Enzyme Studies.

by

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I. Enzymic activity of crystallised myosin.

SZENT-GYÖRGYI[1] described a method for the preparation of myosin free of actin. This myosin could be crystallised and purified by repeated recrystallisations. I have found that, in presence of KCl, this myosin, recrystallised twice, has the same enzymic activity towards ATP as earlier impure preparations. This can be seen from Fig. 1.

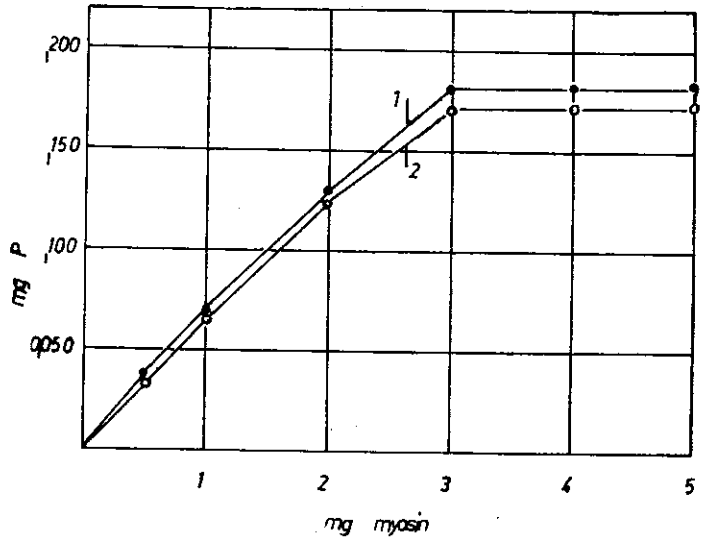

Fig. 1. The splitting of ATP by impure and recrystalliseed myosin. mg P split off at 38° in 5 min. from a mixture of 1 ml of 0.1 *M* veronal acetate buffer of pH 7.0. 0.05 *M* KCl. 3.6 mg ATP as neutral K salt and varying amounts of myosin. Total volum 3 ml.
Curve 1: purified myosin before crystallisation.
Curve 2: the same myosin recrystallised twice.

The reaction mixture was always deproteinised by 1/5 volume of 20% trichloroacetic acid and P was determined in the filtrate according to the method of FISKE and SUBBAROW, modified by JENDRASSIK and LOHMANN.

ATP has two phosphate groups which are readily hydrolysed in 15 minutes at 100° by *N* HCl. Only one of these is split off by crystallised myosin. This process of splitting off of phosphate is activated by KCl (see page 69.) and is inhibited by Mg. The Mg inhibition is shown in Fig. 2. The resulting ADP

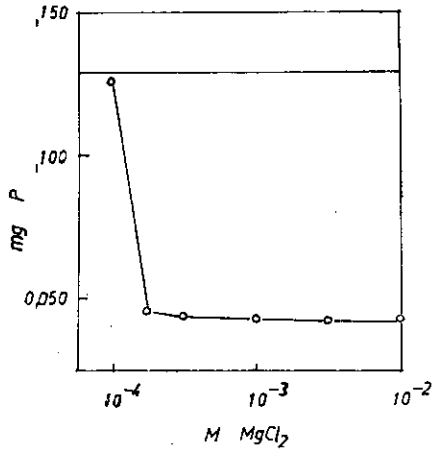

Fig. 2. Effect of $MgCl_2$ on splitting of ATP by crystallised myosin. mg P split off at 38° in 5 min. from a mixture of 1 mg myosin, 0.1 *M* KCl, 3.6 mg ATP and varying concentrations of $MgCl_2$. Total volume 3 ml.

was isolated from the reaction mixture. The Ba salt contained 3/2 Ba atom per molecule of ADP. From the two phosphate groups only one was readily hydrolysable.

The ADP was isolated by precipitating the inorganic phosphate by ammoniacal $MgCl_2$ and precipitating the ADP by $BaCl_2$. I proceeded as follows: 100 mg crystallised myosin and 1400 mg of ATP, in the form of neutral K salt, were dissolved in 300 ml 0.1*M* KCl. Incubation at 38° until samples taken showed that one P had already been split off. The protein was then removed by adding 30 ml of 20% trichloroacetic acid. Thereafter I added 3 g $MgCl_2$ and NH_4OH until the solution began to turn red in presence of phenolphtalein. The resulting mixture was stored overnight at 0° during which period the inorganic P had separated quantitatively in the form of $Mg(NH_4)PO_4$. In order to precipitate the neutral Ba salt of ADP. 3 g of $BaCl_2$ and 1/5 volume of alcohol were added to the liquid. The precipitate was washed first in 50% alcohol, then in

absolute alcohol and dried. The yield was 65 % of the theoretical value.

In order to obtain the ADP free of Mg, the Ba salt was dissolved in 5 % acetic acid and precipitated by Hg acetate. The salt was dissolved in 0.5 *N* HCl and the Hg removed as HgS. H_2S was removed by ventillation. By adding $BaCl_2$ and alcohol to the resulting liquid at neutral reaction the ADP was precipitated as a Ba salt.

Analysis of P: 100 mg Ba salt of ADP were dissolved in presence of HCl in 8 ml of H_2O and K_2SO_4 was added (one molecule of K_2SO_4 to every molecule of Ba salt). Thereafter I neutralised with KOH and brought the volume up to 10 ml with water and centrifuged. 0.1 ml of this solution contained 0.002 mg of inorganic P, 0,046 mg readily hydrolysable P and a total P of 0.093 mg. The proportion of the readily hydrolysable P to the stable one, was found to be 1 : 1.

II. ADP-isomerase.

When adenosine triphosphate was added to impure actomyosin (myosin B)[2] then both of the readily hydrolysable phosphate groups were split off. When all the soluble protein had been removed from actomyosin by four consecutive precipitations, it was found that only one of the two readily hydrolysable phosphates was split off. The other phosphate would split off upon addition of a muscle extract from which all substances, insoluble in water, had previously been removed. K. LAKI[3] showed that in addition to an enzyme system, insoluble in water and liberating only one phosphate group from ATP, a soluble protein and Mg were necessary to split off a second phosphate. My investigations confirmed LAKI's findings. The soluble factor must have been a protein since it was thermolabile and could be precipitated by trichloroacetic acid. In presence of this protein actomyosin readily attacked ADP. This protein was named ADP-isomerase for reasons to be shown hereafter.

By the following method the ADP-isomerase can be obtained 50 times purer than the original muscle juice. Freshly minced muscle of rabbit was suspended in 0.1 *M* KCl, 1.5 ml being taken per g of the muscle. Two hours later the insoluble

parts were removed by centrifugation. Ammonium sulfate was added to 0.5 saturation and the precipitate discarded. The liquid was neutralised and ammonium sulfate added to 0.7 saturation. The resulting precipitate contained the ADP-isomerase. It was dissolved in 0.1 *M* borate buffer of pH 8.5, dialysed for 24 hours and the insoluble parts removed by centrifugation. The isomerase was adsorbed to γ $Al(OH)_3$. From the latter it was eluted with 0.1 M borate buffer of pH 8.5. From this solution the isomerase was purified by precipitating it between 0.5—0.6 saturation of ammoniumsulfate. 50 γ of this protein in 3 ml. activated the splitting of ADP by actomyosin.

The question might be raised whether this new protein is not identical with the myokinase described by KALCKAR,[4] which dismutates ADP into adenosinetriphosphate and adenylic acid. My experiments showed that this is not the case since the new protein when incubated with ADP, did neither produce ATP nor adenylic acid. The product of incubation was not split by crystallised myosin, thus it was not ATP, nor was it reacted upon by deaminase, therefore the product could not have been adenylic acid either. When ADP had been incubated with isomerase, the product was split by actomyosin which suggested that isomerase had changed the molecular structure of ADP. It was for this reason that the name isomerase was given to this new protein since its effect consisted not in dismutation but in isomerisation. There was no appreciable change observed in the relation between the labile and the stable phosphate of ADP if incubated with isomerase.

Actomyosin is able to split off phosphate from the compound formed by the action of isomerase on ADP, whereas it is unable to attack ADP itself. If therefore ADP is incubated first with isomerase and the reaction product is added afterwards to actomyosin, inorganic phosphate will be formed to the extent to which the isomerisation reaction has proceeded. By this method I was able to study the isomerisation reaction itself.

Isomerisation of ADP: 3 mg K-ADP were incubated at 38° for 10 minutes at pH 8.5 in the presence of 50 γ isomerase in 1 ml. I deproteinised with 2 ml of 20% trichloroacetic acid, centrifuged and neutralised it by addition of *M* KOH.

Splitting of the isomerised product: The reaction mixture contained 2 mg of 25% actomyosin produced from cryst. myosin and actin, 1 ml 0.1 *M* veronal acetate buffer. To this mixture I added the substances noted in Col. I. Total volume 3 ml. Incubation for 10 min. at 38°. The results are shown in Table I.

Table I.

Splitting of the isomerised product of ADP by actomyosin

	mg P split off	Percentage ADP converted
Actomyosin + 3 mg ADP	0.00	
" + 3 mg ADP + 50 γ isomerase	0.104	(100)
" + 3 mg ADP previously incubated with isomerase	0.022	20
" + 3 mg ADP previously incubated with isomerase + Mg	0.064	60
" + 50 γ isomerase + 3 mg ADP previously incubated with isomerase + Mg	0.107	

Upon addition of Mg, the effect of the isomerase could be increased by 200 per cent. Thus while in a certain interval isomerase alone turned only 20% of ADP into a product which was split directly by actomyosin, 60 % were transformed in the presence of 0.001 *M* $MgCl_2$.

As to the question what molecular changes ADP had undergone upon the effect of isomerase, we must remember on one hand that isomerisation did not affect the relation of the labile and stable P of ADP. It is seen on the other hand from Table II. that only one half of the readily hydrolysable phosphate of ADP is split off by actomyosin + isomerase, i. e. only one quarter of the total P.

Table II.

10 mg of 25% actomyosin, 0.5 mg isomerase, 10 ml 0.1 M veronal-acetate buffer of pH 8.5, 30 mg ADP containing 2.25 mg readily hydrolysable P and 4.50 mg total P. The mixture was made up with water to 30 ml. Incubated at 38°. At definite time intervals samples of 3 ml were taken out and deproteinised with 1 ml of trichloroacetic acid. The experiment was finished when no more P was liberated. This usually was reached at about the end of 20 min. No more P was liberated even if the experiment was protracted to an hour.

	Inorg. P	Readily hydr. P	Total P
At the outset of experiment in 3 ml	0.00	0.225	0.450
At the end of incubation in 3 ml	0.115	0.110	0.450

III. Adenosinediphosphatase.

When ADP was added to crystallised myosin no reaction whatever took place. ADP was not split by crystallised myosin, not even when isomerase had been added. But if a third protein, — extracted in a soluble form from a washed, acetone

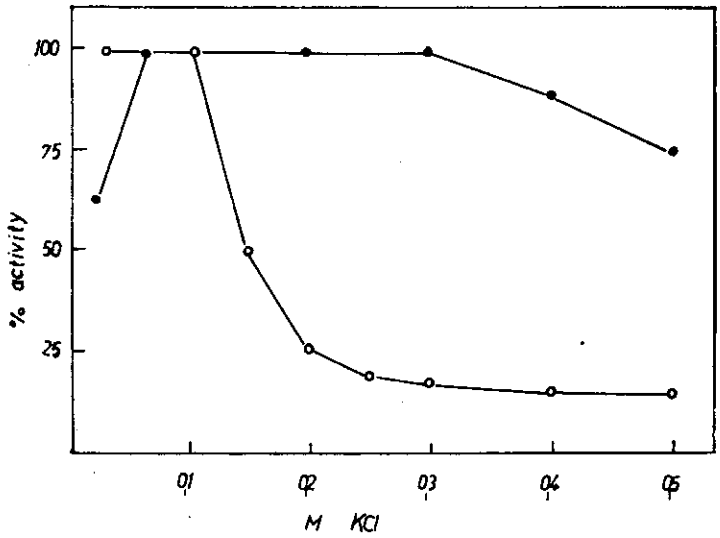

Fig. 3. Effect of KCl concentration on splitting of ATP and ADP. 100% is the maximal amount of P split off under optimal conditions.
○ — ○ — P liberated from ADP
● — ● — P liberated from ATP
2 mg of myosin, 3 mg of K ATP or ADP, KCl of varying concentrations. Total volume 3 ml. Incubated for 5 min. at 38°. In the case of ADP, isomerase and third protein were added, too.

dried muscle — was added to a mixture of crystallised myosin + isomerase, then one half of the readily hydrolysable phosphate of ADP was liberated. The optimum pH of the ADP splitting was at 8.5. The reaction was very sensitive to the KCl concentration. While the ATP-phosphatase activity of crystallised myosin was constant along a wide range of KCl concentration, the ADP splitting of the above three-enzyme system was optimal only within a narrow range of KCl concentration. (Fig. 3, curves 1, 2.)

The third protein could be replaced by actin prepared according to F. B. STRAUB's[5] method, therefore it seemed likely that the two proteins were identical. The experiments, numbered 1—6 below, seem to indicate, that the protein which splits

ADP is actomyosin itself. This, however, could not be proved conclusively.

1. No solution of the third protein could be extracted from a fresh muscle in water or in weak salt solution, since it was always the insoluble part of the fresh muscle which contained the third protein. But, similarly to actin, this protein became soluble in water when the muscle had previously been washed, treated with acetone and dried. When this washed and acetone dried muscle had been extracted in a tenfold volume of water for about 10—20 minutes, a 0.1% solution of protein was obtained of which 0.1 mg manifested the third-protein-activity. In case of small amounts the activation was

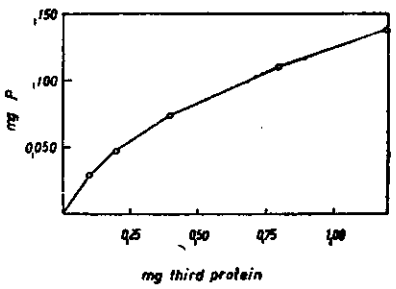

Fig. 4. Effect of varying amounts of the third protein on the splitting of ADP by myosin in presence of isomerase. 1 mg of crystallised myosin, 50 γ isomerase, 1 ml veronal acetate buffer of pH 8.5, 3 mg ADP as K salt and varying amounts of third protein. Total volume 3 ml. Incubated for 5 min. at 38°.

proportional to the quantity of the protein, whereas with greater amounts the curve for the activity flattened as shown by Fig. 4.

Actin prepared by the method of F. B. STRAUB shows a third-protein-activity proportional to its actin content.

2. If 0.01 *M* $CaCl_2$ had been added to a solution of the third protein, the Ca precipitated the third protein quantitatively just as it precipitated actin.

3. When I added the third protein to crystallised myosin in a 0.5 *M* KCl, actomyosin was formed which could be precipitated upon a 5—10 fold dilution. This actomyosin could again be dissolved in a 0.5 *M* of KCl and once more precipitated by dilution. Actomyosin, thus produced, splits ADP in the presence of isomerase and shows the same activity as myosin + actin, or myosin + third protein. Since the third protein as well as the myosin were soluble in water, it was evident that a combination had taken place between myosin and the third protein, making the former insoluble in water.

It is clear from all this that the third protein produced the same effect as actin.

4. Similarly to actin the third protein could be precipitated from its solution by addition of weak acids (pH of about 4.8).

5. The KCl sensitivity (Fig. 3) of the ADP splitting, also corroborated the surmise that it was the actomyosin, which played a part in the reaction since actomyosin, in a 0.2 *M* KCl solution, dissociates into myosin and actin, as proved by the experiments of F. GUBA.[6]

6. The various impure myosin preparations split ADP in the presence of isomerase proportional to their actin content.

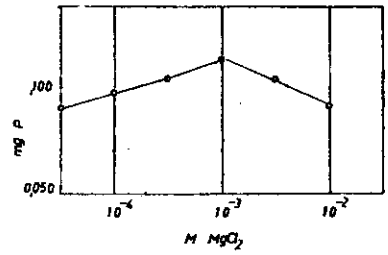

Fig. 5. Effect of varying amounts of $MgCl_2$ on the splitting of ADP by the three-enzyme-system. 1 mg of crystallised myosin, 50 γ isomerase, 0.5 mg third protein 1 ml 0.1 *M* veronal acetate buffer of pH 8.5 and varying concentrations of Mg Cl_2. Total volume 3 ml. Incubated for 5 min. at 38.°

The ADP splitting of the three-enzyme system was activated by $MgCl_2$ to about 30% (see Fig. 5) whereas isomerisation itself was activated to a considerable greater degree (200%) upon addition of Mg. The reason of this is probably that the whole reaction is limited by the relatively slow splitting off of phosphate, which is not activated by Mg.

The ADP splitting in presence of actomyosin and isomerase was inhibited by 0.01 *M* pyrophosphate to an extent of 50—75%, depending on the length of incubation with the inhibitor. Initial velocities were inhibited by 75%, whereas in case of longer incubations the inhibition fell to 50%.

References.

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