerizing tendency is suppressed (16), the turbidity returns to approximately its original level or even somewhat above this. Thus it appears possible, by careful choice of the conditions, to keep actomyosin dissociated into its constituents for a prolonged time without marked alteration of the actin component.

Effect of Graded Amounts of ATP.—The continuous regeneration of ATP in the present system creates the possibility of measuring the effects of small amounts of ATP without complication due to its rapid breakdown. At pH 7.4, however, the complication occurs that no steady response is obtained, but that the turbidity continues to decrease for long periods of time. Frequently, no steady state level is reached at all, but the drop continues until it gradually changes back into the recovery part of the curve. Since such instances always show incomplete recovery of the original turbidity after exhaustion of the substrates, it is concluded that this drift is due to a progressive depolymeri-

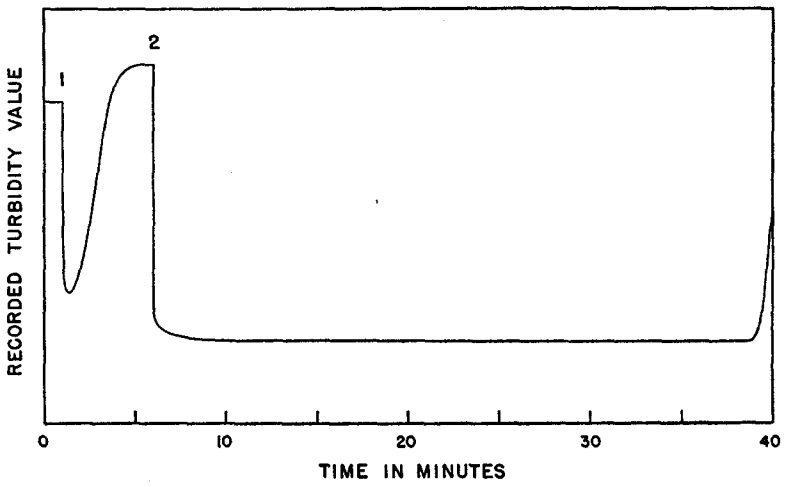

FIG. 4. Turbidimetric response of actomyosin to the addition of ATP in the multienzyme system (as in Fig. 3) first in the absence of Mg^{++} (1), then (at 2) after the addition of $MgCl_2$ to 0.002 M concentration.

zation of F-actin at this pH and Mg^{++} concentration. This case is, therefore, not suitable for further study.

At pH 6.9, however, this complication does not enter since the depolymerization is now suppressed. The stepwise response with increasing amounts of ATP is shown in Fig. 5. At low concentrations of the common substrate one deals not only with incomplete saturation of the actomyosin with ATP, but also with a lower saturation of the pyruvate kinase with ADP, which corrects itself to the speed requirements in the enzyme chain by leaving a greater fraction of the common substrate dephosphorylated. This can be overcome by adding more kinase until the response is not further increased (Fig. 7, curves 1 and 2). The experiment of Fig. 5 was performed with a sufficient excess of kinase to assure approximately complete rephosphorylation of the common substrate. When the fractional response of the actomyosin is plotted against the ATP concentration (Fig. 6) we obtain an ATP–actomyo-

sin dissociation curve of the type described earlier (14), but it is clear that the present methodology offers a means of obtaining such curves accurately whereas previous attempts only permitted a rough approximation. We shall deal with these dissociation curves at a later date when more quantitative measurements are available.

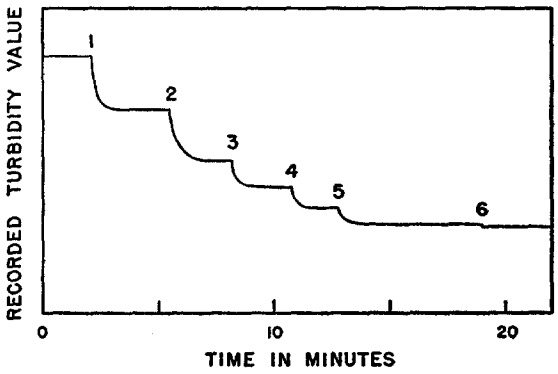

FIG. 5. Stepwise response of actomyosin to the graded addition of ATP to the multienzyme system at 0.01 M $MgCl_2$. Conditions as in the other figures, but with a larger excess, 1.0 ml., of pyruvate kinase. Total cumulative ATP amount present after each addition: curve 1, 0.0048; curve 2, 0.0096; curve 3, 0.0143; curve 4, 0.0238; curve 5, 0.0714; and curve 6, 0.167 μM.

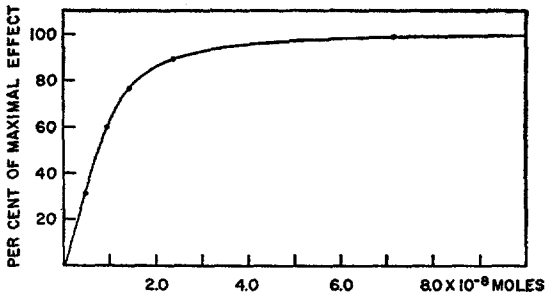

FIG. 6. Actomyosin—ATP dissociation curve derived from the partial responses in Fig. 5.

Breakdown of Phosphopyruvate When Actomyosin Is Only Partly Saturated with Substrate.—From elementary enzyme kinetic theory it is obvious that the total reaction velocity in the system should be decreased when the rate-limiting ATPase is working below its maximal velocity due to partial saturation with substrate. Attempts to confirm this expectation in the present system are illustrated in Fig. 7. Comparison of curves 1 and 2 shows that a sufficient amount of pyruvate kinase is required to keep the common substrate fully phosphorylated. This is true, of course, at all ATP levels. In each case, the

ADP concentration will adjust itself so as to maintain this rate, and it is to be expected (Dixon (8, p. 15)) that the concentration of ADP is inversely proportional to the pyruvate kinase concentration. This relation is borne out approximately by our measurements. However, the saturation of actomyosin with ATP is more sensitive to the pyruvate kinase level at low than at high ATP concentration since in the latter case the actomyosin saturation is not sensitive to the actual ATP level. Correspondingly, at high ATP concentration it is found that the magnitude of the turbidity response is hardly affected by

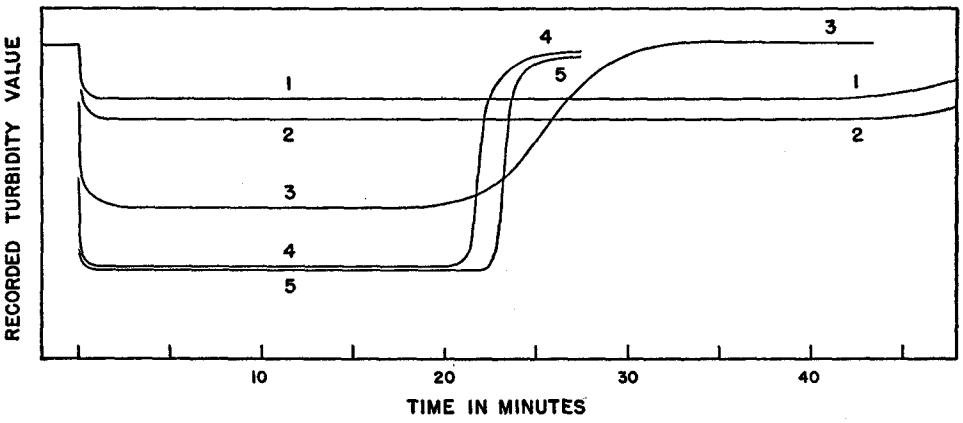

FIG. 7. Actomyosin response and breakdown of the reservoir substrate in the presence of graded amounts of ATP. In all experiments, 0.53 mg. actomyosin in 12 ml. medium, pH 6.9, 0.002 M $MgCl_2$, with 1 μM of PEP. Curve 1, 0.0096 μM of ATP with 0.2 ml. pyruvate kinase; curve 2, same with 0.5 ml. pyruvate kinase; curve 3, 0.0192 μM of ATP with 0.3 ml. pyruvate kinase; curves 4 and 5, with 0.25 ml. pyruvate kinase, 0.048 and 0.146 μM of ATP respectively. The difference in reaction time between curves 4 and 5 is approximately due to the extra ATP added; the other amounts of ATP are too small to be of stoichiometric importance.

changing the pyruvate kinase level unless this is greatly reduced. Comparing experiments (curves 2, 3, and 4) in which the actomyosin saturation is varied by changing the amount of ATP, one would expect the total reaction times to vary inversely with the actomyosin saturation; *i.e.*, with the extent of the turbidity drop. This expectation is approximately fulfilled in the range of low saturations (curves 2 and 3), but breaks down when the actomyosin saturation approaches completion (curves 3 and 4). It is to be stated that the absolute amount of ATP (except for curve 5) is in all cases so small that it does not significantly increase the total reservoir of high energy phosphate and cause a protraction of the response for that reason. A similar kinetic anomaly appears when the actomyosin saturation is changed by reducing the amount of pyruvate kinase (curves 1 and 2).

DISCUSSION

The significance of the quantitative study of coupled enzyme systems has recently been stressed by Dixon (8). The present work appears to be the first effort to study these phenomena in a system of known composition and has the added feature of enabling one to observe the degree of substrate saturation of the terminal enzyme by means of a continuous physical measurement.

In the present system, composed of an adenosinetriphosphatase, a common substrate (ATP), a rephosphorylating enzyme, and a reservoir substrate (PEP), the two enzymes are oppositely affected by the bivalent cations, Ca^{++} and Mg^{++}. In the range of compositions here employed, the maintenance of a finite ATP level in a stationary state is contingent on the presence of Mg^{++} ions. Experiments on the removal or addition of this obligatory activator (Fig. 4) may be illustrative of certain basic mechanisms in cellular function, in which a biologically active ion affects the steady state level of phosphorylation of a biologically potent metabolite, not by any influence upon equilibria, but by regulating the enzymes which determine its formation and breakdown.

The kinetic anomaly noted in those cases in which the terminal ATPase was partly saturated with substrate will require further study before being interpretable. Apart from other possibilities, it appears feasible that the combination between actomyosin (M) and ATP can proceed in two different ways (compare references 4 and 18) denoted by the following reaction equations, in which M*–ATP denotes an actomyosin–ATP complex which has undergone the usual physical change, whereas M–ATP is the same complex not physically altered:

$$\begin{array}{lcl} & & \text{M*–ATP} \rightarrow \text{M} + \text{ADP} + \text{P} \\ & \nearrow\swarrow & \uparrow\downarrow \\ \text{M} + \text{ATP} & & \\ & \searrow\nwarrow & \downarrow\uparrow \\ & & \text{M–ATP} \rightarrow \text{M} + \text{ADP} + \text{P} \end{array}$$

Unknown factors determining the ratio between M*–ATP and M–ATP might be responsible for the observed anomaly.

The complete exploration of the experimental possibilities offered by this system for the study of problems related to the mechanism of muscular contraction will require further investigations.

SUMMARY

Pyruvate kinase and phosphoenolpyruvate, added to actomyosin, cause a maintenance of the response of the actomyosin to stoichiometric amounts of ATP. This steady state maintenance depends on the presence of Mg ions.

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