Immunology 1976 **30** 419

Antibody to *Physarum* myosin

I. PREPARATION AND FUNCTIONAL EFFECTS

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Received 30 *July* 1975; *accepted for publication* 26 *September* 1975

Summary. Preparation of antibody to *Physarum* myosin is described, and evidence is presented that the antibody is specific for this molecule. A diffusion coefficient of 1×10^{-7} cm^2/s is estimated. The antibody interfered with myosin enzyme activity and with superprecipitation of actomyosin. It did not cross-react with rabbit striated muscle myosin.

INTRODUCTION

Specific antibody has been widely used for the localization of proteins within cells and, in the study of striated muscle, this has proved to be a very valuable approach (Pepe, 1966, 1967; Lowey and Steiner, 1972). It has become evident that many eukaryotic cells contain proteins analogous to actin, myosin, and tropomyosin of muscle. The use of antibody techniques for study of these proteins is already yielding valuable data. However, it is important that these techniques be used critically, since the presence in an antigen of small amounts of a highly immunogenic impurity can result in an antibody directed primarily against the impurity rather than the designated antigen.

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We wanted to use antibody directed against *Physarum* myosin to study its concentration and functional effects within the organism, and its cross-reactivity with myosins of related species. To do this, it is first necessary to be certain that the antibody is directed against the antigen alone. In this paper we document the specificity of the antibody by several criteria, and show that it can have functional effects *in vitro*. Elsewhere (Kessler, Nachmias and Loewy, 1976) we use this antibody in conjunction with a quantitative complement-fixation technique. We there estimate the actual myosin–actin ratio in the plasmodia and also examine the immunological relationship between the myosins of several species of myxomycetes, providing evidence for non-conservation of a number of myosin antigenic sites.

MATERIALS AND METHODS

Organism

Physarum polycephalum, a strain originating in the laboratory of Dr H. P. Rusch, McArdle Laboratory, University of Wisconsin, was grown as microplasmodia at 22° in semi-defined sterile medium (Daniel and Rusch, 1961; Daniel and Baldwin, 1964) and plated out after 3 days' growth onto pre-

sterilized rolled oats. Migrating large plasmodia free of slime were collected from plastic wrap after several days and were used as the source for the myosin.

Actomysin

A twice-reprecipitated actomyosin was prepared by a modification (Nachmias, 1974) of the method of Hatano and Tazawa (1968).

Myosin

The *Physarum* myosin used for the preparation of specific antibody was prepared from two fractions obtained from the actomyosin. These are termed myosin supernatant fractions and myosin-enriched actomyosin (MEAM) fractions. Both fractions were further purified by gel filtration. The procedure was as follows: the actomyosin was dissociated with 5 mM magnesium adenosine triphosphate in 0·5 M KCl, 0·05 M imidazole pH 7, and centrifuged for 2 h at 100,000 *g*. The pellet was discarded. The supernate was dialysed against 100 volumes of 0·05 M KCl, 0·01 M imidazole pH 7 containing 0·1 mM dithiothreitol (DTT). The white flocculent precipitate was myosin-enriched actomysin (MEAM) which was collected at 10,000 *g* for 15 min. The supernate was crude myosin left in solution, which contains several bands on SDS gels.

The crude myosin was fractionated directly on Agarose 4B as previously described (Nachmias, 1972) or the MEAM precipitate was redissolved in 0·5 M KCl, 0·05 M imidazole, pH 7, 0·1 mM DTT, but this time containing 2·5 mM magnesium pyrophosphate and fractionated on Agarose 4B pre-equilibrated with and eluted with this solvent. After column purification the crude myosin fraction resulted in 100–500 μg protein per original 100 g myxomycete. On 10 per cent SDS gels, only a single slowly moving band was seen (Nachmias, 1972). The gel filtration of the MEAM fraction gave a higher yield; several milligrams of protein appeared in the ATPase peak of these columns, but often significant amounts of actin contamination could be seen by electron microscopy (filaments) or on gels. The amount of actin contamination was variable. Other contaminating material was not, however, seen on SDS gels. The fractions used for antibody production were therefore free of material expected to be active in antibody production, since native actin has been found to be relatively non-immunogenic. As will be seen below, the use of one fraction for the initial injection and the other for the booster may also have helped to minimize antibody production by minor amounts of any contaminating proteins.

Antibody production and analysis

Four rabbits were immunized. Two were injected with 50 μg each of myosin protein peak from myosin supernatant column and then boosted 4 weeks later with 135 μg each of the peak fraction containing myosin from a column purification of the MEAM. Two other rabbits were treated in reverse order: injected first with myosin purified from MEAM and boosted with myosin supernatant column material. All injections were into the foot-pad with Freund's complete adjuvant. The first two rabbits gave the highest titres and were used for the majority of experiments. Bleedings were taken 2–4 weeks after the booster dose. Titres were routinely run using one-dimensional double diffusion in agar (Preer, 1956; Finger, 1971) in agar-coated glass tubing of 1·7 mm i.d. Ouchterlony, or double diffusion in two dimensions was carried out using standard methods but with a modified LKB instrument (Finger and Heller, 1962). As it was not convenient to make myosin routinely, we frequently tested the antibody against actomyosin or myosin-enriched actomyosin. The agar was then made up for 'dissociating conditions' and 2 mM magnesium pyrophosphate with 0·5 mM EGTA was incorporated into the agar.

Adenosine triphosphatase

Assays were run on column effluents in 0·5 M KCl by a semi-micro version as previously described (Nachmias, 1974).

Spectrophotometry

Absorbance of column fractions was measured in a Gilford spectrophotometer at 320, 280 and 260 nm.

Superprecipitation assays

These were carried out by rapidly diluting actomyosin 9-fold from a high salt buffer (0·5 M KCl, 0·05 M imidazole, pH 7) to low salt (0·055 M KCl, 0·005 M imidazole), pre-equilibrating the suspension at room temperature in cuvettes for 5 min and then starting the reaction with a small volume of 10 mM MgATP (pre-neutralized) so that the final concentration was of the order of $1–4 \times 10^{-4}$ M MgATP. After rapid mixing, the optical density at 600 nm

was recorded at 30-s intervals. For experimental cuvettes, actomyosin was pre-mixed with pre-immune or immune serum before dilution.

RESULTS

Evidence that the antibody is directed against myosin

Several experiments were performed using double diffusion techniques. First, one-dimensional double diffusion in agar (Preer method) was run using the myosin supernatant fraction as antigen. In all the runs, only one band appeared as a distinct precipitin line, with the exception of a single early run which showed a second, diffuse, rapidly moving band which was not seen in any of the later runs. Secondly, double diffusion was run against crude actomyosin or against MEAM. In such cases, no bands were seen unless magnesium pyrophosphate was included in the agar, in which case one band again appeared whose position was shifted with dilution of antibody or antigen (Figs 1 and 2). The MEAM or actomyosin used for these runs contains several polypeptide bands on gels, so that a number of proteins are present in such preparations. Fig. 3 shows an SDS gel of an MEAM preparation; the actomyosin contains additional bands. Thirdly, when double diffusion was run on a series of fractions from an HP column, the position of the precipitin band shifted with antibody dilution and with fraction number (Fig. 4) to indicate that the peak ATPase and 280 nm optical density, coincided with the

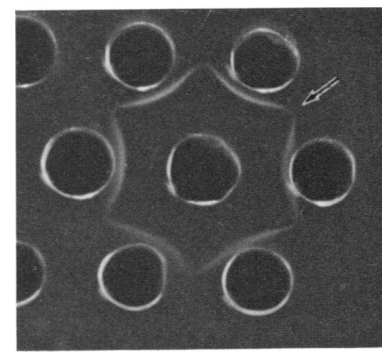

Figure 2. Two-dimensional double diffusion of KR3 antibody (in centre well) and *Physarum* myosin-enriched actomyosin (in outer wells). Starting at the arrow and going counter-clockwise the outer wells are: two wells with undiluted antigen; two with 1:2 dilution of antigen, two with 1:4 dilution of antigen. In this case, the position of the precipitin line moves toward the antigen wells as antigen is diluted. This agar contains 2 mM magnesium pyrophosphate and 1 mM EGTA.

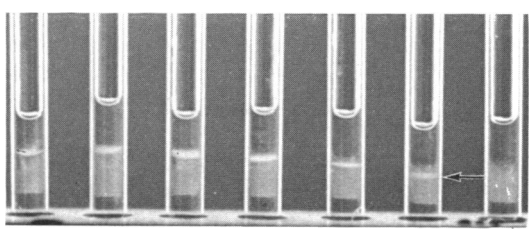

Figure 1. One-dimensional double-diffusion of KR3 antibody (in lower part of tube) and *Physarum* crude myosin supernatant (in upper part of tube). Reading from left to right the antibody is diluted 2-fold in each tube. The position of the single precipitin line moves toward the antibody to a point above midposition as expected from a high molecular weight antigen. Arrow marks the position of the final dilution (1:27) of antibody giving a visible precipitate.

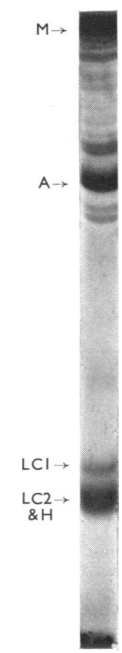

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Figure 3. Polyacrylamide gel in 12 per cent sodium dodecyl sulphate of *Physarum* myosin-enriched actomyosin. H denoted position of marker haemoglobin run with the sample. This comigrates with the second light chain of myosin. M = myosin heavy chain; A = actin; LC1 = heavier light chain of *Physarum* myosin; LC2 = lighter light chain of the myosin.

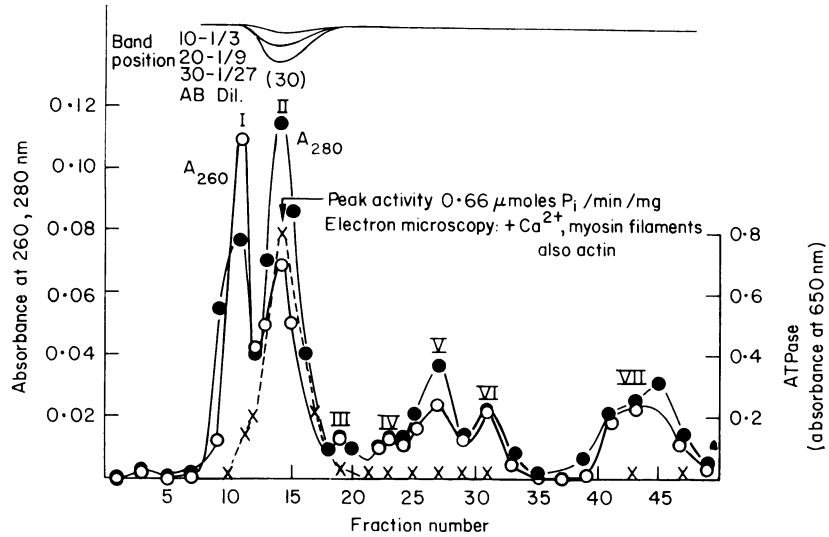

Figure 4. Agarose 4-B column run under dissociating conditions. Voided peak (I) shows a high 260/280 ratio and little ATPase activity. Peak II shows coincidence of ATPase peak activity with a 280/260 peak of a ratio about 1·6. Fractions from this peak showed maximal migration in Preer tubes as in Fig. 1 at antibody dilution of 1/27. All other fractions tested, including the void fraction, gave no detectable precipitin lines, as indicated by level bar at the top of the graph. The peak fraction also formed 0·5 μm long bipolar filaments in the presence of 10^{-4} M calcium chloride at low ionic strength. Actin filaments were detectable in the peak fraction by electron microscopy.

greatest concentration of antigen. Fourthly, the behaviour of the precipitin band as a function of concentration of antigen and as a function of time showed that the antigen was a large molecule with a low diffusion coefficient. The rate of change of band position with time was very slow, but examination over several days showed that the maximum position of migration of the precipitin band from the antigen position was 35 per cent, when equivalence ratios were used. This was estimated with a millimetre rule and a low power microscope. If one calculates an approximate diffusion coefficient for myosin from the band position at this approach to equivalence from the formula (Preer, 1956):

$$\left[\frac{X_{Ab}}{X_{Ag}}\right]^2 = \left[\frac{D_{Ab}}{D_{Ag}}\right], \text{ or } \frac{D_{Ag}}{\sqrt{D_{Ag}}+\sqrt{D_{Ab}}} = P$$

where X_{Ag} and X_{Ab} are the distance of the precipitate to the respective interfaces with antigen or antibody, D_{Ab} and D_{Ag} are the diffusion coefficients respectively of antibody and antigen and P is the position of the band as a ratio of the distance from the top of the agar to the band divided by the length of the agar $\times 100$.

Thus 35 per cent gives a D_{Ag} of $1{\cdot}04 \times 10^{-7}$ cm²/s assuming a D_{Ab} of $3{\cdot}5 \times 10^{-7}$ cm²/s. This agrees reasonably well with the estimate of Adelman and Taylor (1969) of $1{\cdot}26 \times 10^{-7}$ cm²/s for *Physarum* myosin, which would give a band position at equivalence of 37 per cent. Muscle myosin, with a diffusion coefficient of $1{\cdot}12 \times 10^{-7}$ cm²/s would give a band at 35·8 per cent. Band positions between 35 and 37 per cent cannot be differentiated by this technique; however, what can be said with confidence is that the antigen behaves as if it were a slowly diffusing molecule with a diffusion coefficient close to that of muscle myosin.

Fifthly, for evidence that antibodies to other antigens were unlikely to be present in our antiserum, we obtained no bands when the crude actin pellet (obtained by centrifuging actomyosin under dissociating conditions at 100,000 *g*) was tested. No bands appeared in double diffusion when rabbit striated muscle myosin was run against two different antisera.

Sixthly, we compared the behaviour of actomyosin with that of myosin-enriched actomyosin as antigens. We found that for half the protein concentration the myosin-enriched fractions showed a greater content of antigen. As an example, the precipitin band for the myosin-enriched fraction at 5 mg/ml formed at 5 per cent into the agar at 1:16 dilution,

while actomyosin at 9 mg/ml formed a precipitin line at or above the meniscus at the same dilution. Through all the dilutions this relative difference in position was maintained.

Functional effects

The antisera had a definite inhibitory effect on myosin ATPase activity at high ionic strength.

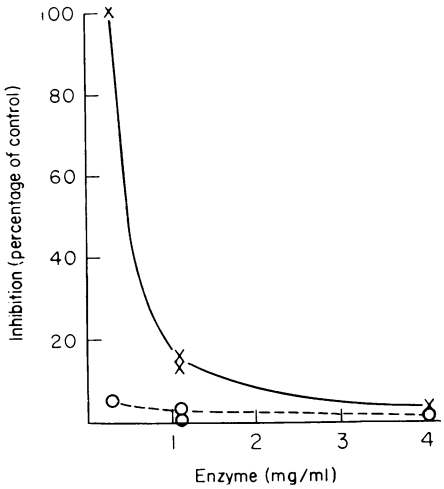

Figure 5. Inhibition of myosin K^+, Ca^{2+}-activated ATPase by immune serum KR4-1. After 10 min pre-incubation of enzyme in 0·5 M KCl buffered with 1/10 volume of antiserum, the reaction was run at room temperature as described in the Materials and Methods section. The estimated *in vivo* myosin level (arrow) is calculated from Kessler, Nachmias and Loewy (1976). (×) Immune serum; (○) pre-immune serum.

With the most active serum, the effect was strong enough at 1/10 dilution to inhibit about 60 per cent of the ATPase activity at the *in vivo* concentration estimated (Kessler *et al.*, 1976) to occur in the plasmodium (Fig. 5). The antisera also had striking but different effects on the course of superprecipitation (Figs 6 and 7). One antiserum caused rapid clumping of the actomyosin following the addition of ATP (Fig. 6) while the other delayed the course of absorbance increase (Fig. 7).

DISCUSSION

We believe that the several critical tests used here establish two facts: (1) that the antibody contains

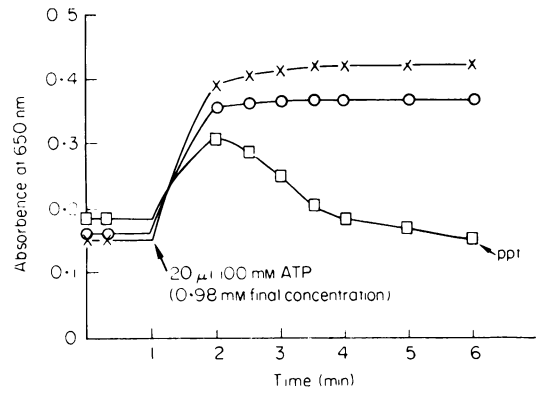

Figure 6. Effect of immune serum KR4-1 on superprecipitation of *Physarum* actomyosin. The actomyosin (0·3 mg/ml) was mixed in buffer at low ionic strength (0·05 M KCl) with pre-immune or immune serum diluted 1:100. After 2 min pre-incubation, superprecipitation was started with MgATP. (×) Control; (○) pre-immune serum; (□) immune serum.

combining sites directed against *Physarum* myosin; (2) that it does not contain detectable combining sites against any other components of crude actomyosin. In addition to the evidence for purity from double diffusion, the effects on myosin's enzymatic activity make it unlikely that the antibody could be

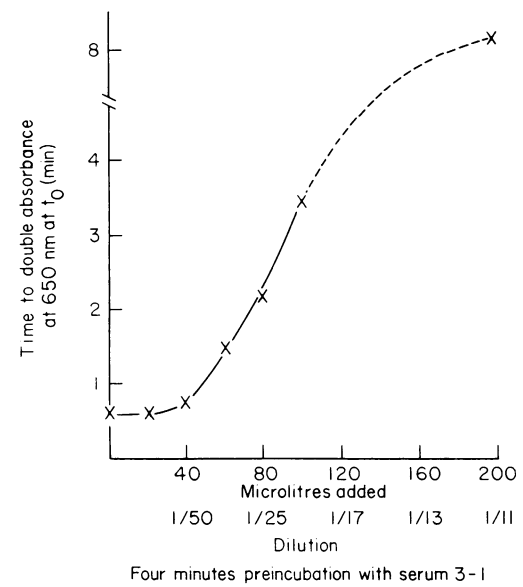

Figure 7. Effect of immune serum KR3-1 on superprecipitation of *Physarum* actomyosin. Different amounts of serum were pre-incubated for 4 min with the actomyosin in 0·05 M KCl before the run.

directed against an adhering contaminant. Finally, the estimate of the diffusion coefficient of the antigen shows that it must be a large slowly diffusing molecule. The antibodies can therefore be justifiably used as cytochemical probes for myosin.

We might conjecture that the different effects of the antisera on the course of superprecipitation reflect some differences in the number of antibodies directed against antigenic sites in head and tail portions of this myosin. Thus an antibody which reacts with head primarily may delay superprecipitation by affecting the enzymatic site. Another population of antibodies which react as well with tail as with head portions of the myosin molecule may alter the course of the reaction so that the actomyosin no longer remains in suspension but precipitates rapidly. The functional results, then, show that with some antisera sites on myosin against which the antibody is directed are close to enzymatic sites and therefore open up the possibility that the antibody approach may be used to affect actomyosin interaction *in vivo*. It is remarkable that the physical form of the *Physarum* myosin molecule, indicated here by the diffusion of the antigen and in agreement with other observations on the shape of the molecule and its ability to form filaments (Adalman and Taylor, 1969; Nachmias, 1974) is so remarkably similar to muscle myosins, although the evolutionary distance is so great, and although the actions of one are manifest in cytoplasmic streaming and the other in contraction in a linear array.

ACKNOWLEDGMENTS

This work was supported by grants numbered AM-17492 and AI-08732 from the National Institutes of Health, U.S. Public Health Service. We thank Dr Irving Finger for advice on several aspects of the immunology. Ms Joanna Sloane helped with careful technical assistance.

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