PROTEOLYTIC SEPARATION OF AN ENZYMIC ACTIVE SUBFRAGMENT FROM THE MYOSIN-SUBFRAGMENT (S-1)

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Received September 25, 1967

Direct visualization of myosin molecule by electron microscopy has shown it to be a long rod with a globular head at one end (Rice (1961), Huxley (1963)). The use of proteolytic enzymes such as trypsin and chymotrypsin can cleave myosin into well defined and high molecular weight fragments, heavy and light meromyosins (Szent-Gyorgyi (1953), Gergely (1955)). The globular head was found in the heavy meromyosin (HMM), and the light meromyosin was regarded as a main component of the rod. Both the ATPase activity and the actin binding ability, which are the characteristics of myosin, are found with HMM and also with the myosin-subfragment, so called S-1 (Mueller and Perry (1962)), but not with the light meromyosin. It is then generally accepted that the two characteristics of myosin were confined to the globular head, and the S-1 has been assumed to be a primary component of the globular head. In this report, isolation of an active subfragment from the S-1 is presented. The most remarkable difference of the new subfragment from the S-1 has been shown in the effect of pH on the ATPase and ITPase activities.

Experimentals The ATPase activity was measured at 25°C in the presence of 5 mM $CaCl_2$, 0.5 M KCl, 20 mM tris-maleate buffer (pH 7.0) and 2 mM ATP, unless otherwise stated. Specific ATPase activity was expressed as unit (U), which is given as μmoles Pi

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The study was aided by a grant from Muscular Dystrophy Association of America, Inc.

liberated in 1 minute per 1 mg of protein. Pi liberated was determined by Fiske-SubbaRow's method.

Fractionation of proteins was performed by employing DEAE-cellulose column chromatography and Sephdex G-100 or G-200 gel filtration. DEAE-cellulose column was developed by 20 mM tris-HCl buffer, whose pH was adjusted to 7.6 at room temperature, combined with increasing KCl concentration upto 0.2 M. A solution of 0.15 M KCl and 20 mM tris-HCl, whose pH was adjusted to 8.0 at room temperature, was used for the elution in Sephdex gel filtration.

The S-1 was prepared by the tryptic digestion of HMM by the method of Mueller and Perry (1962) with slight modification. The S-1 once fractionated through DEAE-cellulose column was concentrated by the precipitation using ammonium sulfate between 48 and 60 per cent saturation. After dialysis of the precipitate against 0.15 M KCl and 20 mM tris-HCl (pH 8.0) over night, it was further purified by Sephadex G-200 gel filtration. ATPase activity of the purified S-1 was in the range between 1.0 and 1.2 U. $OD^{1}_{1\%}{}^{cm}$ at 280 mμ was 7.9. The purified S-1 was applied for the fragmentation using nagarse.

The sedimentation coefficient was determined by a Spinco, model E, analytical ultracentrifuge. Trypsin, chymotrypsin, trypsin inhibitor, ATP, ITP and diisopropylfluorophosphate were purchased from Sigma Chemical Company. Nagarse was kindly supplied by Dr.T.Sekine (Juntendo University) and Dr.Y.Tonomura (Osaka University).

Results and discussion Total ATPase activity of the reaction mixture containing the S-1 was kept constant during the proteolysis using nagarse for 20 minutes in 0.1 M KCl and 20 mM tris-HCl (pH 8.0), at 25°C. At 150 minutes after the addition of nagarse, the ATPase activity was about 60 per cent of the original one under the condition. Weight ratio of nagarse to HMM was 1:50. After 30 minutes of the incubation, the reaction was stopped by the addition of diisopropylfluorophosphate in a final concentration of 1×10^{-4} M. The mixture was fractionated using Sephdex G-100. One example is shown in Fig. 1. Two major and a minor peaks were obtained. Approximately one-half of the applied amount of S-1 was found in the first peak, whose ATPase activity was 1.4 U. The third peak showed no

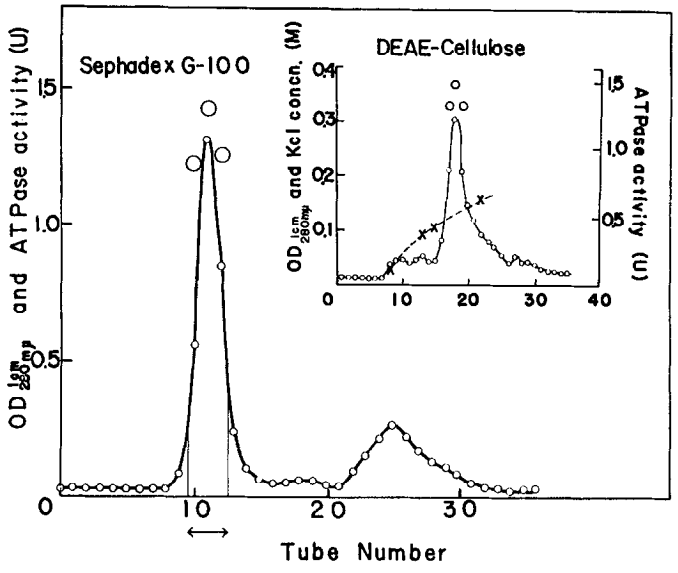

Fig. 1. Fractionation of a nagarse digest of S-1.
A 6.6 mg per ml solution of S-1 (5 ml) was digested with nagarse (0.8 mg), under the condition described in text. The solution was applied to a column (2 x 38 cm) of Sephadex G-100 equilibrated with 0.15 M KCl - 20 mM tris-HCl buffer (pH 8.0). The column was developed at 4°C with the same solvent at a flow rate of 20 ml per hour, and 5 ml fractions were collected. Inset is a rechromatography of fractions (tube number 10 - 12) isolated from S-1. Protein solution was applied to a column (1.8 x 6 cm) of DEAE-cellulose. The column was developed with KCl gradient elution at a flow rate of 25 ml per hour, and 5 ml fractions were collected. Solid line, optical density at 280 mμ ; ○, ATPase activities ; ×, concentrations of KCl.

ATPase activity. After the rechromatography of the first peak using DEAE-cellulose, a main peak was eluted at 0.12 M KCl and the ATPase activity remained at nearly 1.5 U. It was concluded from these results that the first peak contained a new subfragment keeping the active site of S-1. This fraction is tentatively designated as S-n. $OD^{1\,cm}_{1\%}$ at 280 mμ of the S-n was nearly 8.0.

Sedimentation coefficient of S-n, whose $OD^{1\,cm}_{280m\mu}$ was equal to 2.27, was found to be 4.7s in 0.15 M KCl and 20 mM tris-HCl (pH 7.6), while that of S-1 giving the same optical density was 5.5s (Yagi and Yazawa (1966)). S-n was distinguished from S-1 by the disc gel electrophoresis of polyacrylamid, at pH 8.5. When the cross-linkage of polyacrylamid was 10 per cent, a

single band of S-1 was found half way down from that of S-n. S-n and S-1 were also separated to each other by DEAE-cellulose column chromatography with KCl gradient ; S-n was eluted at 0.12 M KCl as shown above, while S-1 was at 0.14 M.

The pH-activity curve of myosin-ATPase, which has a maximum at pH 6.5 and a minimum at 8 in the presence of 5 mM $CaCl_2$ and 0.5 M KCl (Banga (1941)), was also obtained with HMM-ATPase and S-1-ATPase (Yagi and Yazawa (1966)). Since the characteristic pH-activity curve was obtained with myosin, HMM and S-1, it was assumed that the local structure of active site of myosin-ATPase was maintained in HMM and S-1 after the tryptic digestions. As shown in Fig. 2, however, the maximum and the minimum were not detected in the pH-activity curve of S-n, but a plateau was shown

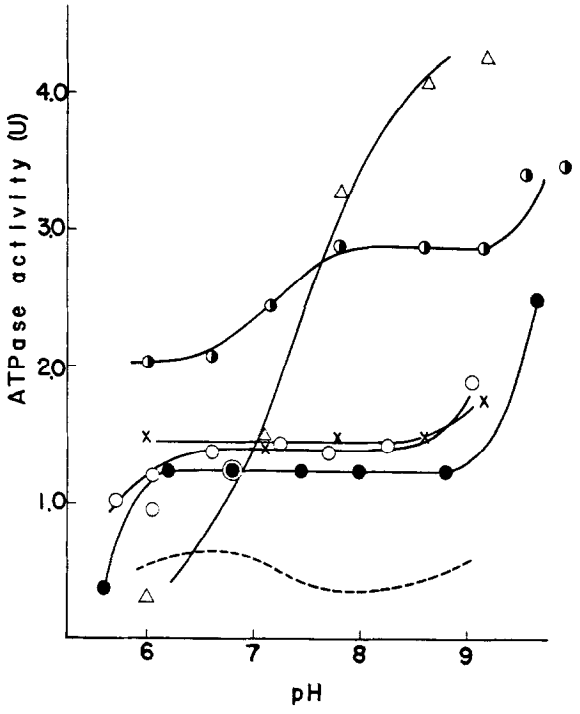

Fig. 2. pH-activity curves of S-n-ATPase and S-n-ITPase. Reaction mixture contained 0.04 mg S-n, 2 μmoles ATP or ITP, 5 μmoles $CaCl_2$ or 10 μmoles EDTA, 0.5 mmoles KCl, and 20 μmoles histidine or tris-maleate buffer in a total volume of 1 ml. At pH above 9, 20 μmoles glycine-NaOH buffer was used instead of histidine or tris-maleate buffer. ○ and ●, ATP, $CaCl_2$, tris-maleate buffer ; ×, ATP, $CaCl_2$, histidine buffer ; ◑, ITP, $CaCl_2$, histidine buffer ; △, ATP, EDTA, histidine buffer. The broken line is HMM-ATPase activity given as reference, which was measured in the presence of $CaCl_2$.

between pH 6 and 9. The ATPase activity decreased below pH 6 and it raised above 9. When ITP was used as the substrate instead of ATP, a difference was also obtained between the pH-activity curve of S-n and those of the mother proteins, i.e., the curve of S-n showed a shift of about one pH unit towards basic region than those of the latters. Therefore, the local structure of active site, which appeared to be maintained after the successive tryptic digestions, may be modified by the nagarse digestion.

On the other hand, the pH-activity curve of ATPase in the presence of 10 mM EDTA fitted to the first order sygmoid curve giving the pK of 7.3, and it agrees well with those of mother proteins. Therefore, it indicates that the difference in the effect of pH on ATPase and ITPase of S-n from that of mother proteins was disappeared by the addition of EDTA. This phenomenon may throw s light on the function of EDTA in the myosin-ATPase.

When myosin, HMM and S-1 are treated with lower concen-

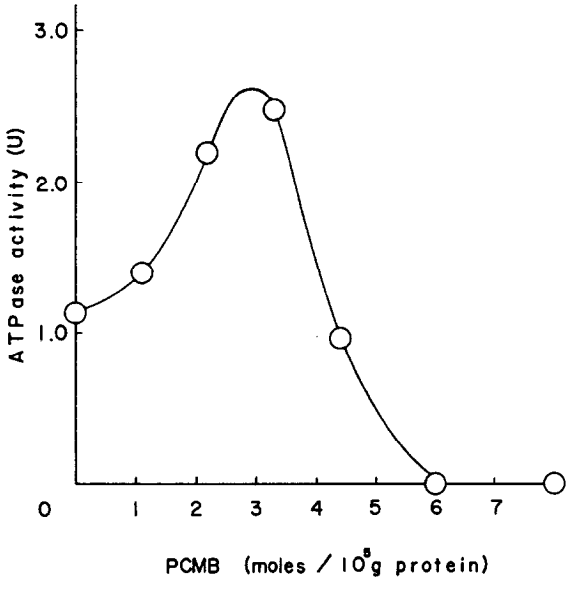

Fig. 3 Effect of PCMB on S-n-ATPase activity.
A 0.125 mg per ml solution of S-n was incubated with various amount of PCMB in 20 mM tris-maleate buffer (pH 7.0) for 25 minutes, at 25°C. 1.75 ml of the solution was diluted to 4.5 ml with KCl and tris-maleate bufer and, then, 0.5 ml of 20 mM ATP was added to start the reaction. Final concentrations of KCl and tris-maleate buffer (pH 7.0) were 0.5 M and 20 mM, respectively.

trations of PCMB, stimulation of the Ca-ATPase occurs instead of inactivation obtained at higher concentrations (Kielly and Bradley (1956), Yagi and Yazawa (1966)). When about one-half of the amount of reagent was added to that of SH-group on molar basis, the maximum activation was obtained. As shown in Fig. 3 , this was also the case with S-n. The amount of PCMB required for the maximum activation was 3 moles per 10^5 g of the protein.

Nagarse could not be replaced by trypsin, chymotrypsin or pronase. During the reactions using the latter proteolytic enzymes, the total ATPase activity decreased accompanying the decrease in the amount of S-1 and the increase in the amount of dialyzable components.

An active subfragment was also isolated directly from HMM by nagarse digestion and it resembled S-n in the values of sedimentation velocity and in the mobility of disc gel electrophoresis.

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