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STUDIES ON THE SPECIAL PROPERTIES OF ACTOMYOSIN IN THE GEL FORM

II. AN ANALYSIS OF THE TURBIDITY CHANGES SEEN IN GEL SUSPENSIONS

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Summary

The turbidity changes induced by MgATP in suspensions of actomyosin gel particles have been studied systematically over a wide range of MgATP concentrations at different temperatures with and without the regulatory proteins An analysis of these changes distinguishes three separate protein-protein interactions in the gel

(1) The transient cyclic interactions between actin and myosin involved in the hydrolysis of MgATP and contraction

(2) Cross-links that cause turbidity in the original suspension.

(3) Cross-links that cause the high turbidity usually associated with superprecipitation.

In general, there was a good correlation between the half-time for reaching maximum turbidity during superprecipitation and the rate of hydrolysis. On the other hand, the actual magnitude of the turbidity increase was progressively diminished as the concentration of substrate was raised in the millimolar range. It appears that some essential phase of the superprecipitation process is limited by the same enzymatic step that limits the rate of hydrolysis. However, whether or not this phase leads to an increase in turbidity depends on the concentration of MgATP. Apparently, high physiological levels of MgATP (1—5 mM) inhibit the formation of the specific cross-links that cause high turbidity in the superprecipitate. It is proposed that MgATP interferes with these links

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Abbreviation EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid

when it can bind to a low-affinity site on myosin that is separate from the high-affinity active sites for hydrolysis. Thus, in contracting muscle, interfilament interactions analogous to those that increase turbidity and cause isodimensional shrinkage in the gel would be prevented by the high level of MgATP in the sarcoplasm. Observations relating the shortening of isolated myofibrils to their turbidity in suspension lend support to this interpretation.

Introduction

Since the discovery by Albert Szent-Gyorgyi [1] that actomyosin gels superprecipitate during the hydrolysis of MgATP, this phenomenon has been used in various forms as an in vitro model of muscle contraction. For example, H.H Weber and Portzehl [2] prepared filaments of actomyosin (partially dried to give the structure more resistance to tearing) and were able to measure the development of some tension when the threads superprecipitated and shortened. In many studies, superprecipitation has been observed and measured by changes in the appearance or the volume of the gel [3—5]. More recent studies have focused on the turbidity changes that occur during superprecipitation of actomyosin gel particles in suspension, because of the ease by which this can be measured [6—20]. In general, over the years, it has been established that superprecipitation occurs during actin-activated hydrolysis of MgATP by myosin, and that the conditions for this physical transformation are, for the most part, similar to those that support muscle contraction.

Yet, there is no direct indication that the primary contractile events in muscle or in model systems include reactions analogous to the obvious physical changes associated with superprecipitation. In particular, superprecipitation measured by a decrease in volume of the gel or an increase in turbidity of a gel suspension requires much lower concentrations of MgATP and lower ionic strength than are found in muscle. In the work reported here, we measured the turbidity changes induced by MgATP in suspensions of actomyosin gel particles under a variety of conditions. Records of the turbidity change with time were obtained at different temperatures, with and without the regulatory proteins in the gel, over a wide range of MgATP concentration. An analysis of the results indicates that there is a primary coupling between hydrolysis and an essential step in the process of superprecipitation, but that this leads to a secondary increase in turbidity only when the concentration of MgATP is relatively low.

Experimental Procedures

The experimental procedures and preparations used in these studies are described in the preceding paper [22].

Results and Discussion

In general, when MgATP is added to a mechanically stirred suspension of actomyosin particles, three different physical changes can be distinguished that alter the turbidity of the mixture.

1. Clearing of the initial gel The hydrated opalescent particles of the original suspension swell, and dissolve in the extreme, causing a fall in turbidity.

2. Superprecipitation. The original particles, or those cleared by reaction 1 above, can shrink to an opaque dense form, causing a large rise in turbidity.

3. Swelling of the superprecipitate. The superprecipitated particles formed by reaction 2 above, can swell (as observed in the phase contrast microscope), causing a fall in turbidity. Although this reaction and reaction 1 cause the turbidity to change in the same direction, it is important to our analysis that we make a sharp distinction between them, therefore, we will arbitrarily refer to the one as clearing and the other as swelling.

Figs. 1 and 2 show how the turbidity of an actomyosin gel suspension changed in response to various serial additions of ATP and Ca^{2+}. The reaction mixture contained Mg^{2+} (required for all the reactions) at 5 mM, and a low level of KCl, 25 mM. The sequence of additions was different for the various curves but the final conditions were the same for all. The curves in Fig. 1 are typical of many that have been obtained with many preparations of actomyosin in the course of various studies The particular records were chosen to demonstrate certain salient features of the system that are important to discussions later.

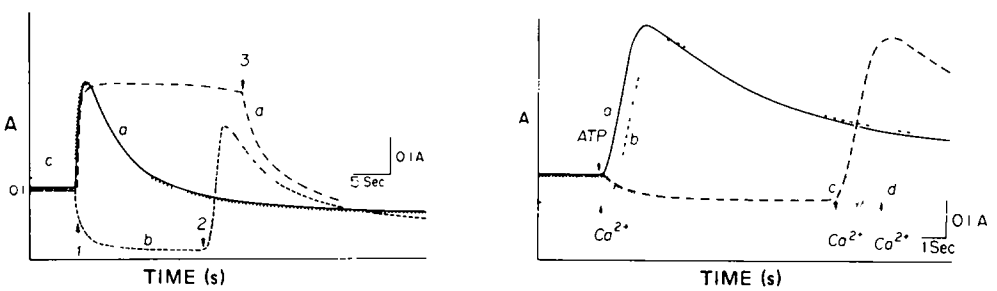


Fig 1 Records of turbidity change with the addition of Ca^{2+} and ATP in varying sequence (Curve a) With Ca^{2+} present, ATP was added at time 1. Superprecipitation proceeded rapidly, followed by clearing of the original gel turbidity and swelling of the superprecipitate (Curve b) With Ca^{2+} absent, ATP was added at time 1 In response, the gel cleared to a steady-state level When Ca^{2+} was then added at time 2, the protein superprecipitated and then swelled to the same end-point as in curve a (Curve c) A low level of MgATP (10^{-5} M) was present without Ca^{2+} prior to time approx 1, allowing a slow rise in turbidty (superprecipitation) At approx 1, Ca^{2+} together with the full complement of ATP was added and the turbidity then followed the same course as in curve a (Curve d) With Ca^{2+} present, a low (10^{-5} M) concentration of ATP was added to fully superprecipitate the gel, at this level of nucleotide no swelling of the superprecipitate occurred Thus the peak turbidity was maintained until the full complement of ATP was added at time 3, causing the superprecipitate to swell to the same end-point as in curve a The gel contained the regulatory proteins with or without 10^{-5} M Ca^{2+} as described above The normal complement of ATP gave a concentration in the reaction mixture of 2 5 10^{-3} M General conditions 25°C, 25 mM Tris-HCl (pH 7 4), 5 mM $MgCl_2$ and 25 mM KCl 0 1 mg gel/ml was used When Ca^{2+} was present, the mixture contained 1 mM CaEGTA and when Ca^{2+} was absent the mixture contained 1 mM EGTA in excess of Ca^{2+}

Fig 2 Typical records of turbidity change showing the independence of superprecipitation from clearing of the original gel (Curve a) Ca^{2+} was present in the reaction mixture when ATP was added to initiate superprecipitation In this case, the peak turbidity apparently includes some turbidity from the original gel (Curves b, c and d) Ca^{2+} was absent when the addition of ATP cleared the original gel Then, after different time periods in the cleared state, the addition of Ca^{2+} caused superprecipitation, followed by clearing to the same end-point In curve b, ATP was added 2 min prior to the zero time point shown

From records of the kind shown in Fig. 1, it has become evident that the final turbidity reading, no matter how simple or complex the approach to it, depended on the final conditions and was independent of the order in which Ca^{2+} and ATP were added. For example, in curve a of Fig. 1, Ca^{2+} was added to activate the regulatory system and then ATP was added to induce superprecipitation. In response, the turbidity rose sharply to a transient peak and then fell to the final steady-state level.

For comparison, in curve b the same amount of ATP was added first before the calcium. The initial effect of the substrate in the absence of calcium was to clear the starting gel to a lower steady-state value. Then when calcium was added to activate the system, the gel superprecipitated rapidly, with the turbidity (as in curve a) first rising to a peak and then settling to a final value. Although the peak value in curve b was lower than in curve a, because of the initial clearing of the gel, the final turbidity value was the same.

In curve c, this same final turbidity value was reached by still another pathway. In this case, a low level of ATP was added first. Under these conditions, even without calcium, there was a very slow rate of superprecipitation. Then, at the point where calcium was added to fully activate the system, rapid superprecipitation started and followed the same course as in the other two curves (a and b).

It was also possible to reach the same end-point by first superprecipitating the gel to a maximum turbidity with a low level of ATP that did not clear the gel or the superprecipitate. This could be done with one addition of sufficient ATP to complete the superprecipitation (curve d), or as we have previously shown [6,7], by the serial addition of smaller amounts causing the turbidity to rise in steps. Whatever the procedure for reaching maximum turbidity, the suspension could then be brought down to the final steady-state value by adding ATP, all at once or in steps to the final concentration. However, there were certain time limits on the reversibility of these changes. In particular, the protein tended to lock into a high-turbidity state on standing at low levels of MgATP or on being washed free of nucleotide, and swollen particles of superprecipitate tended to aggregate with time causing a slow downward drift in the turbidity. Since these phenomena were relatively slow, taking minutes, we were able to avoid or correct for them in our analysis of the system

It is often considered that an initial clearing of the gel suspension, or some microscopic counterpart of that phenomenon, might be a prerequisite for superprecipitation. This assumption stems from the fact that superprecipitation at moderate to high concentrations of MgATP, or high salt, can be preceded by a clearing phase [5]. However, turbidity records at low ionic strength do not support this interpretation. At 25°C and the low salt concentration of the experiments reported here, much higher concentrations of MgATP (1—5 mM) were required to clear the original gel than to saturate the active sites and attain the maximum rate and extent of superprecipitation. At low concentrations of MgATP, the turbidity of the starting gel appeared to persist after superprecipitation and contribute to the maximum turbidity reading. At intermediate levels of MgATP, gel clearing apparently occurred after superprecipitation coincident with swelling of the superprecipitate. Only at high levels of MgATP was any clearing evident prior to superprecipitation.

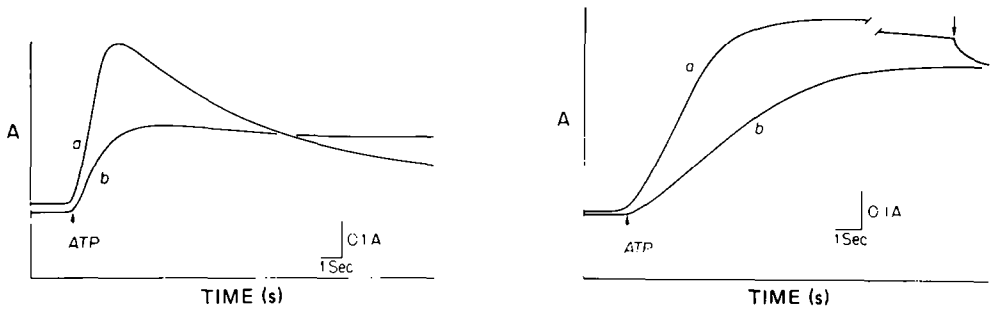


Fig 3 Turbidity changes in the actomyosin gel with and without the regulatory proteins (Curve a) The gel contained the regulatory protein complex with Ca^{2+} Superprecipitation was initiated by the addition of ATP at the time indicated (Curve b) Same as for curve a, except the regulatory proteins were absent General conditions were the same as for Fig 1, except ATP was at 1 25 10^{-3} M

Fig 4 Turbidity changes at 10° C with and without the regulatory proteins. (Curve a) The gel contained the regulatory protein complex with Ca^{2+} Superprecipitation was initiated by the addition of ATP at the time indicated. (Curve b) Same as for curve a, except the regulatory proteins were absent The general conditions were the same as for Fig 1 except the temperature was 10° C and the ATP concentration was 10^{-5} M The break in the top curve represents 1 min, at the upper arrow the record speed was reduced to one-eighth of the original Both curves came to the same end-point

Swelling of the superprecipitate, like clearing of the initial gel turbidity, required concentrations of MgATP well above those required to saturate the active sites for MgATP hydrolysis. When swelling was extensive, at concentrations of MgATP above 1 mM, it appeared on the turbidity records as a fall from a high transient peak to the final steady state, and both the peak and the final turbidity were lower the higher the level of MgATP.

The swelling of superprecipitate by high levels of MgATP is apparent in the records reported by Matsunaga and Noda [6]. They found a relatively low max-

TABLE I

EFFECT OF THE REGULATORY PROTEINS ON SUPERPRECIPITATION AS MEASURED BY CHANGES IN TURBIDITY

Values are duplicates taken from two different records

ATP concentration (mM)	Half-time to peak turbidity (s)		Peak turbidity		End turbidity	
	With TT	No TT	With TT	No TT	With TT	No TT
0 01	17, 19	19	45, 42 5	45 5	45, 42 5	45
0 025	6, 5	8	57, 53	49	54, 43	48
0 05	4, 3 5	5	57, 56	50	40, 39	41
0 1	5, 4	8	53, 51 5	44	33, 29 5	33
0 25	3 5, 4	7	54, 47	39 5	30, 29	29
0 5	3, 4	8 5	51 5, 50	36	27, 26	28
1 0	4, 6	8	46, 43 5	31 5	22, 22	27
2 5	7, 5	16	38, 37	26	14, 16	24
5 0	18, 20	*	24, 22 5	−6 **	14, 9 5	6

* Clearing and very slow rise

** Minimum value during clearing

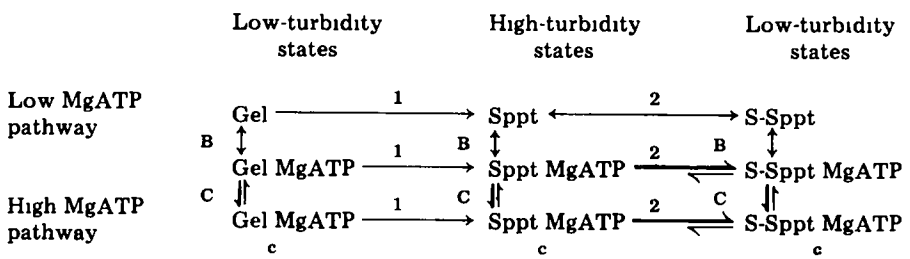
TT, the tropomyosin-troponin complex with Ca^{2+}

imum turbidity reading when superprecipitation took place in a high concentration of MgATP and suggested that the protein showing this was an intermediate form between the starting gel and high-turbidity superprecipitate formed in low salt.

When EGTA was added to the suspension of regulated actomyosin after it had reached a steady state in the presence of calcium, the turbidity fell to a new steady-state level, the effect was reversed by calcium in excess of the chelator. Apparently, the superprecipitate swells to a greater extent when calcium is removed from the regulatory protein. Maruyama and Kominz [7] some years ago presented records that indicated this influence of calcium on the turbidity of superprecipitate. They interpreted the effect of removing calcium as a partial reversal of the overall superprecipitation process.

The rate of superprecipitation slows markedly when the temperature is lowered. On the other hand, low temperature apparently favors clearing of the gel and swelling of the superprecipitate. The net result of these effects can be summarized by describing the changes apparent in the turbidity record when the temperature is set lower without changing the MgATP concentration. (a) Gel clearing becomes more apparent in the first part of the record; this is seen as an initial lag or even a fall in turbidity. (b) The rate of superprecipitation decreases, this is seen as a slower rise in turbidity (a longer $t_{1/2}$ to the peak turbidity. (c) The rate of superprecipitate swelling decreases; this is seen as a slower fall from the peak turbidity. (d) The extent of superprecipitate swelling increases, this is seen as a lower final steady state turbidity reading.

When the regulatory protein complex of tropomyosin-troponin with Ca^{2+} was added to the actomyosin gel, the effect depended on the level of substrate used. (a) At low levels of MgATP when only the high-affinity active sites were saturated, the tropomyosin-troponin-Ca^{2+} complex increased the rate of superprecipitation at all temperatures, (Fig. 3 and Table I) as we reported for 25°C [8]. (b) On the other hand, at far higher levels of MgATP (above 3 mM), especially at low temperatures, the tropomyosin-troponin-Ca^{2+} complex inhibited superprecipitation. When this occurred, it was associated with the substrate-inhibition of hydrolysis discussed in the preceding paper [22]. The turbidity changes observed in the gel suspension are summarized in Scheme I.



Scheme I

The MgATP indicated in this scheme is bound to a hypothetical low-affinity site; binding to the high-affinity active sites for hydrolysis is implicit. The reversible binding of MgATP to the low-affinity sites (reaction B) has two effects indicated by the thicker arrows it clears the turbidity present in the

original gel by reaction C and causes the superprecipitate to swell (S) and lose its turbidity by reaction 2. The final steady-state turbidity in this scheme is determined by the relative proportions of the various protein forms.

Thus, for example, at intermediate concentrations of MgATP, when the substrate is added to the gel, the suspension will partially lose its original turbidity (by reaction C), superprecipitate (by reaction 1) and then swell (by reaction 2) to a degree dependent on the substrate level. When reaction 1 is relatively fast, e.g at high temperature with the tropomyosin-troponin-Ca^{2+} complex, then clearing (C) will be masked by a rapid accumulation of superprecipitate (Sppt) (to a peak turbidity) followed by its swelling (to a final steady-state turbidity.) On the other hand, when reaction 1 is relatively slow, e.g at low temperature, then clearing by reaction C will be the first apparent effect of substrate, followed by the other reactions In general, heat markedly increases the rate of reaction 1; it also increases reaction 2, but to a far lesser extent. Therefore, high temperature records show a sharp transient peak reflecting the accumulation of Sppt. that subsequently swells. At low temperature, the turbidity often shows no peak but rises monotonically to the final steady state (Fig. 4). A transient peak in turbidity is also more evident in the presence of the regulatory proteins since these increase the rate of reaction 1 while decreasing the rate of reaction 2

Although gel clearing and superprecipitation are essentially separate reactions in this scheme, they are indirectly related because gel clearing is associated with swelling or dissolution of the original protein particles. This extension or disruption of the gel matrix decreases the effective actin concentration around the myosin active sites. Up to a point, even after the original turbidity has completely cleared, the swelling that occurs does not significantly inhibit hydrolysis or superprecipitation. However, beyond that point, with extensive expansion of the gel at high levels of MgATP, the rates of hydrolysis and superprecipitation fall as these processes become limited by the low effective concentration of actin in the reaction mixture

In general, despite the apparent complexities of the turbidity changes, there was a good correlation between the half-time for the turbidity increase associated with superprecipitation and the rate of hydrolysis. For example, in Table I although the peak turbidity and the final steady-state turbidity declined progressively as the concentration of MgATP was raised from 0 025—2.5 mM, the half-time for superprecipitation did not change significantly. Over this entire range, hydrolysis and superprecipitation occurred at their maximum rate. At low levels of ATP, where the active sites for hydrolysis are unsaturated, the rates of the two processes were correspondingly lower; the same was true at far higher levels of MgATP when substrate-inhibition occurred.

It seems most probable that some essential phase of superprecipitation that determines the half-time for the turbidity change is limited by the same step in the enzymatic pathway [22] that limits the rate of hydrolysis. However, whether or not this leads to an increase in turbidity depends on the level of MgATP.

At low salt concentration and low levels of MgATP that just saturate the high-affinity active sites for hydrolysis, the turbidity change associated with superprecipitation is maximal. Under these conditions, the actual rate of the

turbidity increase, i.e the absorbance change with time, can be related to the rate of hydrolysis [10,11]. However, under conditions of high salt and high MgATP that depress the extent of the turbidity increase, it is the half-time for the turbidity change that relates to hydrolysis, since this measure of the rate of superprecipitation is not affected by its extent Thus, in the work reported here, the minimum half-time for superprecipitation, even at high substrate levels, and the V for hydrolysis changed in parallel with temperature ($\triangle H^{\ddagger} \cong$ 25 kcal).

At 25°C, the apparent rate constant of about 2—3 s^{-1} for superprecipitation was close to the rate of approx 5 s^{-1} per myosin head for the actomyosin Mg^{2+}-ATPase during the burst phase of hydrolysis. This indicates that the coupling between the two processes was tight with one molecular event of superprecipitation occurring for every 2 or 3 molecules of MgATP hydrolyzed per myosin head. Our earlier kinetic data suggest that an essential step in superprecipitation cannot occur unless both heads on a single cross-bridge of the myosin filament have MgATP at their active site with at least one of them moving through the rate-limiting step of hydrolysis [9,10,18]. Apparently, the cross-bridge is not free to move in a way that allows superprecipitation unless MgATP binds to both heads of the myosin molecule at the same time.

It seems probable that some essential step that leads to superprecipitation is closely related to a primary step in the mechanism of contraction. However, the turbidity change that follows that step in vitro, when conditions allow, probably does not normally occur in muscle with its high level of MgATP. This interpretation gains some support from observations we have made on the shortening of myofibrils in relation to their turbidity in suspension. In general, it appears that the turbidity change associated with contraction of the myofibrils depends on whether or not the cross-section of the sarcomere shrinks or swells For example, at low ionic strength and low ATP, the activated myofibril not only shortens but also shrinks by side-to-side interactions of its filaments, the fibrils appear in the phase contrast microscope as small dense and separated spots or sticks. And associated with these changes, the turbidity increases and the suspension takes on a crystalline look that is strikingly similar to a suspension of superprecipitated particles at high turbidity. On the other hand, when the MgATP level is high (e.g. 3 mM) and the ionic strength is high (e.g. 0.1 M KCl) when conditions are closer to those that prevail in living muscle, then the activated myofibril shortens without side-to-side shrinkage; instead, its cross-section swells and this is associated with a decrease in turbidity. Interestingly, the appearance of the short and swollen myofibrils in the phase microscope resembles the small clear bubble-like elements that result when superprecipitated actomyosin particles swell at high levels of MgATP.

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