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Stringent requirement for Ca^{2+} in the removal of Z-lines and α-actinin from isolated myofibrils by Ca^{2+}-activated neutral proteinase

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Treatment of isolated myofibrils with Ca^{2+}-activated neutral proteinase (CANP) results in specific removal of Z-line and of α-actinin. To investigate the ionic requirement for these processes, we measured Z-line removal by phase-contrast and interference microscopy and α-actinin removal by sodium dodecyl sulphate/polyacrylamide-gel electrophoretic analysis of myofibrillar proteins. The proteolytic digestion of native purified proteins was measured directly on polyacrylamide gels and by the fluorescamine technique. We found that the removal of Z-line and α-actinin as well as the release of proteolytic degradation products from isolated myofibrils by CANP occur only in the presence of Ca^{2+}; Sr^{2+}, Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} are all ineffective. In contrast with this stringent requirement for Ca^{2+}, the proteolytic activity of CANP measured with denatured casein, native and denatured haemoglobin, native actin and tropomyosin also occurs in the presence of other bivalent cations, in the following order: $Ca^{2+} > Sr^{2+} > Ba^{2+}$. These data suggest that only Ca^{2+} can produce the conformational change in myofibrils that renders them susceptible to the action of CANP, whereas its proteolytic activity is stimulated by several bivalent ions.

It is now well established that a variety of tissues, including skeletal (Huston & Krebs, 1968; Reddy *et al.*, 1975; Dayton *et al.*, 1976*a*,*b*; Ishiura *et al.*, 1978) and cardiac (Waxman & Krebs, 1978; Toyo-Oka & Masaki, 1979) muscle, uterus (Puca *et al.*, 1977), brain (Inoue *et al.*, 1977) and platelets (Phillips & Jakabora, 1977), contain Ca^{2+}-activated neutral proteinase. This enzyme was first discovered by Huston & Krebs (1968) when they studied the mechanism by which phosphorylase kinase is activated. They demonstrated that the protein factor, KAF, which is required for kinase activation, is a Ca^{2+}-dependent proteinase. The activation consists of limited proteolysis, which can be accomplished either by treatment with KAF or by trypsin.

In subsequent studies, Busch *et al.* (1972) isolated, also from skeletal muscle, CASF, which removed Z-lines when incubated with isolated myofibrils. The isolation scheme for CASF was similar to that used for KAF. Again, the removal of Z-lines could be duplicated by treatment with trypsin. Busch *et al.* (1972) did not study the proteolytic nature of CASF at that time. In the following years, however, several laboratories, including our own, demonstrated that the Z-line-removing factor is indeed a proteinase. Various abbreviations have been used for this enzyme, such as CaAF (Reddy *et al.*, 1975), CAF (Dayton *et al.*, 1976*a*,*b*) and CANP (Ishiura *et al.*, 1978). We have chosen to adhere in this publication to CANP as an abbreviation of the name that most closely describes the known properties of this enzyme.

In the present paper, we report studies on the ionic requirement of CANP for Z-line and α-actinin removal, as well as for its proteolytic activity as measured with purified myofibrillar proteins and with intact myofibrils. The results indicate that Z-line and α-actinin removal and digestion of intact myofibrils are activated by Ca^{2+} only, whereas the proteolytic digestion of purified proteins can be activated by a variety of bivalent ions. The results are interpreted to indicate that the bivalent ions are

Abbreviations used: CANP, Ca^{2+}-activated neutral proteinases; CASF, Ca^{2+}-activated sarcoplasmic factor; KAF, kinase-activating factor; DTT, dithiothreitol.

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required for activation of CANP, whereas only Ca^{2+} can bring about the conformational change in myofibrils that makes them susceptible to CANP attack.

Experimental

Materials

β-Glycerophosphate, EGTA, EDTA, DTT and haemoglobin were purchased from Sigma Chemical Co., [^{3}H]acetic anhydride (sp. radioactivity 2 Ci/mmol) was from Amersham/Searle Corp., casein was from Nutritional Biochemical Corp., Amido Black-10B was from J. T. Baker Chemical Co., fluorescamine was from Roche Diagnostics and AcA_{44} and Sephadex G-200 were from Pharmacia Fine Chemicals.

Methods

Preparation of myofibrils and myofibrillar proteins. Myofibrils were prepared from rabbit skeletal muscle as described previously (Zak *et al.*, 1972). Myosin and actin were purified by the methods of Shiverik *et al.* (1975) and Spudich & Watts (1971) respectively; α-actinin was prepared by the method of Arakawa *et al.* (1970), followed by chromatography on Sephadex G-200. Sources of tropomyosin and troponin were myofibrils after removal of myosin (Spudich & Watts, 1971); the proteins were extracted and purified by the method of Hartshorne & Mueller (1969). Myofibrils were stored at −20°C in 50% glycerol in the presence of 0.1 M-KCl and 0.25 mM-thioglycollic acid; purified proteins were stored in the same way, except that the appropriate buffers contained 1 mM-DTT.

Measurement of proteolytic activity on polyacrylamide gels. The method originally described by Eguchi & Yoshitake (1967) was modified so that native proteins could be used as substrates. Protein was mixed into polyacrylamide solution at a concentration of 0.5 mg/ml, and the mixture was then polymerized by addition of a catalyst. Under these conditions the proteins were not covalently bound to the gels nor were they found to undergo denaturation. The enzyme was electrophoresed into the gels at 2.5 mA per tube in a cold room. After the tracking dye had migrated 1 cm from the bottom of the tube, the gels were removed and incubated in an appropriate buffer containing either Ca^{2+} or EGTA for 1 h. The gels were stained with Amido Black-10B (0.5%) in acetic acid, methanol and water (1:4:5, by vol.) for 15 min and destained with the same solvent. The decreased intensity of staining was evaluated by densitometric tracing at 540 nm with a Gilford spectrophotometer.

Assay of proteolytic activity by fluorescamine reaction. The enzymic reaction was stopped by dilution (1:10), and the undigested substrate was removed by 800 *g* centrifugation for 10 min in the case of myofibrils, or by isoelectric-point precipitation in the case of haemoglobin and purified myofibrillar proteins. The fluorescence of the supernatants produced by addition of fluorescamine was assayed by the method of Schwabe (1973) with an Aminco-Bowman Spectrofluorometer whose excitation and emission wavelengths were set at 390 and 475 nm respectively. Alanylglycine was used as a standard.

Gel electrophoresis. For sodium dodecyl sulphate/polyacrylamide gel electrophoresis, 5% acrylamide gels containing 1:37 (w/w) cross-linker were used with Tris/glycine buffer, pH 8.8. Gels were stained with Coomassie Brilliant Blue in 25% isopropyl alcohol, 10% acetic acid for 18 h and destained in an isopropyl alcohol/acetic acid solution. Densitometric tracings were obtained at 540 nm with the Gilford spectrophotometer.

Phase-contrast and interference microscopy. A Zeiss microscope fitted with appropriate optics was used. The negatives of pictures taken with even-field interference optics were scanned in a spectrodensitometer (model SD 3000; Schoeffel Instruments Corp.) fitted with an SDC 300 density computer.

Analytical methods. Proteins were determined either by the biuret method, when the concentration exceeded 10 mg/ml (Gornall *et al.*, 1949), or by the method of Lowry *et al.* (1951) when the concentrations were below this value. Radioactivity was measured with a model 332 Packard Tri-Carb liquid-scintillation spectrometer, in an Instagel (Packard) counting solution. A correction for quenching was made by use of an external standard. The radioactivity of ^{3}H was measured with an efficiency of 38%.

Results

Purification of CANP

The procedure that we described previously (Reddy *et al.*, 1975) for the purification of CANP was used with several modifications. During DE-52 DEAE-cellulose chromatography, the linear gradient of KCl or NaCl (0.2–0.4 M) was started after washing of the column with 800 ml of 0.15 M-salt and the CANP activity was eluted at 0.25 M-KCl concentration. The enzymic activity, which eluted as a single peak containing some additional proteins, was concentrated by precipitation at 50% saturation of $(NH_4)_2SO_4$ and then subjected to molecular sieving on an AcA_{44} column. The enzymic activity and the 280 nm absorbing material was eluted as a symmetrical peak with one additional protein peak. The enzyme was 90–95% pure, as judged from electrophoresis in the presence of non-denaturing buffer systems evaluated densitometrically after

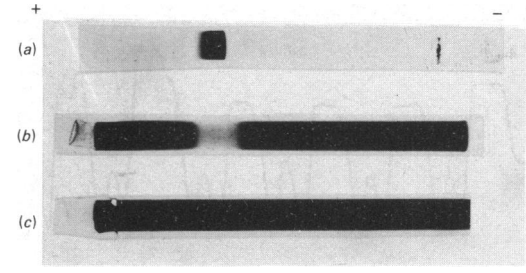

Fig. 1. *Proteolytic activity of highly purified CANP measured on polyacrylamide gels*

CANP (final purification step) was subjected to electrophoresis in non-denaturing buffer (Tris/glycine, 0.05M, pH 7.6). The polyacrylamide concentration was 5%. (*a*) 30μg of CANP. (*b*) Casein (0.5mg/ml) was added to the polyacryalmide solution before polymerization. After polymerization, 10μg of enzyme was loaded per gel. After electrophoresis the gels were incubated in the presence of 5mM-Ca^{2+}, 10mM-DTT and 50mM-Tris/maleate buffer, pH 7.0, for 60min at 25°C. (*c*) As for (*b*), except that 5mM-EGTA was substituted for Ca^{2+}.

staining with Amido Black-10B. The single visible protein band was shown to contain the proteolytic activity (Fig. 1). Only one subunit with a mol.wt. of 80000 was observed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown).

Effect of bivalent metallic ions on haemoglobin splitting by CANP

The trichloroacetic acid-soluble radioactivity derived from [^{3}H]acetylhaemoglobin as substrate was measured as an index of proteolytic activity (Table 1). The enzymic activity was activated by the bivalent metallic ions in the following order: $Ca^{2+} > Sr^{2+} > Ba^{2+} = Mn^{2+}$. Co^{2+} and Zn^{2+} were without effect.

Effect of bivalent metallic ions on Z-line removal

The effect of the presence of various bivalent ions on Z-line removal by CANP is shown in Table 1. The time necessary for complete removal of Z-lines from isolated myofibrils was recorded during observation under the phase-contrast microscope. Unlike the effect on proteolytic activity, of all bivalent ions tested, only Ca^{2+} activated Z-line removal. With the rest of the bivalent ions, the Z-lines remained intact even after 24h of incubation.

Effect of bivalent metallic ions on α-actinin release from myofibrils by CANP

We have shown previously (Reddy *et al.*, 1975) that, as CANP removes Z-lines, α-actinin dis-

Table 1. *Effect of bivalent metallic ions on Z-line removal from myofibril, haemoglobin-splitting activity and of α-actinin release by CANP*

The assay mixture contained 1mg of myofibrils, 100mM-KCl, 1mM-NaN_3, 10mM-DTT, 10μg of CANP and 1mM-bivalent ion or EGTA per 1ml of final volume. The temperature was 25°C. The Z-lines were observed at regular intervals under the phase-contrast microscope. — indicates that the Z-lines remained intact after 24h of incubation. The A_{280} of the supernatant after pelleting the myofibrils at 1000*g* for 10min was measured by using the incubation medium without myofibrils and enzymes as a blank. The optical densities obtained from supernatants of myofibrils incubated in the presence of 1mM-Ca^{2+} without enzyme were subtracted from the A_{280} values shown. The assay mixture contained 10^5c.p.m. of denatured [^{3}H]acetylhaemoglobin (corresponding to 0.4mg of protein), 1mM-metal ion, 10mM-DTT, 50mM-Tris/maleate buffer, pH 7.0, and 8μg of enzyme. The assay was performed at 25°C for 10min. The values obtained in the presence of 1mM-EDTA were subtracted from the numbers shown. The release of α-actinin from the myofibrils was determined after 30min incubation at 25°C, with enzyme and the appropriate bivalent ions; the myofibrils were washed by one cycle of pelleting and resuspended as described under 'Methods'. Next, 100μg of myofibrillar protein was loaded on to 5% polyacrylamide gels. The conditions of electrophoresis are described under 'Methods'. After staining with Coomassie Blue and destaining, the gels were scanned at 540nm with a Gilford spectrophotometer (model 2400) that had a linear transport attachment. The area corresponding to α-actinin was measured by planimetry. Each number is a mean from ten determinations ± S.D.

Metal ion (1mM)	Z-line removal: Time for complete removal	Z-line removal: A_{280}	Proteolytic activity (c.p.m. released)	α-Actinin content (arbitrary units)
None	—	0.15	0	40 ± 3
EDTA or EGTA	—	0.13	0	40 ± 2
Ca^{2+}	30min	0.60	1870	8 ± 1
Sr^{2+}	—	0.13	1605	38 ± 0.5
Ba^{2+}	—	0.14	572	40 ± 1
Mg^{2+}	—	0.14	535	38 ± 1
Mn^{2+}	—	0.14	345	38 ± 1
Co^{2+}	—	0.13	0	—
Zn^{2+}	—	0.13	0	—

appears from the profiles of myofibrillar proteins displayed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The area of Coomassie Blue-stained α-actinin relative to other myofibrillar proteins as determined by planimetry is shown in Table 1 (Reddy *et al.*, 1975). The myofibrils treated with CANP in the presence of Ca^{2+} show considerable loss of α-actinin band intensity. Other bivalent ions tested, even Sr^{2+} and

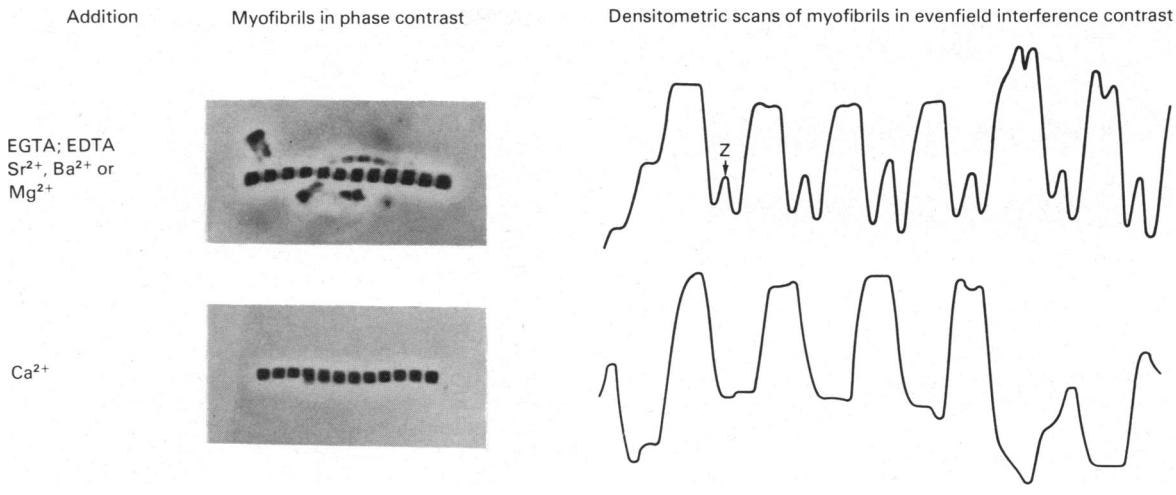

Fig. 2. *Phase micrographs and scans of interference micrographs of myofibrils treated with CANP in the presence of bivalent ions and their chelators*

Myofibrils were incubated with CANP (100:1) in the presence of 100mM-KCl, 10mM-DTT, 50mM-Tris/maleate buffer, pH 7.0, 1mM-NaN_3 and 1mM-bivalent ion or chelator at 25°C for 30min. Magnification, 1000×.

Table 2. *Effect of various amounts of SR^{2+} on Z-line removal by CANP*

The experimental conditions were as described in the legend to Table 1.

Additions	State of Z-line after 6h of incubation	A_{280} of the supernatants
Blank	Intact	0.14
EGTA	Intact	0.135
Ca^{2+} (1mM)	Lost	0.65
Ca^{2+} (1mM); Sr^{2+} (1mM)	Lost	0.66
Ca^{2+} (1mM); Sr^{2+} (2mM)	Lost	0.65
Ca^{2+} (1mM): Sr^{2+} (10mM)	Lost	0.65
Sr^{2+} (1mM)	Intact	0.14
Sr^{2+} (10mM)	Intact	0.135

Ba^{2+}, which usually replace Ca^{2+} in their effect on activities of other enzymes (Seidel & Gergely, 1963; Brostrom *et al.*, 1971), did not influence the enzymic removal of α-actinin.

In a different approach to assessing the removal of Z-lines, we obtained interference micrographs after 30min of treatment with CANP. The densitometric scans of even-field interference contrast pictures are shown in Fig. 2. The intensity of the Z-band region decreased only if Ca^{2+} was present; Sr^{2+}, Ba^{2+} and Mg^{2+} were without effect.

To determine whether there is any synergistic action of bivalent ions, we studied the effect of various concentrations of Sr^{2+} in the presence of a constant amount of Ca^{2+} on the removal of Z-lines by CANP. The state of the Z-lines as seen in the phase-contrast microscope is listed in Table 2 together with the A_{280}-absorbing material released into the incubation medium 4h after the addition of CANP to isolated myofibrils. No additive effect of Sr^{2+} on either activity was detected.

Proteolytic activity of CANP with isolated myofibrils as a substrate

The proteolytic digestion by CANP of isolated rabbit skeletal-muscle myofibrils and of native haemoglobin is compared in Fig. 3. Proteolysis was followed by the production of fluorescent compounds of the peptides released during the reaction; their quantity is expressed as alanylglycine equivalents. The data show that the hydrolysis of native haemoglobin is activated by bivalent metallic ions in the following order: $Ca^{2+} > Sr^{2+} > Ba^{2+}$. In contrast, no peptides were released except in the presence of Ca^{2+} when myofibrils were treated with CANP. Even increasing the concentrations of Sr^{2+} and Ba^{2+} to 5mM (results not shown) did not result in detectable digestion of myofibrils.

Proteolytic digestion of purified myofibrillar proteins

Myosin, α-actinin, actin and tropomyosin and troponin complex isolated from rabbit skeletal muscle were used as substrates for CANP. The proteins were 95–98% pure as judged from densitometric scans of Coomassie Blue-stained sodium dodecyl sulphate/polyacrylamide gels.

The semi-quantitative method used for evaluation

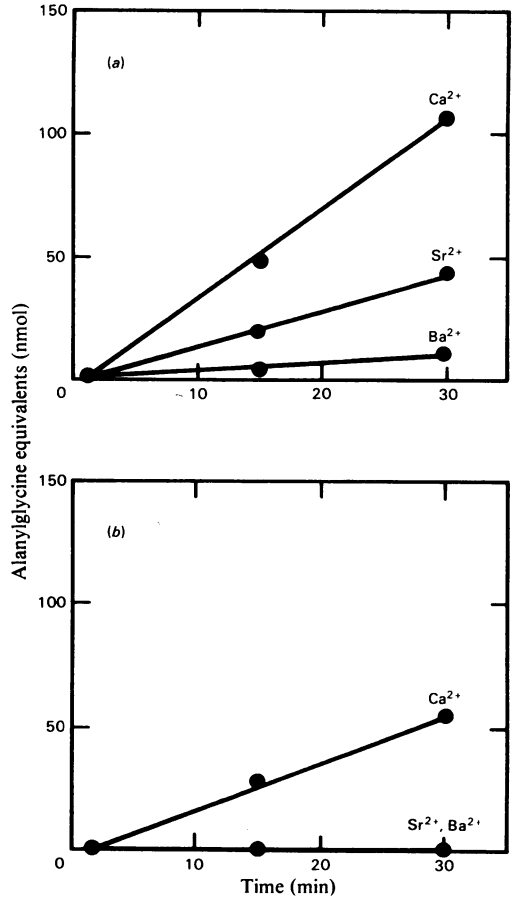

Fig. 3. *Effect of alkaline-earth metals on the proteolytic activity of CANP on native haemoglobin (a) and myofibrils (b)*

The reaction mixture containing 1 mg of substrate, 50 mM-sodium maleate buffer, pH 7.0, 10 mM-DTT, 8 μg of enzyme and 1 mM-metal ion was incubated at 25°C. The fluorescence of the degradation products produced by addition of fluorescamine was assayed as described under 'Methods'.

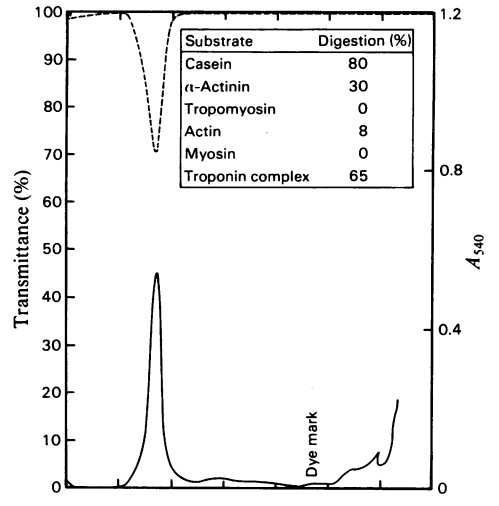

Fig. 4. *Proteolytic activity of CANP measured with different substrates on polyacrylamide gels*

——, Densitometric tracing of Amido Black 10B-stained CANP; 30 μg of protein was loaded per gel. – – – –, Tracing of gel containing substrate after its incubation with CANP and 5 mM-Ca^{2+}. The decrease in absorbance corresponds to the digested area. The conditions of electrophoresis were as given in the legend to Fig. 1. The proteins used as substrates were 95–98% pure, as determined from densitometric scans of sodium dodecyl sulphate/polyacrylamide gels. The inset table gives a list of proteins employed to test the substrate specificity of CANP.

of proteolytic digestion of myofibrillar proteins is shown in Fig. 1. Gel (*a*) corresponds to purified CANP analysed on polyacrylamide gels. In gels (*b*) and (*c*), the individual myofibrillar protein was intermixed with polyacrylamide solution before polymerization. After CANP was introduced electrophoretically into the gels (conditions the same as used for gel *a*), the gels were incubated for assessment of proteolytic activity. The densitometric tracing of the remaining proteins, stained with Amido Black, is shown in Fig. 4. The continuous line corresponds to the intensity of CANP, and the broken line indicates the area on the gel that was digested by the action of CANP. A planimetric analysis of the digested area is given in the insert Table. The data suggest substantial proteolysis of casein, α-actinin and troponin, some digestion of actin, and no detectable activity when myosin or tropomyosin were used as substrates.

Since the above method is only semi-quantitative, we investigated the proteolytic digestion of isolated myofibrillar proteins by the fluorescamine assay, which is more accurate and sensitive. The effect of alkaline-earth metals on the hydrolysis of myosin, actin and tropomyosin is shown in Fig. 5. For reference, the digestion of native and denatured haemoglobin is given in (*a*). it can be seen that denaturation leads to enhancement of proteolytic digestion in the presence of Sr^{2+} and Ba^{2+}; in the presence of Ca^{2+}, on the other hand, the denaturation has a relatively small effect. For the native myofibrillar proteins, a different pattern was observed (*b*). Hydrolysis of native actin and troponin was activated by all three alkaline-earth metals, but in the case of native myosin, only Ca^{2+} was effective as an activator.

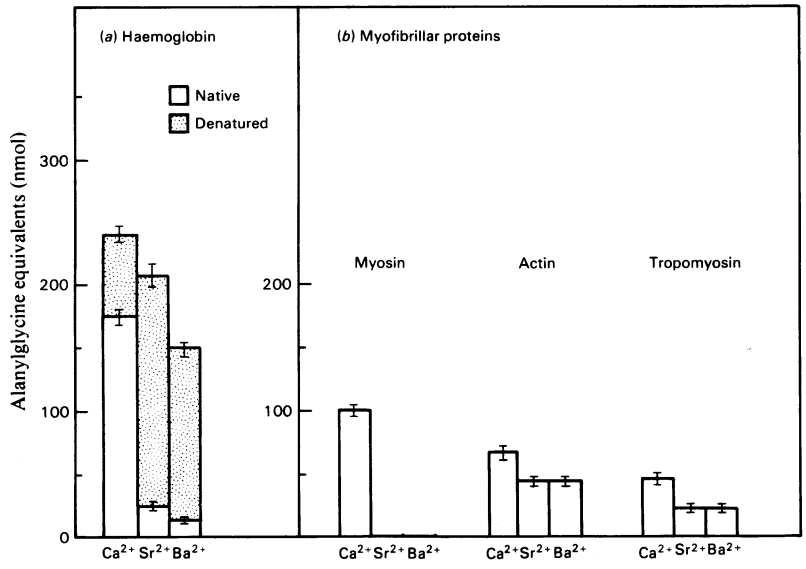

Fig. 5. *Effect of alkaline-earth metals on the hydrolysis of purified proteins by CANP*
The experimental conditions were as described in the legend to Fig. 3, except that the incubation time was 30 min.

Discussion

The post-mitochondrial supernatant of muscle homogenate contains a CANP that removes the electron-dense material associated with Z-lines during incubation with isolated myofibrils (Busch *et al.*, 1972; Reddy *et al.*, 1975; Dayton *et al.*, 1976*a*,*b*). Concomitantly with the loss of Z-lines, α-actinin is removed (Reddy *et al.*, 1975; Dayton *et al.*, 1976*a*,*b*). We have reported here the results of studies designed to elucidate the mechanism of action of CANP on digestion of purified myofibrils and their constituent proteins.

It is now well established that, in the presence of millimolar concentrations of Ca^{2+} and thiol reducing agents, CANP hydrolyses denatured proteins, such as haemoglobin and casein, to degradation products that remain soluble after protein denaturation. In a manner similar to most other biological effects, Ca^{2+} can be substituted by other bivalent ions in the following order of decreasing activity: $Ca^{2+} > Sr^{2+} > Ba^{2+}$.

In the case of native proteins, however, the substrate and ionic requirements of this enzyme are less clear. In the present study we have used two different procedures to assess proteolytic activity. In the first one protein substrates were incorporated into the polyacrylamide gels, and the amount of protein and probably the large peptide fragments remaining after incubation of the gel with electrophoretically introduced CANP was recorded. In the second method, the fluorescamine reaction was used, in which the increase in the number of primary amino groups is measured, irrespective of the size of cleavage product.

The data obtained with the two methods used in our study indicate that the hydrolysis of casein, α-actinin and troponin complex by CANP leads to peptides of low molecular weight, whereas the digestion of myosin, tropomyosin and actin results in fragments of high molecular weight only. This conclusion is consistent with the analysis of digestion products by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Reddy *et al.*, 1975; Toyo-oka & Masaki, 1979). However, in the case of troponin subunits, the degradation into small peptides occurs simultaneously with the formation of large fragments (Reddy *et al.*, 1975; Toyo-oka & Masaki, 1979).

The ionic requirement for the digestion of myofibrils by CANP is of considerable interest. The removal of Z-lines and the release of α-actinin from isolated myofibrils occur only in the presence of Ca^{2+}. No other bivalent metallic ions such as Sr^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} can substitute for Ca^{2+} as the activator of CANP. This stringency was demonstrated by several experimental approaches, such as evaluation of the densitometric scans of myofibrils, measurement of the time needed for complete removal of Z-lines, production of material absorbing at 280 nm during digestion of myofibrils and finally by measurement of the content of α-actinin remaining in myofibrils after treatment with CANP. Moreover, primary amino groups appear in CANP-treated myofibrils only in the presence of Ca^{2+}; Sr^{2+} and Ba^{2+} are without effect.

When purified myosin was used, its digestion also proceeded only in the presence of Ca^{2+}, similar to the case of intact myofibrils. In contrast, the proteolysis of purified actin and tropomyosin was activated by Sr^{2+} and Ba^{2+} as well as by Ca^{2+}. Thus it appears that, in intact myofibrils, actin and tropomyosin are sterically hindered so that their hydrolysis cannot occur in the absence of Ca^{2+}.

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