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A Peptide Material from Myosin Containing Sulfhydryl Groups

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It has been shown that a peptide material is easily split off from myosin when firmly bound bivalent metal ions are removed. The reaction is accompanied by the appearance of a high ultra-violet absorption.

From oxidized myosin the same peptide material has been isolated as two peptides differing only in aspartic acid occurring as N-terminal group in one of them. The sequence in the peptide containing aspartic acid is Asp.CySH.Tyr.Arg.Lys.Val.Gly.Glu. Several pieces of evidence suggest that the peptide material might be part of the ATPase-active centers of the myosin.

In our investigations on myosin, we have observed, that treatments removing all calcium ions from protein cause the stability to be lost. A peptide material is gradually split off and at the same time a strong ultra-violet absorption appears. The latter has generally a maximum between 260—270 mμ but can change with treatments and also with time so that alterations can occur from one day to another. In a large number of different treatments, a release of a peptide material has been observed. This occurs, for example, after such mild treatments as an extensive washing of the rabbit myosin with 0.025 M KCl, or at mere precipitation by dilution of heart myosin. In those cases the ATPase activity will gradually be lost as the peptide material is given off.

It has also been possible [1] to divide myosin into two fractions by treatment with ammonium sulphate at 20°C. One of the fractions is stable and soluble in salt solutions only. This fraction has no phosphatase activity. When the fraction is denaturated by heat, only minute amounts of peptide material will be given off. The other fraction, which is soluble in water, is unstable and gradually gives off a strongly ultra-violet-absorbing material. It does not show any phosphatase activity. In properly designed experiments the fraction can be stabilised by calcium or magnesium hydroxide but not by potassium or sodium hydroxide [2]. This stabilisation must be achieved before the peptide

material is split off. If this is done, the fraction gives an ultra-violet spectrum in which tyrosine and tryptophan are visible, and the phosphatase activity appears again.

In denaturation by heat, certain amounts of the peptide material are given off from this fraction associated with a strong ultra-violet absorption. A peptide material can be isolated which contains the following amino acids: Asp, CySH, Tyr, Arg, Lys, Val, Gly, Glu, and has aspartic acid as its N-terminal group.

On account of certain difficulties in the preparation of the peptide material in a well defined state, other ways, to be reported here, have been tried for this isolation.

MATERIAL AND METHODS

In all experiments myosin from rabbit skeletal muscle was used and prepared according to Mommaerts and Parrish [3]. The myosin obtained was freeze-dried.

Zone electrophoresis was performed in a column according to Porath [4] with stabilizing powder according to Flodin and Kupke [5]. As buffer 0.05 M triethylamine-carbonic acid buffer at pH 7.5 or 0.05 M pyridinium acetate-acetic acid buffer at pH 3.6 was used. The eluate after the electrophoresis was taken up with a fraction collector, generally in 10 ml portions. The concentration in the samples was determined either at 270 mμ in a Beckmann D.U. spectrophotometer or with the ninhydrin reagent according to Moore and Stein [6].

One dimensional paper-chromatographic determinations were performed on the peptide material using either 70 % ethanol, or phenol buffered with citrate, or butanol:acetic acid:water (5:1:4). The last two systems were also used for the two dimensional determination of the amino acids after hydrolysis of the peptides in 5.8 N HCl at 105° for 24 h.

In the experiments with oxidized myosin an ionic exchanger (kindly supplied by Dr. P. Flodin, Pharmacia Ltd, Uppsala) was used. The resin was a special aliphatic polyamine which has a much higher purity than IR4B and did not interfere with the determinations.

TRYPSIN-DIGESTED MYOSIN

In the first experiments a trypsin digestion was directly tried on the myosin.

The myosin was dissolved in a 0.25 M triethylamine-carbonate buffer of pH 8.4 and digested with trypsin (Merck); 2 mg trypsin was used for 100 mg myosin. The digestion was performed during 2 days at 37°C. Dark bottles were used and the solution was saturated with chloroform. After the digestion the solution was evaporated *in vacuo*. The residue was dissolved in 5 ml 0.05 M triethylamine-carbonate buffer and a zone electrophoresis was made on the solution.

A diagram of such a zone electrophoresis is shown in Fig. 1, giving the ultra-violet absorption of the fractions at 270 mμ. Of the components obtained, the

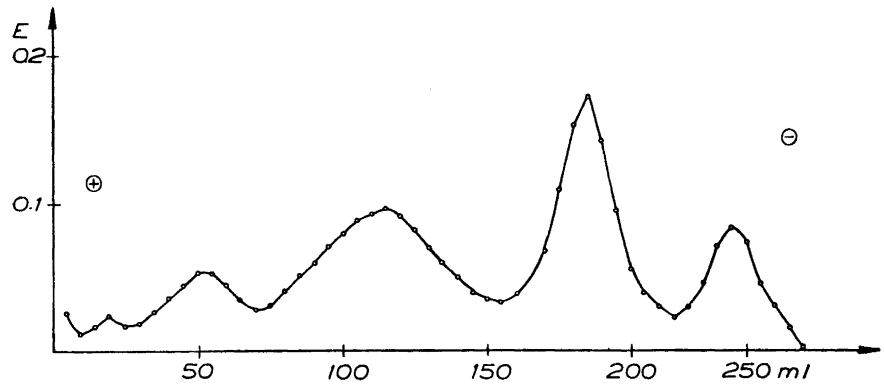

Fig. 1. Electropherogram of trypsin-digested myosin. The run was performed with 40 mA for 11 h in a 3 × 150 cm column in 0.05 M triethylamine carbonate buffer at pH 7.4. Ordinate is the optical density at 270 mμ.

two basic fractions, and sometimes also the neutral one, showed an anomalous ultra-violet absorption (see Fig. 2). The most basic component comprised about 3—4 % of the material and had an ultra-violet absorption at 270 mμ which was about 5—10 times that of any of the other components. In paper-chromatography in different solvents only one spot appeared. The amino acids occurring in this fraction were: CySH, Tyr, Arg, Lys, Val, Gly, Glu.

In the other basic fraction, several components occurred. This fraction comprised 20—25 % of the material while the neutral fraction contained about 50 % of the material.

However, the experiments did not give good reproducibility. In many cases the first component seemed to have disappeared into the second component, and also in the neutral one a strongly ultra-violet absorbing material could occur. In addition other reactions, which partly destroyed the material, appeared several times.

Another preparative method was therefore tried and gave results with a high degree of reproducibility.

PEPTIDES FROM OXIDIZED MYOSIN

The myosin was oxidized according to the method given by Harrington and Schellman [7]. The oxidized myosin was either digested with trypsin as before or treated with 11.6 N HCl at room temperature for 3 days. The solution was dried over sodium hydroxide pellets in a vacuum desiccator. In both cases the same peptide material could be isolated.

The partially broken-down myosin was dissolved in water of pH 4 and put on the ion-exchange column of the polyamine. The greatest part passed through but components containing cysteic acid were adsorbed. The adsorbed

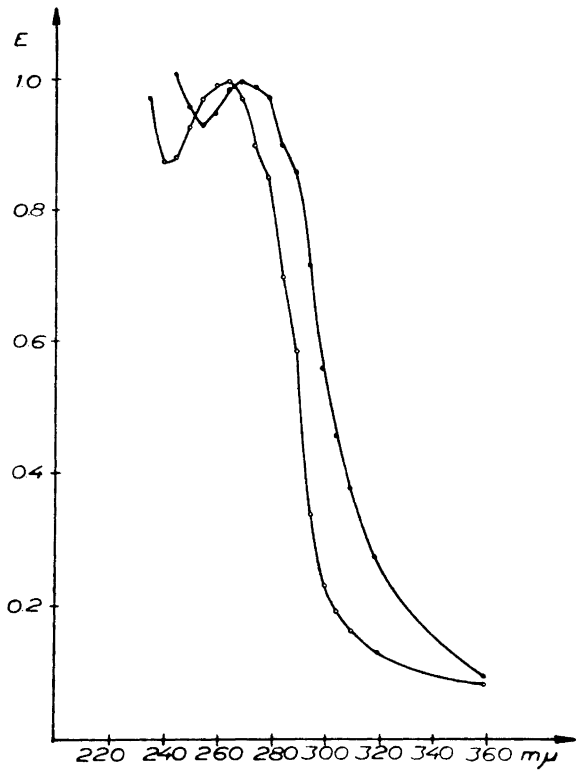

Fig. 2. Ultra-violet absorption spectrum of the most basic component. The extinction at the max. is set equal to 1.

material was eluted with water acidified with formic acid to different pH levels.

Two fractions were obtained, one at pH 2.4 and the other at pH 2.2. The two fractions were of about the same magnitude and comprise together about 8—9 % of the material.

The fraction eluted at pH 2.4 gave in zone electrophoresis one component which moved slowly to the anode. In paper-chromatography only one spot was visible.

The fraction eluted at pH 2.2 gave one component in zone electrophoresis moving a little slower than cysteic acid. In paper-chromatography only one spot was visible.

A qualitative amino acid analysis showed for the fraction at pH 2.4 the following amino acids: $CySO_3H$, Tyr, Arg, Lys, Val, Gly, Glu, and for the fraction at pH 2.2 the same amino acids and aspartic acid.

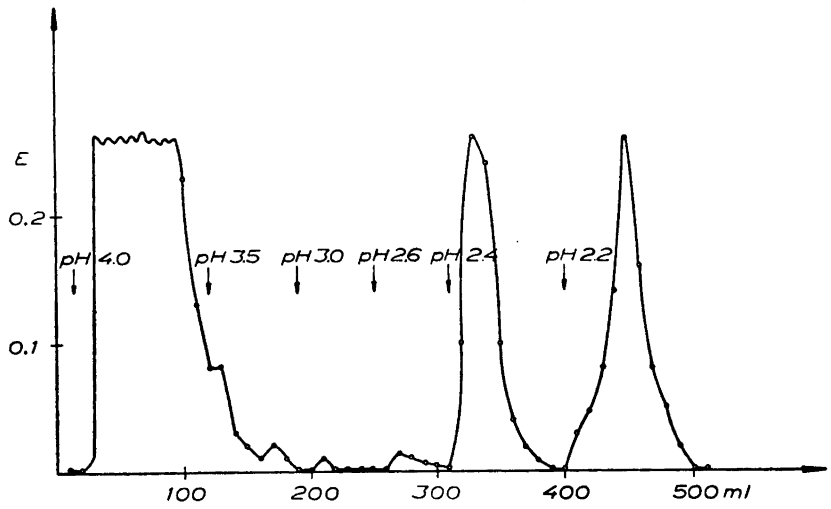

Fig. 3. Elution diagram of oxidized myosin treated with 11.6 N HCl on the polyamine column. The ordinate is the optical density as obtained with the ninhydrin reaction.

As N-terminal groups, determined according to Sanger[8], the component at pH 2.4 gave cysteic acid and the one at pH 2.2 aspartic acid. With carboxypeptidase Gly.Glu appeared in the C-terminal end in both cases.

The peptide obtained at pH 2.2 was digested with a small amount of pepsin at pH 2 for one day. The material was dried *in vacuo* and dissolved in water at pH 4 and then fractionated on the polyamine resin.

Three different main fractions were obtained. One fraction (I) that passed through; a second fraction (II) was eluted at pH 2.4, and a third one (III) in the interval pH 2.2—1.9.

The following amino acids were found in the different fractions.
Fraction I: Tyr, Arg, Lys, Val, Gly, Glu and free Asp
Fraction II: $CySO_3H$, Tyr, Arg, Lys, Val, Gly, Glu
Fraction III: Asp, $CySO_3H$, Tyr, Arg, Lys.

From these observations the sequence Asp.$CySO_3H$ (Tyr, Arg, Lys) Val. Gly.Glu can be deduced.

By zone electrophoresis of fraction III, the following peptides could be isolated

Asp.$CySO_3H$.Tyr.Arg; $CySO_3H$.Tyr.Arg; Asp.CySO/H.Tyr.Arg.Lys.

And from fraction I

Arg.Lys; Tyr.Arg.Lys.Val.Gly; Lys.Val.Gly.Glu; Gly.Glu.

The sequence then ought to be

Asp.$CySO_3H$.Tyr.Arg.Lys.Val.Gly.Glu.

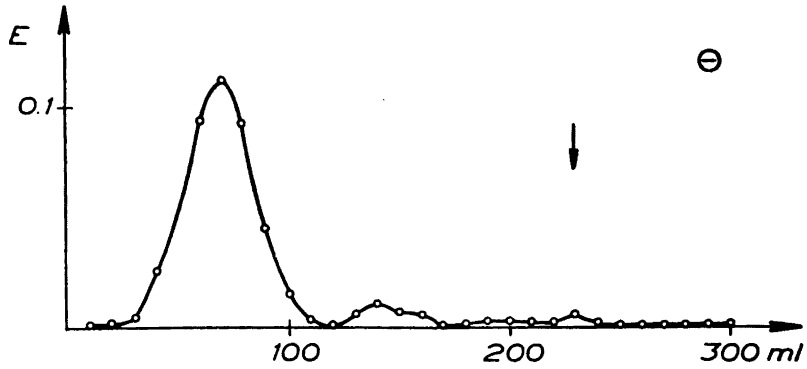

Fig. 4. Electropherogram of the component obtained at pH 2.2 from the polyamine in 0.05 M pyridinium acetate. The ordinate is the optical density as obtained with the ninhydrin reaction.

DISCUSSION

The peptide material so easily split off from myosin when firmly bound calcium is removed consists of two peptides which seem to differ only in one amino acid (aspartic acid) occurring in a N-terminal position in one of the two peptides. Several features indicate that the peptide material ought to belong to the active center of myosin which gives the ATPase activity.

The indirect evidence which support this hypothesis is:

1) Of the two fractions obtained from myosin by treatment with ammonium sulphate at 20°, only the one in which the phosphatase activity can be restored will give off substantial amounts of the peptide material on heat denaturation.

2) Obviously the peptide material is intimately connected with firmly bound calcium and the splitting off of the peptide material occurs only when the calcium is removed. Gelotte [9] has observed that the disappearance of calcium and the appearance of a strong ultra-violet absorption run parallel. The strong ultra-violet absorption is associated with the peptide. Friess [10] and Kielley and Bradley [11] conclude from their investigations on the phosphatase activity in the presence of chelating agents that a bivalent metal ion occurs in the active center. Kielley and Bradley say: "The characteristic properties of EDTA further suggest that the unidentified groups interacting with the SH group is a metal."

3) Singer and Barron [12] demonstrated the inactivation of the ATPase activity by sulfhydryl reagents. Later Kielley and Bradley [11] have studied the inactivation of the SH groups more thoroughly. From their investigation they postulate that at least two SH groups occur in the active center. We have not found any peptide with two SH groups and there does not seem to occur any other SH peptides than those two observed by us. It may therefore be that at least two such peptides occur in one active center.

According to Kielley and Bradley one of the SH groups ought to be active and the other inactive. It is very difficult to say, if there is any real difference between the two peptides obtained in regard to their state in myosin since there is not sufficient information about the mechanism for splitting off the peptides. Some slight indications exist that the difference in aspartic acid content might be real. The two peptides are obtained in the same amounts from myosin and, in an unfinished investigation about the sulfhydryl peptides in L- and H-meromyosin, mostly the peptide without aspartic acid has been found in the phosphatase-inactive L-meromyosin. The question is, however, as yet not settled.

4) Also the tyrosine group might function in the hydrolysis of ATP. Ravikowitch, Syetkina and Lyontyvea [13] have found alterations in the ultraviolet spectrum when actomyosin reacts with ATP. The absorption peak at 277 mμ in actomyosin moves and disappears into the adenine peak. This alteration was only seen in enzymatically active actomyosin, and ADP and AMP did not show this effect. The authors postulate that tyrosine and/or tryptophan takes part in the reaction. Taver and Morales [14] have also observed a similar effect.

5) Laidler and Ethier [15] have interpreted their results on the kinetics of the ATPase activity as indicating a neutralization of charge when the enzyme-substrate complex is formed and a separation of charges when the breakdown occurs. If the two basic groups of the peptide are considered to bind phosphate groups of ATP, this also fits well with the amino acid sequence of the peptide.

Thus there are several indirect pieces of evidence suggesting that the peptides belong to the ATPase-active center.

If that is so, *i. e.* the peptides are parts of the active center, they must be so situated on the myosin that at least one of the two peptides is freely exposed and can form a complex with the substrate in which most of the amino acids residues in the peptide ought to take part. For the activity the bivalent metal ion bound to the peptide is also necessary.

The fact that the treatment with ammonium sulfate can give two different and defined fractions of the myosin might indicate that the active centers, to a large extent, are located near the places where the two parts are connected. In fact, in the water soluble and calcium-hydroxide treated part aspartic acid and cysteine appears as N-terminal groups. However, further studies are necessary to confirm this tentative conclusion.

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