The crystallisation of myosin and some of its properties and reactions.

by

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It has been shown in the previous papers of this laboratory (1. 2.) that the contractile substance of striated muscle is „actomyosin", a compound of two proteins: actin and myosin.

The methods, hitherto employed by different authors for the preparation of „myosin" always yield a myosin more or less heavily (1—3%) contaminated with actin. All the data of literature relate to such undefined mixtures. Since even small traces of actin greatly modify the properties and reactions of myosin it seemed desirable to obtain myosin free of actin.

In this paper I will describe the preparation of actin-free myosin, its crystallisation, will describe some of its properties and reactions.

The preparation of myosin.

In all our experiments only chemicals of high purity were employed. The distilled water was redistilled from glass vessels. As material the striated muscle of the rabbit was used.

The animal was killed by decapitation, quickly skinned, eviscerated and dipped into ice-water. After a few minutes the body-muscles were cut out and packed between ice. Then they were minced on a cooled meat mincer with a sieve plate of holes of 2 mm diameter.

Principle. The separation of myosin from actin is based

on the observation that ATP will precipitate, in presence of different salt concentrations, actomyosins of different composition. The higher the salt concentration the less myosin is taken down by a given amount of actin but at the same time the less complete the precipitation. We have to work at the salt concentration at which the precipitation is fairly complete and not too much myosin is taken down by the actin. So for instance, in presence of 0,05 *M* KCl and at pH 6,5 a 0,5 % actomyosin will precipitate as such, that will say that one part of actin will take down with it 200 parts of myosin. In presence of 0,1 *M* KCl a 1,5 % actomyosin will be precipitated from the same solution and one part of actin will take down only 66 parts of myosin. Above 0,2 *M* KCl the precipitation becomes incomplete. The separation of actin involves always the loss of a relatively great quantity of myosin. It is thus essential to start with myosin of possibly little activity.

This precipitation with ATP will not yield a completely actin-free preparation (0.1 % actomyosin). The muscle extract seems to contain also some inactive actomyosin which is not precipitated by ATP. This actomyosin can be precipitated by diluting the KCl to 0.04 *M* at a slightly alkaline reaction. Myosin is soluble, actomyosin insoluble in this solvent. The relative loss in myosin in still bigger than in the case of the ATP precipitation and still more myosin is taken down by the same amount of actin. This method can thus be used only with extracts which have been rendered very poor in actin by the preceding ATP-precipitation.

Myosin cannot be freed from actin by crystallisation since actomyosin behaves as an individual substance. As shown by F. GUBA (oral comm.) even a 2,5% actomyosin can be brought to crystallisation as such.

First step. The muscle is suspended in the ice-cold KCl-Phosphate mixture of GUBA and STRAUB which contains 0,3 *M* KCl and 0,15 *M* K-phosphate of pH 6,5. The muscle is extracted for ten minutes under constant stirring and then centrifuged at 0°. The precipitate is discarded, the fluid diluted with four volumes of water of room temperature (22°) and stirred gently. After one or two hours suddenly a flocculent precipitate is formed. What happened was that the ATP present was used up to such an extent that it caused no

more a dissolution but a precipitation of the actomyosin. (If this precipitation takes place without stirring a very fine colloidal precipitate is formed which cannot be separated on the centrifuge.)

Myosin is rather sensitive to heat and even short (10 min.) incubation at 37° causes a rise of its viscosity and partial loss of its enzymic activity. Therefore it is important to work at low temperature. Unfortunately this is not possible throughout, for in two of the steps the precipitaion would be incomplete at 0°. Fortunately myosin is stabilized by ATP, as observed by ENGELHARDT and LJUBIMOWA (3) and corroborated by myself. In the first steps of our preparation the myosin is protected by the ATP present. The fluid contains even at the moment of the precipitation some ATP.

The precipitate thus formed is separated by rapid centrifugation at room temperature and the opalescent fluid is diluted with 1,5 vols. of ice-cold water. This water is run in slowly, in about 10 minutes under constant energetic stirring. The myosin separates in the form of fine, needle-shaped crystals. If the water is added suddenly and without stirring an amorphous precipitate is obtained.

The fluid is allowed to stand for an hour or two at 0°, decanted and the myosin separated on the centrifuge.

If necessary this crystalline precipitate can be washed by suspending it in 0,04 *M* KCl and separating the myosin again on the centrifuge.

Example: 357 g minced muscle. Extract 750 ml. Contains 7 g 0,35% actomyosin. The first precipitate contains *2* g 1,5% actomyosin. The final myosin precipitate contains 3,4 g myosin. Thus only about ½ of the myosin present in the extract is isolated. The other half is lost as actomyosin or is left behind in the fluid.

Second step. The crystalline myosin precipitate of step 1 is dissolved in a 0,02 *M* K_2CO_3 containing 10 ml of 1% alcoholic phenolphthalein in every liter. We add the carbonate solution in small quantities and homogenise carefully with strong stirring. Carbonate is added till the fluid retains a faint rose colour (pH 8,3) and add for every g of myosin present 4 ml of *2 M* KCl. Then we dilute with water adding 50 ml for every ml of KCl solution. This water is of room tempera-

ture (22°) and contains 0,001% phenolphtalein and sufficient K_2CO_3 to give it a faint rose colour. The water is added under strong stirring. A voluminous, loose precipitate is formed which is separated on the centrifuge. The faint rose coloured opalescent fluid is poured off and cooled. The precipitate is treated once more in the above way, *i. e.* if its colour has faded out we restore it by adding K_2CO_3, then we add KCl and finally water and centrifuge, the only difference being that this second time we add only half as much KCl and water as the first time. The precipitate is discarded and the fluids united. From this point on the preparation is continued at 0°.

The fluid is stirred energetically and 1% acetic acid is run in very slowly till the fluid is neutralised. The myosin separates in the form of somewhat irregular needles and is centrifuged.

Recrystallisation.

The precipitate is dissolved by adding 2 *M* KCl in small kuantitites. The fluid is carefully homogenised after each addition. KCl is added till the concentration of the KCl reaches 0.6 *M*. Then we dilute further with 0,6 *M* KCl till the fluid loses its very high viscosity and contains about 3 % myosin.

The myosin solution is stirred very energetically and water is run in very slowly till the KCl concentration drops to 0,04 *M*. The addition of this amount of water should take about one hour. The myosin separates in the form of beautiful needleshaped crystals. About half of the myosin isolated in the first step will be obtained in this form. This myosin contains no actin or only negligible traces of it (0—0,02 %).

Some properties and reactions of myosin.

Myosin crystallises in the form of very fine needles (Fig. 1.) with a strong tendency of lateral association. Needles, observed with high power, will often be found to be a bundle of finer needles. Sometimes the needles associate into fine, long threads, fibrils (Fig. 2.).

Analysis. Recrystallised myosin was precipitated with alcohol and extracted with boiling abs. alcohol for two hours.

The alcohol was evaporated, the residue extracted with chloroform. The chloroform was evaporated and the residue

Fig. 1. Myosin crystals. Magn. 1:90.

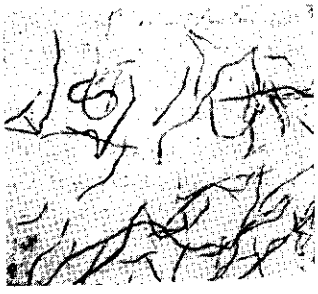

Fig. 2. Myosin threads. Magn. 1:40.

extracted with ether. After evaporation of the ether the residue was weighed. The lipoid matter was partly insoluble in acetone and constituted 3% of the dry weight of myosin.

The lipoid-free myosin was dried *in vacuo* at 120° C. The elementary analysis was kindly made by DR. M. KOVÁTS OSKOLÁS. It showed the following result:

C	50,04%
H	7,70%
N	16.15%
S	1.14%
Ash	1.23%

According to this analysis myosin is a protein. It contains 6 atoms of S for every 17000 g.

Stability. Myosin, if dissolved in 0,5 *M* KCl and neutralised, can be kept at 0° C without loss of enzymic activity for a fortnight. Finer colloidal properties, however, seem to be impaired after a few days storage, as indicated by the sluggishness of the fall of viscosity of an actomyosin, prepared from it, on addition of ATP (oral comm. of F. B. STRAUB).

Solubility. If the crystalline myosin is suspended in water and is rendered salt-free by dialysis it swells up to a glassy mass which shows only a slight opalescence. On dilution it dissolves in water giving a clear and very viscous solution from which no myosin can be separated by centrifugation. According to the water-solubility in entire absence of salts myosin is not a globulin. On the other hand, on adding ammoniumsulfate, the myosin precipitates as we pass half saturation. In this respect myosin conforms with globulins.

Myosin is precipitated by small concentrations of neutral salts and dissolves in presence of higher salt concentrations. If dissolved in water or in 0,1--0,2 *M* KCl it shows a very strong DRF. This DRF, however, is not due to the single myosin particles but to their coaxial association, for the DRF becomes weaker at 0.3 *M* KCl and disappears entirely at 0.4 M KCl. It also disappears if the solution is rendered alkaline. Evidently the higher salt concentration or pH prevents association. At the low velocity gradients of our experiments myosin shows, in 0,6 *M* KCl, no DRF.

Hand in hand with the association the limpid myosin solution becomes more and more opalescent.

If a sufficiently strong 0.5 *M* KCl-solution of myosin is diluted with water and is stirred, the turbidity assumes a silky appearance as the concentration of the KCl falls below 0,4 *M*. Between crossed Nicolls the fluid shows a strong double refraction. This indicates that the myosin particles aggregate

coaxilly to anisodimensional particles. As we dilute the solution more and more the aggregation becomes stronger and as we reach the limit of the solubility of myosin about 0,05 *M* KCl the particles become visible in the form of crystals. Strong mixing is essential if we want to obtain well formed crystals, for this mixing provides the coaxial orientation of particles which is necessary for crystallisation.

Reaction with salts. The most striking property of myosin is that it is readily precipitated by small concentrations of alkali salts. It is almost quantitatively precipitated by 0,05—0,01 *M* KCl; even 0,0015 *M* KCl causes precipitation.

In table 1. the precipitating action of different salts is compared.

Table I.

	0,2	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	0,0004
KCl	0	0	+	++	++	++	+	+	0	0
KF	+	+	+	++	++	++	++	++	+	0
KJ	0	0	+	++	++	+	0	0	0	0
LiCl	0	0	+	+	++	++	++	+	+	0
NaCl	0	+	+	++	++	++	+	+	0	0
$MgCl_2$	0	++	++	++	++	++	++	++	++	++
$CaCl_2$	0	++	++	++	++	++	++	++	++	++

0,5 ml. of the salt solution was added to 2 ml of a 0,1% salt free myosin solution. Upper line: the final molar concentration of the salt. O means no change, + means turbidity or precipitation.

The table shows that the action of the different alkali halogens is rather similar. Bivalent cations give a rather voluminous precipitate through the whole range without a well defined maximum. This makes it evident that the precipitation is rather the action of the cation than that of the anion. The valency of the anion has less influence and even the trivalent phosphate as K salt has about the same action as KCl.

It was interesting to know whether the precipitation of myosin by salts is connected with a loss of charge. On my request K. LAKI has kindly undertaken to investigate this question. His report reads as follows:

„The migration of charcoal particles coated with myosin was followed in the microscopic cataphoretic apparatus described by J. H. NORTHROP und M. KUNITZ (J. gen. Physiol. 7. 729, 1925). It was found that the myosin, dissolved in dist. water, is negatively charged and readily migrates under the influence of an electric field. In KCl solution of 0,015 *M* the migration is slowed down. At 0,025 *M* the migration is still detectable but at 0,05 *M* the particles ceased to move. Tests were made also at higher KCl concentrations up to 0,2 *M* but no migration was detectable.

From these preliminary experiments the conclusion can be drawn that the charge of the myosin particle is diminished or lost at KCl concentrations where the myosin precipitates. The results obtained are presented in the following table:

M KCl	Conc. of the myosin	
0,00	6,0 mg/cc	Moves to the pos. pole.
0,012	0,6 „	Moves slowly „ „
0,025	3,0 „	Moves slowly „ „
0,05	0,6 „	No migration
0,05	0,3 „	„
0,08	2,0 „	„
0,10	6,0 „	„
0,10	3,0 „	„
0,20	2,0 „	„

Casein, treated in the same way, migrates in 0,2 *M* KCl to the positive pole".

The effect of pH on precipitation is shown in Tab. II. The buffer acted as salt.

The table shows that at a higher pH the precipitation is limited to a smaller range and is weaker. If we raise the pH further there will be no precipitation at all. At low pH (the three lowest lines), where we approach the isoelectric point, the myosin separates in form of a gelatinous mass instead of giving a precipitate. The maximum precipitation is observed at pH 6,4 and 6,7.

The asterisks mean that the precipitate, on mincing, has a silky appearance and shows a strong DRF, is thus crystalline.

Table II.

$\frac{Na_2HPO_4}{KH_2PO_4}$	0,1	0,05	0,025	0,0125	0,006	0,003	0,0015	0,0008	pH
1/0	0	0	0	0	+	+	+	0	
16/1	0	0	0	++	++	+	+	0	8
8/1	0	0	0	++	++	+	+	0	7.7
4/1	0	0	+	++	++	+	+	0	7.3
2/1	0	0	+*	++	++	+	+	0	7
1/1	0	0	++**	+++	+++	+	+	0	6.7
1/2	0	+**	++**	++*	++	+	0	0	6.4
1/4	+	+	++	++	++	+	0	0	6.1
1/8	+	+	+	+	+	+	0	0	5.8
1/16	+	+	+	+	+	+	+	0	5.5
0/1	+	+	+	+	+	+	+	0	

Isomolar Na_2HPO_4 and KH_2PO_4 were mixed in different proportions (Col 1). The final molar concentration of PO_4 is given in the upper line.

This shows that crystallisation is limited to a narrow pH range and has its maximum about pH 6,5.

This pH of 6,5, at which the myosin precipitates and crystallises most readily, at which the most inactive myosin can be extracted (see GUBA and STRAUB), seems to correspond to the pH of thoroughly washed myosin, as determined by SLATER (5), the flocculation maximum as found by COLLIP (6) and the minimum of acid-base binding capacity as given by EDSALL (4).

It seemed to be interesting to know whether myosin binds any K at those KCl concentrations at which it is precipitated. This question was answered in the following way: the crystalline myosin, after its recrystallisation, was centrifuged, K was estimated in the supernatant fluid. The crystalline precipitate was weighed, dried, weighed again, combusted and its K estimated. I am indebted to Prof. E. ERNST for the K estimations.

The precipitate contained 4,5---7% crystalline myosin and a KCl solution the concentration of which was identical with that of the supernatant fluid and varied between 0,3—0,1 *M*. Knowing the quantity of the water in the precipitate and the

KCl concentration in the supernatant fluid, it can be calculated how much of the total K of the precipitate falls to the water and how much to the myosin. If the K of the water is subtracted from the total K the difference is the amount held by the myosin These data show that one g atom of K is held in the different experiments by the following quantities of myosin: 10000, 12000, 12000, 12000, 14000. On average 12000 g myosin bind one atom of K. This K can be removed by dialysis.

Reaction with ATP. If ATP is added to the watery solution of myosin the viscosity will decrease and the DRF will become somewhat weaker. This indicates that ATP disaggregates to some extent the associated myosin.

ATP will show a solvatising action also in presence of salts. If ATP is added along with the salts the precipitate formation will be weaker, or no precipitate will be formed at all. So, for instance, 0,05 mg ATP per ml will be sufficient to prevent any precipitate-formation by neutral potassium-phosphate.

If the salt precipitates the myosin in amorphous form the DRF disappears and can be brought back by the addition of ATP which dissolves the precipitate.

These experiments indicate that the myosin particles are present in their solution in a straight, distended form. They do not change their shape not even if discharged or precipitated or if ATP is added. This conclusion is also supported by our earlier observation that myosin threads do not contract.

References.

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