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Review

Calcium-Binding Measurement of Troponin by a Calcium Ion Sensitive Electrode

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Ca^{+2}-binding to rabbit skeletal troponin C and triponin was studied by using a Ca^{2+}-sensitive electrode. We can measure Ca^{2+}-binding in the presence or absence of Ca^{2+}-chelating agents such as EGTA to adjust pCa in a wide pH range and ionic strength comparing the results with those obtained by an equilibrium dialysis method. Troponin C has two high affinity sites and two low affinity sites and the Ca^{2+}-affinity of both sites was influenced by pH and ionic strength. The change of the Ca^{2+}-affinity in a pH range from pH 5.6 to 10.4 can be explained by the electrostatic interactions between Ca^{2+} ions and troponin C. In alkaline pH, four Ca^{2+}-binding sites bind Ca^{2+} with the same affinity and the distinction between the high affinity sites and the low affinity sites vanished. This result shows that the difference of the Ca^{2+}-affinity is owing to differences of the secondary or the tertiary structure of the Ca^{2+}-binding sites, not owing to the difference of the primary structures of the Ca^{2+}-binding sites. The two high affinity sites bound two Ca^{2+} ions cooperatively in neutral pH. The cooperativity diminished both in acidic and alkaline pHs.

EGTA or EDTA influenced Ca^{2+}-binding to troponin. In the absence of EGTA or EDTA, troponin binds four Ca^{2+} ions with strong cooperativity and seemed to bind four Ca^{2+} ions simultancously. On the other hand, in the presence of EGTA or EDTA, two Ca^{2+} ions bind to the high affinity sites and two Ca^{2+} ions bind to the low affinity sites with strong cooperativity in weakly alkaline pH where EGTA or EDTA was saturated with Ca^{2+} ions. In neutral pH, EGTA and EDTA interact with troponin and the Ca^{2+}-affinity of EGTA (EDTA) increased in the presence of troponin. Troponin I and troponin T interact with troponin C at pH 12 and stabilize the structure of troponin C in such a high pH. It is concluded that an equilibrium dialysis, unless it was supported with some complementary studies, is not a suitable method for the present system.

KEY WORDS: Calcium electrode/ Calcium titration/ Troponin/ Tropronin C/ Electrostatic interaction/ Equilibrium dialysis/

I. INTRODUCTION

Contraction of skeletal muscle is regulated by Ca^{2+} released from sarcoplasmic reticulum.[1] Ca^{2+} ions bind to troponin located on the thin filament and bring conformation change on troponin and the conformation change may transmit to thin

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Abbreviation used: EGTA, ethylene glycol bis (β-aminoethyl ether) N,N,N′,N′-Tetraacetic acid; MES, 2-(N-Morpholino)ethanesulfonic acid; PIPES, Piperazine-N,N′-bis(2-ethanesulfonic acid); Tris, Tris(hydroxymethyl)aminomethane; CAPS, 3-Cyclohexylaminopropane-sulfonic acid; A_{Ca}, Activity of Ca^{2+} ion; pCa, $-\log[A_{Ca}]$

filament and regulate actomyosin ATPase. Troponin is composed from three subunits, *i.e.* troponin *C*, troponin I, and troponin T. Potter and Gergely reported Ca^{2+}-binding to troponin C, troponin, and myofibril by an equilibrium dialysis method.[2] Troponin C has four Ca^{2+}-binding sites; two high affinity sites and two low affinity sites. Potter and Gergely interpreted that Ca-binding to the low affinity sites regulated contraction of skeletal muscle.[2] But the Ca^{2+}-titration curve of the low affinity sites was not coincident with the muscle tension development curve. Muscle tension is developed in a quite narrow pCa region[3], and therefore, Ca^{2+} ions should bind to the binding sites cooperatively and conformation changes following the Ca^{2+}-binding should take place cooperatively. Furthermore, skeletal muscle develops the tension more sharply than that of heart muscle, and heart muscle troponin C has two Ca^{2+}-binding sites[3,4] while skeletal troponin C has four Ca^{2+}-binding sites. It suggests cooperative binding of Ca^{2+} to troponin C, but Potter and Gergely reported no cooperative binding of Ca^{2+} to troponin C.[2] Whether the cooperativity is originated from cooperativity of Ca^{2+}-binding sites of a single troponin molecule or cooperativity of the other proteins on the thin filaments is important to interpret the regulation of muscle contraction by Ca^{2+} ions.

Equilibrium dialysis has been used to study the binding of Ca^{2+} to protein, in this method, however it is necessary to adjust pCa by a Ca^{2+}-chelating agent such as EGTA or EDTA. The association constant of Ca^{2+} to a chelating agent was assumed unchanged under those conditions, then it required that the chelating agent did not interact with the protein molecule. Furthermore, it is necesary that Ca^{2+}-affinity constant (pK_{Ca}) of the chelating agent is close to a pCa value where Ca^{2+}-binding is studied. Since Ca^{2+}-affinity of a chelating agent is very sensitive on pH, it is not possible to choose pH and ionic strength freely. These difficulties can be avoided by using a Ca^{2+} ion-sensitive electrode, a Ca^{2+}-titration curve can be obteined both in the presence or absence of a Ca^{2+}-chelating agent and also in a wide range of pH.[5,6]

II. Ca^{2+}-BINDING TO TROPONIN C

Troponin C is a subunit which shows a big conformation change upon Ca^{2+}-binding.[7,8] It is interesting to study a relationship between the conformation and Ca^{2+}-binding. Ca^{2+}-binding to troponin and troponin C was determined by using a Ca^{2+}-sensitive electrode as described.[5,6]

Troponin C binds four calcium ions, *i.e.* two ions to two high affinitay sites and two ions to two low affinitay sites in neutral pH as shown in Fig. 1. The Ca^{2+}-affinity of both of the high and low affinity sites increased with increasing pH. The results of the titrations are listed in Table 1. Troponin C contains as many as 46 carboxyl groups in a total of 159 amino acid residues[9] and the ionization of these groups should influence the conformation and the Ca^{2+}-bindign of troponin C.[10] The electrostatic effect on the Ca^{2+}-binding is expressed as follows:[10]

$$k_i = k_i^0 \exp(-2\,wzZ) \qquad (1)$$

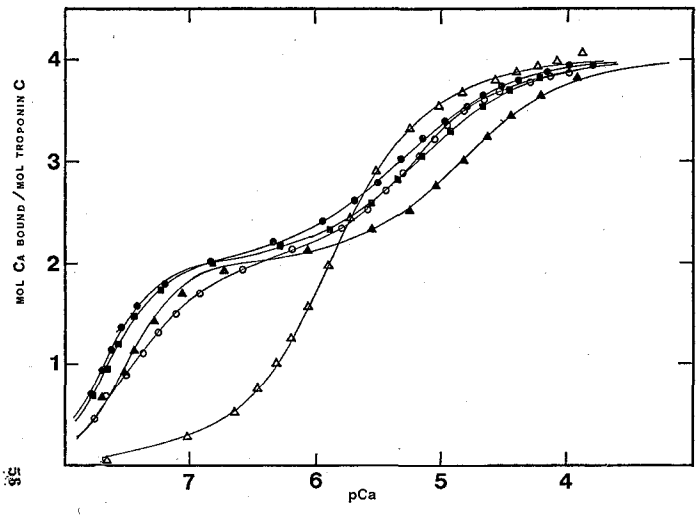

Fig. 1. Ca^{2+} binding to troponin C. Ca^{2+} binding was measured by using a Ca^{2+} sensitive electrode. Solid lines are calculated curves assuming the two high affinity sites bind Ca^{2+} with cooperativity and the low affinity sites bind Ca^{2+} independently. The association constants and the cooperativity were obtained by least square method and listed in Table 1. (▲): 0.1 M KCl, 20 mM Mes, pH 5.60. (■): 0.1 M KCl, 20 mM PIPES, pH 6.68. (●): 0.1 M KCl, 20 mM Tris-HCl, pH 8.10. (○): 0.1 M KCl, 20 mM CAPS, pH 10.0. (△): 0.1 M KCl, pH 12.0.

Tabl I. Ca^{2+}-binding constant of troponin C.

pH	$<pK_1>$	$(<pK_1>_0)$	pK_2	(pK_2^0)	L	solvent condition
5.6	7.49	(6.43)	4.79	(3.85)	110	0.1 M KCl, 20 mM MES
6.7	7.64	(6.10)	5.18	(3.85)	>200	0.1 M KCl, 20 mM PIPES
6.8	7.59	(6.03)	5.16	(3.81)	>200	0.1 M KCl, 10 mM Imi-HCl
8.1	7.68	(6.01)	5.34	(3.91)	>200	0.1 M KCl, 20 mM Tris-HCl
9.3	7.49	(5.76)	5.18	(3.70)	18	0.1 M KCl, 20 mM CAPS
10.0	7.57	(5.73)	5.29	(3.72)	5	011 M KCl, 20 mM CAPS
10.4	7.44	(5.49)	5.21	(3.52)	4	0.1 M KCl, 20 mM CAPS
12.0	5.94	(5.88[a])	5.80		2.1	0.1 M KCl
5.7	6.59		4.70		89	2 mM $MgCl_2$, 0.1 M KCl, 20 mM MES
8.1	6.85		5.08		>200	2 mM $MgCl_2$ 0.1 M KCl, 20 mM Tris-HCl
6.0	6.90	(6.04)	4.37	(3.64)	>200	1 M KCl, 20 mM MES
6.9	6.93	(5.96)	4.46	(3.63)	>200	1 M KCl, 16 mM PIPES
8.4	6.97	(5.95)	4.40	(3.52)	>200	1 M KCl, 20 mM Tris-HCl

[a]: assumed that the high and the low affinity sites are equivalent and the binding constants are same.

where k_i is a Ca^{2+}-association constant for site i and k_1^0 is an intrinsic association constant of that site, namely the one under the condition that the net charge of the protein is zero, w is an electrostatic interaction factor which is a function of the dimension of the protein and ionic strength, z is the valence of a calcium ion and Z is

the net charge of the protein. The values of k_1^0 and k_2^0 calculated by Eq. 1 are listed in Table 1 and are constant in the pH range from 5.5 to 8.1 as seen in the Table. This result shows that the dependence of Ca^{2+}-affinity on pH was explained by electrostatic interacions between Ca^{2+} and the protein. It means that the carboxyl groups at the Ca^{2+}-binding sites are ionized always in this pH range and the intirnsic Ca^{2+}-binding affinity of the sites is not changed. Circular dichroism measurements showed that helical content of troponin C increased rapidly below pH 6 and this conformational change does not influence the Ca^{2+}-affinity of troponin C. The intrinsic Ca^{2+}-association constant for the high affinity sites was decreased extensively above pH 9.3 but the constant for the low affinity sites decreased only slightly.

Ca^{2+}-affinity of both sites is decreased by increasing KCl concentration. Salt ions shield the electric charges on the protein and Eq. 1 describes the shielding effects. The values of w and the net charge of troponin C in 1M KCl were reported previously.[10] The intrinsic binding constant, k_0, is equal to that in 0.1M KCl as listed in Table 1, and it is shown that K^+ does not bind to the Ca^{2+}-binding sites.

The two low affinity sites bind Ca^{2+} ions independently to each other but the high affinity sites bind two Ca^{2+} ions cooperatively in the presence or absence of Mg^{2+} ion.[6] $<pK_1>$ in Table 1 is an averaged value of the high affinity sites and a paramenter L represents an extent of the cooperativity.[6] Teleman *et al.* reported positive cooperativity[11] but Potter and Gergely repotred no cooperativity in Ca^{2+}-binding to the high affinity sites.[2]

Troponin C binds four Ca^{2+} ions with the same affinity at pH 12 as shown in Fig. 1. Troponin C unfolds slightly at pH 12 and the fact that the Ca^{2+}-affinity of both of the high and low affinity sites is same at this pH shows that the difference of the Ca^{2+}-affinity in neutral pH is due to a difference in the secondary or tertiary structure of the binding sites, but not due to a difference in the primary structure of the sites.

III. CALCIUM BINDING TO TROPONIN

Although Ca^{2+}-binding to tropponin C is not influenced by the presence of EGTA or EDTA, Ca^{2+}-binding to whole troponin is affected by EGTA or EDTA.

The Ca^{2+}-titration curve of troponin in the absence of EGTA or EDTA is different from that of troponin C in neutral and weakly alkaline pH. At neutral or nearly neutral pH, troponin binds Ca^{2+} in very narrow pCa region as shown in Fig. 2 and Hill consttants are large (from 4 to 8). In alkaline pH, difference between the high affinity sites and the low affinity sites of troponin C appeared. Titration curve is not altered in the presence of Mg^{2+} ions. The titration curve shown in Fig. 2 completely different from that reported by Potter and Geigely who measured it by an equilibrium dialysis method.[2] They reported that Ca^{2+}-binding of troponin is same as that of troponin C but the affinity to Ca^{2+} is increased by 10-fold for both of the high and low affinity sites. The major differences between their measurements and ours are in the method used for pCa determination and existence or absence of of chelators. Therefore, we measured the Ca^{2+}-binding in the presence of EGTA next. It was postulated that Ca^{2+}-affinity of EGTA is not changed by the presence

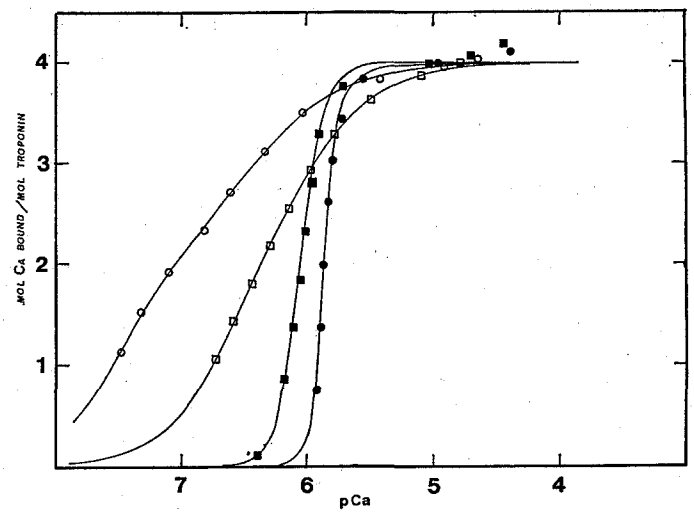

Fig. 2. Ca^{2+} binding to troponin in the absence of EGTA or EDTA. (●): 0.1 M KCl, 20 mM MOPS, pH 7.14. (■): 0.1 M KCl, 20 mM CAPS, pH 9.50. (○): 0.1 M KCl, 20 mM CAPS, pH 11.24. (□): 0.1 M KCl, pH 12.44.

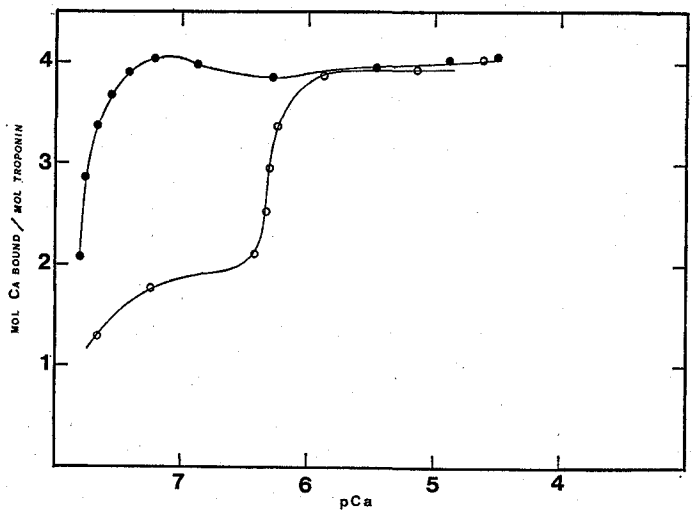

Fig. 3. Ca^{2+} binding to troponin in the presence of EGTA. (●): 0.2 mM EGTA, 0.1 M KCl, 20 mM MOPS, pH 7.17. (○): 0.2 mM EGTA, 0.1 M KCl, 20 mM Tris-HCl, pH 8.64.

of troponin as it was postulated by equilibrium dialysis method. In weakly alkaline pH, such as pH 8.64, where EGTA was saturated with Ca^{2+} ions, troponin binds four Ca^{2+} ions with two high and two low affinity sites as shown in Fig. 3. The result for the high affinity sites is consistent with that of Potter and Gergely, but the low affinity sites bind two Ca^{2+} ions with strong cooperatvity which contradicts with the report of Potter and Gergely.[2] On the other hand, the titration curve in neutral pH is completely different. The curve is similar with that in the absence of EGTA but

shifted to high pCa about one pCa unit. Apparently troponin binds more than four Ca^{2+} ions at the peak, and at the lower pCa side, the number gradually decreased to four mol Ca^{2+} per mol troponin as shown in Fig. 3. Although an exact titration curve was not obtained beyond pCa 7.5 because of a limit of the electrode, the results can be interpreted only when we assume that the Ca^{2+}-affinity of EGTA is increased more than 10 times by its interaction with troponin molecule. EGTA may interact with troponin and influence interactions between troponin subunits to result a change in the Ca^{2+}-affinity of troponin C, or EGTA molecules may be influenced from troponin and the Ca^{2+}-affinity of EGTA is increased by such interactions with troponin. Titration curves in the presence of EDTA are quite similar to those in the presence of EGTA.

To examine the interaction between EGTA or EDTA and troponin directly, EGTA (EDEA)-binding to troponin was measured by an equilibrium dialysis method. Ca^{2+}-binding was measured simultaneously. No EGTA nor EDTA bound to troponin both in the presence or absence of Ca^{2+} ions within the experimental error. The Ca^{2+}-binding determination by an equilibrium dialysis method shows that about two moles of Ca^{2+} ions bind to one mole of troponin even at pCa 9 and the Ca^{2+} affinity of troponin might be larger than $10^{9,5}$ if we assumed that the Ca^{2+}-affinity of EGTA was constant, irrespective of the presence of troponin. Fluorescence intensity changes at the lower pCa region and it contradicts with this result. We can interpret this Ca^{2+}-binding experiment in another way: Ca^{2+}-affinity of EGTA (EDTA) in a dialysis tube was not same as that in the outer solution and Ca^{2+}-binding at high pCa region is contributed by Ca^{2+}-binding to EGTA (EDTA). If EGTA (EDTA) interacts with troponin in such a way, analysis of equilibrium dialysis measurements is very difficult and we cannot conclude whether troponin interacts with EGTA (EDTA) or not by those experiments. The methods other than an equilibrium dialysis are needed to solve the problem unambiguously, and investigations to get more conclusive results are now in progress in our laboratory.

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