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ISOLATION AND ELECTROPHORETIC PROPERTIES OF A CALCIUM-BINDING PROTEIN FROM THE CILIATE TETRAHYMENA PYRIFORMIS

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SUMMARY: A calcium-binding protein (TCBP) was isolated from the ciliate Tetrahymena pyriformis by a procedure which included 8 M urea extraction of the cell homogenate or boiling water extraction of acetone-dried powder of the cell, ammonium sulfate precipitation (60-80% saturation) and preparative electrophoresis on alkaline polyacrylamide gel. The TCBP has a molecular weight of 14,000 daltons and an isoelectric point of pH 4.0 and is easily detected in crude cell extracts by the change of electrophoretic mobility depending on Ca ion concentration. By equilibrium dialysis TCBP was found to bind to 2 moles of calcium per mole of the protein at a free calcium concentration of 10^{-4} M. On alkaline polyacrylamide gel, the TCBP interacts with rabbit skeletal muscle troponin I and with some material(s) of Tetrahymena origin to form Ca^{2+}-dependent complexes.

Calcium ion (Ca^{2+}) plays an important role in the regulation of muscle contraction, cell motility, and activity of the nervous system. Ca^{2+}-binding proteins (CBPs) or Ca^{2+}-dependent regulators (CDRs) corresponding to these Ca^{2+}-dependent physiological phenomena have been isolated from various cells and tissues, and their biochemical characteristics have been investigated by many workers (for review, see 1). These CBPs or CDRs include troponin C (TN-C) from skeletal muscle (2), parvalbumin from fish muscle (3), cyclic nucleotide phosphodiesterase activator (4, 5) and adenylate cyclase activator (6) from mammalian brain. Recently, CDRs have also been found in the lower organism such as coelenterate (7), annelid (8) and fungus (9).

In a ciliate, the existence of CBP is suggested by Ca^{2+}-dependent phenomena, such as ciliary reversal (10) or contraction of contractile fibers (11). In fact, "spasmin" which was supposed to be a CBP was found in the vorticellid ciliate Zoothamnium (12).

As a first step in studies on the mechanism of Ca^{2+} regulation in ciliates we have examined whether a CBP is present in the ciliate Tetrahymena

Abbreviations used were: TCBP, Tetrahymena Ca^{2+}-binding protein; CBP, Ca^{2+}-binding protein; CDR, Ca^{2+}-dependent regulator; TN-C, troponin C; TN-I, troponin I; EGTA, ethylene glycol-bis-(2-aminoethyl ether) N,N'-tetraacetic acid; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride; AU-gel, alkaline-urea gel; AG-gel, alkaline-glycerol gel.

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and have succeeded in the isolation of a CBP. During the course of the study on the characteristics of this protein, it has been found that the protein activates guanylate cyclase of Tetrahymena in the presence of Ca^{2+}. Details of the guanylate cyclase activation by the Tetrahymena CBP (TCBP) shall be reported in the following paper. In this paper, the isolation procedure and electrophoretic properties of the TCBP are reported.

MATERIALS AND METHODS: Tetrahymena pyriformis strain W was grown axenically at 26°C in a proteose-peptone medium as described previously (13). Electrophoresis on alkaline-urea gel (AU-gel, pH 8.3) and alkaline-glycerol gel (AG-gel, pH 8.3) were carried out by the methods of Perrie and Perry (14). Electrophoresis on sodium dodecyl sulfate (SDS)-phosphate gel and isoelectrofocusing were performed as described by Weber and Osborn (15) and Cummins and Perry (16), respectively. TN-C and troponin I (TN-I) were prepared from rabbit skeletal muscle as described previously (17). Tubulin was prepared from bovine brain by the method of Shelanski et al. (18). Calcium binding to the TCBP at 10^{-4} M calcium concentration was determined by equilibrium dialysis. A protein sample (0.3 mg) was dialyzed for 21 hrs against a solution of 5 mM Tris-HCl (pH 7.5), 0.1 M KCl, and 10^{-4} M $CaCl_2$ containing ^{45}Ca. At the end of the time, radioactivity of the outer and inner mediums was determined. Protein determination was based on the measurement of N by micro-Kjeldahl method (19) assuming an N content of 16%. Where the presence of non-protein N was expected, the Folin method (20) as well as the micro-Kjeldahl method was used.

RESULTS AND DISCUSSION: Changes in mobility of CBPs on AU-gel on addition of Ca^{2+} or ethylene glycol-bis-(2-aminoethyl ether) N,N'-tetraacetic acid (EGTA) can easily be observed by ordinary polyacrylamide gel electrophoresis (21), even when a crude extract of homogenate of cells or tissues is used.

For detection of a CBP in Tetrahymena, an 8 M urea extract of Tetrahymena cells and two representative CBPs, rabbit skeletal muscle TN-C and bovine brain tubulin (22), were analyzed by electrophoresis on AU-gel (Fig. 1). The mobility of TN-C was reduced 7.5% by the presence of 2 mM EGTA when compared with the mobility in the presence of 2 mM $CaCl_2$ (Fig. 1b), whereas that of tubulin was not changed at all (Fig. 1c). The urea extract of Tetrahymena showed many bands (Fig. 1a), and one of the fast-moving bands behaved like TN-C: that is, its mobility (arrow) was decreased 3.6% by addition of 2 mM EGTA. Head and Perry (21) proposed that the mobility change of TN-C caused by addition of Ca^{2+} reflects a conformational change of the protein by Ca^{2+}-binding. Although the mobility of a Ca^{2+}-binding protein such as tubulin was not decreased in the absence of Ca^{2+}, a mobility change in the presence or absence of Ca^{2+} is supposed to be a characteristic of some CBPs. Therefore, the protein of Tetrahymena that migrated faster in the presence of Ca^{2+} is most likely to be a CBP, and is provisionally referred to as Tetrahymena CBP (TCBP).

As a first step in purification of this protein, ammonium sulfate fractionation was carried out. Tetrahymena was homogenized in 2 vol. of distilled

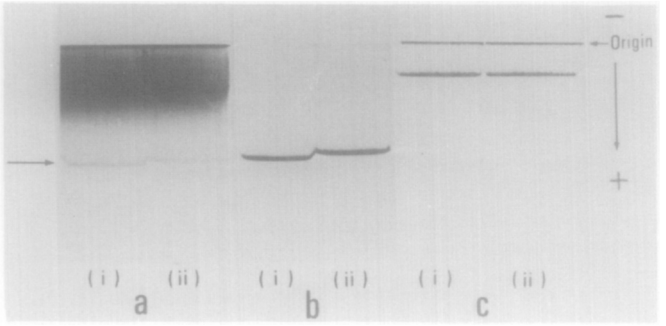

Fig. 1 Effect of Ca^{2+} on the electrophoretic patterns of Tetrahymena urea extract (a), rabbit skeletal muscle troponin C (b) and bovine brain tubulin (c). 10% Polyacrylamide gel was prepared with a solution of 24 mM Tris, 150 mM glycine (pH, 8.3), and 8 M urea (AU-gel). Either 2 mM $CaCl_2$ (i) or 2 mM EGTA (ii) was added to the samples. Tetrahymena urea extract was prepared as follows. Tetrahymena cells were homogenized in a blender with cold distilled water containing 10^{-4} M N-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK, Merck), added to inhibit the protease activity in Tetrahymena. The homogenate was centrifuged at 10,400xg for 20 min., and the precipitate was extracted with 8 M urea containing 1 mM EGTA and 10^{-4} M TLCK for 4 hrs. The suspension was then centrifuged at 45,000xg for 40 min. and the supernatant was used for electrophoresis.

water and the homogenate was centrifuged at 10,400xg for 20 min. The pellet was suspended in 4 vol. of 8 M urea and insoluble material was removed by centrifugation. The supernatant was dialyzed against 24 mM Tris-150 mM glycine buffer (pH 8.3) and then fractionated with ammonium sulfate at 0-60%, 60-80%, and 80-100% saturation. The three fractions obtained were dialyzed extensively against distilled water and lyophilized. Their electrophoretic patterns in the presence and absence of Ca^{2+} are shown in Fig. 2. Most of the TCBP was found in the fraction obtained with 60-80% saturation of ammonium sulfate, but a small amount was also found in the fraction obtained with 80-100% saturation when EGTA was included in the sample solution (Fig. 2c ii).

As shown in Fig. 2b, the TCBP migrated well in front of other proteins on AU-gel. This high mobility of TCBP facilitated its purification by preparative electrophoresis according to the method of Hirabayashi and Perry (17) from the fraction obtained with 60-80% saturation. Other important characteristic of the TCBP, found in the way of our experiment, was that it was quite stable in acetone or at a high temperature (95°C) for longer than 30 min. After the acetone and heat treatments neither the electrophoretic mobility nor regulatory function on the guanylate cyclase activity were changed as far as our experiments were concerned. Therefore, another more efficient procedure for the isolation was made up as follows: acetone-dried powder was prepared by washing Tetrahymena cells 4 times with acetone and extracted with 10 vol. of distilled water for 30 min. at 95°C. After cooled in an ice bath it was centrifuged at 30,000xg

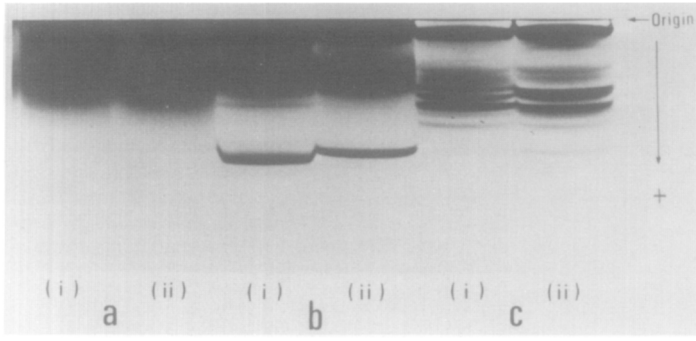

Fig. 2 Alkaline urea polyacrylamide gel (AU-gel) electrophoresis of Tetrahymena urea extract fractionated with ammonium sulfate. The urea extract was dialyzed against 24 mM Tris-150 mM glycine buffer (pH 8.3) and fractionated with 0% to 60%, 60% to 80%, and 80% to 100% saturation of ammonium sulfate. Each fraction was dialyzed extensively against distilled water and lyophilized. Conditions for electrophoresis were the same for Fig. 1. (a), 0-60% Fraction; (b), 60-80% fraction; (c), 80-100% fraction. Either 2 mM $CaCl_2$ (i) or 2 mM EGTA (ii) was added to the samples.

for 30 min. The supernatant was fractionated with solid ammonium sulfate between 60-80% saturation. The precipitate was dissolved in distilled water, dialyzed against it and lyophilized. The powder was used for further purification by preparative electrophoresis on AG-gel or AU-gel.

The preparation of TCBP obtained in these ways appeared pure and its molecular weight was calculated to be 14,000 daltons from its mobility in SDS-polyacrylamide gel electrophoresis. Its isoelectric point was found to be around pH 4.0 by electrofocusing.

Preliminary calcium-binding study of the TCBP performed by equilibrium dialysis showed that TCBP bound to 1.74 ± 0.14 moles of calcium per 14,000 g protein at 10^{-4} M free calcium concentration. Moreover, when a mixture of the TCBP and $^{45}Ca^{2+}$ was subjected to electrophoresis on AU-gel, the band of the TCBP correctly corresponded to the localization of $^{45}Ca^{2+}$ radioactivity (data not shown). This indicates that even in the presence of 8 M urea TCBP interacts with Ca ion.

The interaction of CBP with divalent cations caused some change in the characteristics of the protein; the spectral change of TN-C caused by Ca^{2+} is simulated to some extent by Cd^{2+}, Sr^{2+}, and Mn^{2+}, but not by Mg^{2+}, Co^{2+}, or Zn^{2+} (21). Therefore, we examined the effects of various ions on the electrophoretic mobility of the TCBP on AG-gel, in which urea in AU-gel was replaced by 40% glycerol to keep the native binding ability as much as possible. On AG-gel, unlike on AU-gel, the mobility of TCBP is smaller in the presence of Ca^{2+} than in the presence of EGTA (for details, see later). Divalent cations were added to the TCBP in 1 mM EGTA and analyzed by AG-gel electrophoresis

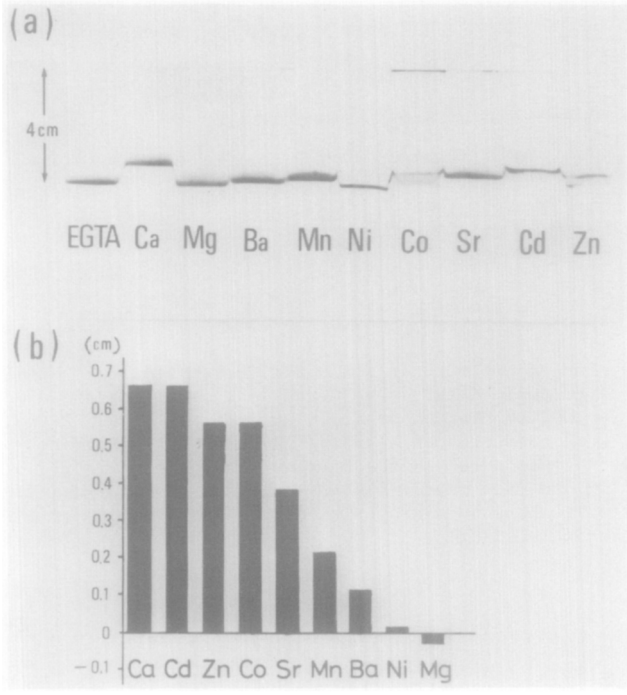

Fig. 3 Effects of divalent cations on the mobility of Tetrahymena Ca^{2+}-binding protein on alkaline glycerol gel (AG-gel). (a) Electrophoretic patterns in the presence of various cations. The protein sample was incubated with 1 mM EGTA, and then with 10 mM concentrations of the various divalent cations indicated. (b) Retardation of mobililty of Tetrahymena Ca^{2+}-binding protein in the presence of various divalent cations. The ordinate is the difference between the migration distance in the presence of EGTA and that in the presence of the cations.

(Fig. 3). The sample to the far left in Fig. 3a contained 1 mM EGTA without any added divalent cation. The retardations of bands in the presence of cations compared to this band of the TCBP + EGTA were measured and are shown in Fig. 3b. Cd^{2+} caused similar retardation to Ca^{2+}; Mg^{2+} caused no retardation, and other ions caused intermediate extent of retardation. Binding of divalent cations to the TCBP does not necessarily produce a retardation of the electrophoretic mobility, but the retardation observed suggests some interaction between the TCBP and the ions.

The mobility changes of TN-C and the TCBP can be observed on both AG-gel and AU-gel. Fig. 4 (a, b and c) shows the electrophoretic patterns of TN-C, the TCBP in a crude fraction, and the urea extract of Tetrahymena. TN-C migrated faster in the presence of Ca^{2+} than in the presence of EGTA on AG-gel as well as on AU-gel. However, the TCBP migrated faster in the presence of EGTA than in the presence of Ca^{2+} on AG-gel, and vice versa on AU-gel (Fig. 2b and Fig. 4b). Hitchman et al. (23) reported that a porcine intestinal CBP mi-

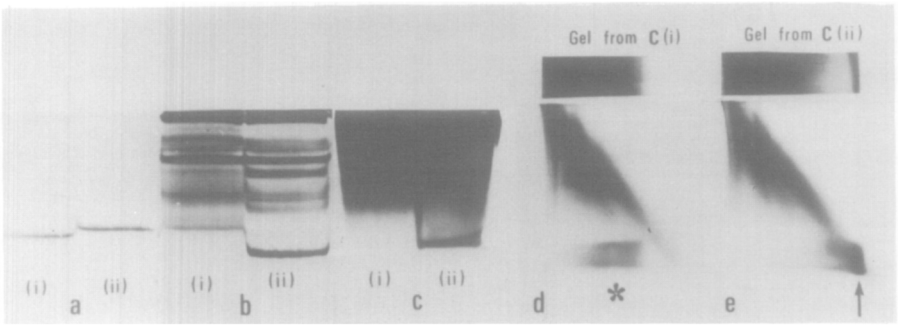

Fig. 4 Mobility changes of troponin C and the Tetrahymena Ca^{2+}-binding protein. Electrophoreses of troponin C (a), the Tetrahymena sample obtained by fractionation with 60% to 100% saturation of ammonium sulfate (b) and Tetrahymena urea extract (c) were conducted on alkaline glycerol gel. Either 2 mM $CaCl_2$ (i) or 2 mM EGTA (ii) was added to the sample. The gels of c (i) and c (ii) were cut vertically and laid on the second slab gels (d and e) horizontally, and the second electrophoresis was carried out in the presence of EGTA. For an asterisk and arrow, see text.

grated faster in the presence of EGTA than in the presence of Ca^{2+} in Davis' polyacrylamide gel electrophoresis system due to increase in the negative charge of the protein. Since as far as we know, TN-C is the only protein known to migrate faster in the presence of Ca^{2+} than in its absence in the gel electrophoresis system without urea, TN-C may have different properties from other CBPs or CDRs.

The electrophoretic pattern of the urea extract of Tetrahymena on AG-gel is interesting (Fig. 4c): in the presence of EGTA the mobility of TCBP increase, but in the presence of Ca^{2+} no band is found in the position where the TCBP should be found (Figs. 4b (i) and 4c (i)). Since TCBP by itself does not form any polymer in the presence of Ca^{2+} (Fig. 3a), the electrophoretic pattern in Fig. 4c suggests the existence of some material(s) which forms a Ca^{2+}-dependent complex with the TCBP. To confirm the existence of the complex, two-dimensional gel electrophoresis was conducted according to the method of Makioka and Hirabayashi (24). After the urea extract of Tetrahymena was electrophoresed in the presence of Ca^{2+}, the slab gel was cut vertically and laid on the second gel horizontally, and the second electrophoresis was carried out in the presence of EGTA (Fig. 4d), in which a broad band (* in Fig. 4d) appeared in a retarded position of the first electrophoresis as compared with that of the control band (arrow in Fig. 4e). Significant function of TCBP might be deduced from the studies on this Ca^{2+}-dependent complex in the urea extract.

Skeletal muscle TN-C and TN-I form a complex in the presence of Ca^{2+} even in alkaline urea solution (21). Furthermore, CDR from coelenterate (7) and

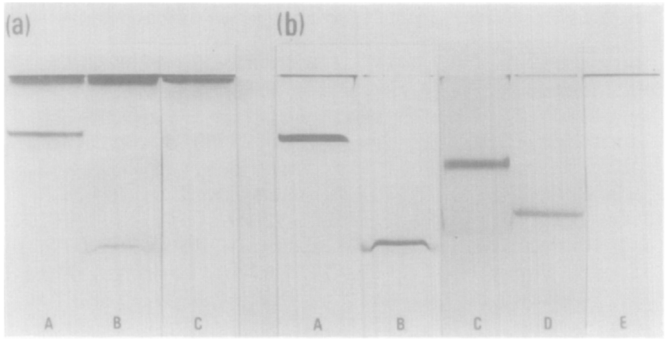

Fig. 5 Ca^{2+}-Dependent complex formation of Tetrahymena Ca^{2+}-binding protein (TCBP). (a) Complex formation between TCBP and rabbit skeletal muscle troponin I on alkaline glycerol gel (AG-gel). A, TCBP + troponin I, in the presence of 2 mM $CaCl_2$; B. TCBP + troponin I, in the presence of 2 mM EGTA; C, troponin I alone. (b) Complex formation between TCBP or rabbit skeletal muscle troponin C and lysozyme on alkaline glycerol gel (AG-gel). A, TCBP + lysozyme, in the presence of 2 mM $CaCl_2$; B, TCBP + lysozyme, in the presence of 2 mM EGTA; C, rabbit skeletal muscle troponin C + lysozyme, in the presence of 2 mM $CaCl_2$; D, rabbit skeletal muscle troponin C + lysozyme, in the presence of 2 mM EGTA; E, lysozyme alone.

TN-C-like proteins isolated from brain (25) and blood platelet (26) can form Ca^{2+}-dependent complexes with skeletal muscle TN-I. These data prompted us to examine the binding of TCBP to skeletal muscle TN-I. As shown in Fig. 5, TCBP bound to TN-I in the presence of Ca^{2+} on AG-gel. Ca^{2+}-dependent binding was also observed on AU-gel (data not shown). However, as shown in Fig. 5b, TCBP and TN-C also bound to lysozyme (Sigma) in the presence of Ca^{2+} on AG-gel. Lysozyme is a basic protein (pI, 11.0) and does not migrate into the gel by itself. These results throw some doubt on the functional significance and specificity of the TN-I-TCBP complex and the similar complex in Tetrahymena cells. However, the existence of some material(s) which forms a Ca^{2+}-dependent complex with TCBP seems reasonably certain. Investigation on this complex might provide information on the authentic function of TCBP in Ca^{2+} regulation as well as in the regulation of guanylate cyclase activity.

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