Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 447-451

CALCIUM REGULATES TROPONIN-TROPOMYOSIN BINDING IN THE RECONSTITUTED THIN FILAMENT

T.-I Lin, P. Lambert, and R. M. Dowben

Medical Research Institute
Baylor University Medical Center
Dallas, Texas 75246

Received June 1, 1983

Steady-state fluorescence anisotropy technique was used to determine the binding constant of troponin for IAEDANS-labeled tropomyosin under various conditions. In the absence of actin, Ca does not affect the binding between troponin and tropomyosin. The presence of actin greatly strengthens troponin-tropomyosin binding in the absence of Ca. However, Ca weakens troponin-tropomyosin binding by about 2.5-fold in the reconstituted filament. It is suggested that the Ca-regulated binding may serve as a molecular switch for the troponin molecule to get "on" and "off" the actin-myosin interaction site regulating muscle contraction-relaxation cycles.

It is well known that calcium regulates muscle contraction mediated via troponin and tropomyosin (1). In the absence of Ca, troponin inhibits the actin-myosin binding, while this inhibition is lifted in the presence of μ M concentration of Ca. Although models (2,3) have been proposed for the Ca-regulation mechanism based largely on studies using X-ray diffraction and three-dimensional image reconstruction from optical diffraction of electron micrographs techniques, it is still unknown how Ca affects the binding interactions between various thin filament proteins.

In this communication, we report the use of fluorescence anisotropy titration technique for the determination of binding constant between troponin and tropomyosin in the presence of Ca and/or actin.

MATERIALS AND METHODS

Hydroxylapatite was from Bio-Rad and 5-(iodoacetaimidoethyl)amino-naphthalene-1-sulfonic acid (IAEDANS) was from Molecular Probes. All other chemicals were of the highest analytical grade available. Ethanol-ether and acetone powders were prepared from the back and leg muscles of rabbits (4). Crude troponin and tropomyosin were extracted from ethanol-ether powder and separated by ammonium sulfate and isoelectric fractionations. Troponin was applied to a 2.5 X 25 cm DEAE-Sephadex column equilibrated with 20mM Tris/HCl at pH 7.5. A 550 ml gradient from 0 to 0.4 M

0006-291X/83 $1.50

LiCl was used to elute troponin (at 0.3 M). Tropomyosin and actin were prepared and purified as described previously (5,6). Before use, troponin and tropomyosin were dialyzed to remove traces of Ca and then in the sample buffer containing 150 mM KCl, 10 mM phosphate, pH 7.0. The purity of proteins was checked by SDS-PAGE. Preparation of IAEDANS-labeled tropomyosin and determinations of concentrations of dye, labeled and unlabeled tropomyosin were described previously (7). The concentrations of troponin and actin were determined by their absorbance using extinction coefficients of 0.234 at 280 nm and 0.63 (mg/ml) at 290 nm respectively.

The binding of troponin to IAEDANS-labeled tropomyosin and to labeled tropomyosin-actin was followed on an SLM Model 8000 fluorescence polarization spectrometer. The apparatus uses dual channel analyzers arranged in a T-configuration with ratio-mode recording. Samples were excited at 345 nm and emission was measured at above 400 nm using a pair of matched long pass filters. Samples were thermostated at 25°C controlled by a circulating water bath. Concentrated troponin, typically 25 μM was added in small increments to a 2 ml sample buffer solution of labeled tropomyosin (1 μM) or actin-tropomyosin. For actin experiments, labeled tropomyosin was mixed with F-actin in a 1:7 molar ratio with 0.02 mM $MgCl_2$-ATP (final concentration) in addition to the regular buffer solution. For Ca and EGTA experiments, reagents were added to both troponin and sample mixtures. Binding curves were obtained by monitoring the fluorescence anisotropy changes of labeled tropomyosin or labeled tropomyosin-actin associated with troponin binding over a titration range of troponin to tropomyosin up to 3.5:1.

The addition of troponin to the labeled tropomyosin caused a substantial increase in anisotropy without noticible change in overall fluorescence intensity. It is assumed that the observed total anisotropy is equal to the sum of the anisotropies of the bound and unbound labeled tropomyosin. The fraction of bound labeled tropomyosin x is determined by eq. (1)

$$x = (A_x - A_f)/(A_b - A_f) \qquad (1)$$

where A_f, A_b, A_x are respectively fluorescence anisotropies of unbound and completely bound labeled tropomyosin and at concentration x of labeled complex. The binding constant of troponin for labeled tropomyosin can be determined by equation (2)

$$K_a = 1/(TN) \cdot x/(1-x) \qquad (2)$$

where (TN) is the concentration of the unbound troponin.

The binding constants are determined by either graphic methods, i.e. direct plot of 1/(TN) vs x/(1-x), Scatchard and Hill plots or nonlinear least squares curve fitting method by computer (see below).

RESULTS AND DISCUSSION

The anisotropy of labeled tropomyosin is typically about 0.066 to 0.07 which varies slightly in different preparations and concentrations. Since tropomyosin is a fairly elongated molecule, its rotational relaxation time is considerably long. The small anisotropy of labeled tropomyosin suggests that the label undergoes rapid local wobbling motion. Addition of troponin causes an overall anisotropy change of 0.038, or a 57 % increase, when labeled

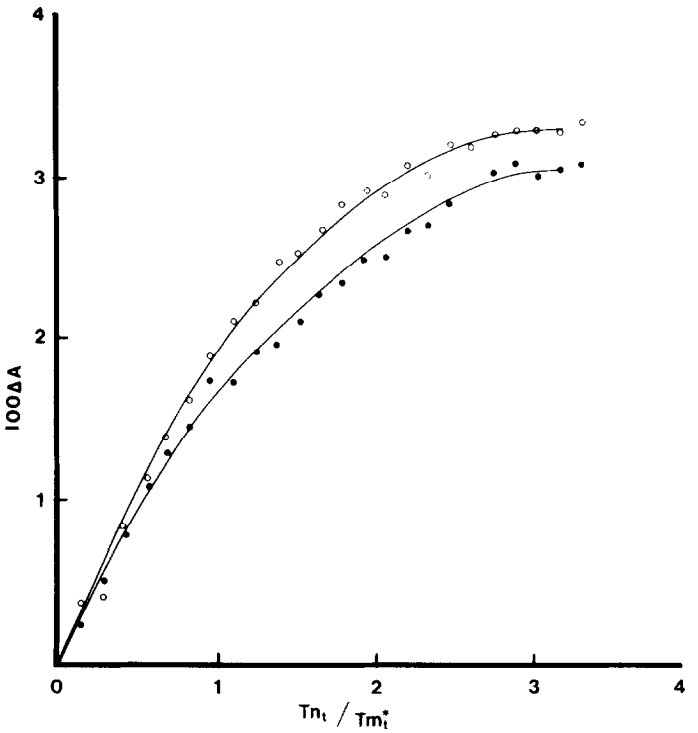

Fig. 1. The effects of Ca (solid circles) and EGTA (open circles) on the binding curve of troponin for 1 μM IAEDANS-labeled tropomyosin in the presence of 7 μM actin. Line-drawn curves are the computer simulated curves with K_a = 2.76 x 10^6 M^{-1} (lower curve) and K_a = 7.14 x 10^6 M^{-1} (upper curve).

tropomyosin is saturated with troponin. Analyses of troponin-tropomyosin binding curve (not shown) by graphic methods reveal a simple bimolecular binding mechanism following eq. (2) for troponin-tropomyosin interaction with a 1:1 binding stoichiometry. Addition of 2.5 mM $CaCl_2$ produced virtually no effect on the binding curve as compared to the addition of 2.5 mM EGTA. This result is consistent with our preliminary study (8) which reports the apparent dissociation constants in the presence and absence of Ca to be 0.532 ± 0.192 μM and 0.587 ± 0.287 μM respectively.

The effect of Ca on the troponin-tropomyosin binding curve in the presence of actin is shown in Fig. 1. Anisotropy of labeled tropomyosin in tropomyosin-actin filament is about 0.084, or about 27 % higher than in the absence of actin. The addition of troponin further increases the anisotropy with the same net increase as in the absence of actin. It seems obvious that Ca weakens the troponin-tropomyosin binding affinity.

The apparent binding constant can be obtained by using eq. (2) once the concentration of bound complex at every given total concentration of troponin and tropomyosin is determined by eq. (1). However, experimentally, it is difficult to obtain A_b accurately since very high concentration of troponin must be employed. The anisotropy for the bound labeled complex can be obtained graphically from a double reciprocal linear plot of net anisotropy changes vs molar ratios of Tn_t/Tm_t and extrapolating a straight line to infinite troponin concentration. Once A_b is obtained, several linear plots are used to calculate K_a by linear regression technique. However we found that in these types of plots, K_a depends too sensitively on the value A_b used in the calculation resulting in large errors. A better way for K_a determination is to treat both K_a and A_b as unknown parameters and the observed A_x, total concentrations of troponin and tropomyosin as three experimental variables and use nonlinear least squares curve fitting technique to fit experimental data with best values of K_a and A_b. Values of K_a and A_b obtained by graphic methods are used as initial values for a computer program which calculates the sum of the squares of the difference between the calculated and the observed anisotropies for all data points. The computer program minimizes the sum by a Gauss-Newton algorithm using an iteration procedure until values of K_a and A_b are converged. The binding constants obtained this way still vary due to the slight inconsistency of A_b values in different experiments. The average value for the binding constant is obtained from three protein preparations and 12 independent measurements. The mean value for the binding constant between troponin and tropomyosin in the reconstituted thin filament in the presence of Ca is $(2.76 \pm 0.62) \times 10^6\ M^{-1}$ and in the presence of EGTA is $(7.14 \pm 0.85) \times 10^6\ M^{-1}$.

Note that although there is a large standard deviation between measurements, the difference in the binding constant between the presence and absence of Ca is consistent; troponin appears to bind tropomyosin about 2.5 times stronger in the absence of Ca than in its presence. Similar

results were obtained when Ca-EGTA buffers were used to repeat the experiment. The binding constant is about 2.5 times higher in a 10^{-9} M Ca buffer than in a 10^{-6} M Ca buffer.

In conclusion, it seems that actin greatly strengthens the binding interaction between troponin and tropomyosin. The binding interaction is strongly dependent on the physiological concentration of Ca. Thus, it is suggested that the Ca-regulated binding may serve as a molecular switch for the troponin molecule to get on and off the myosin binding site on the actin molecule and may also be responsible for the Ca-dependent movement of the tropomyosin molecule in the thin filament observed in the early studies (2,3).

Acknowledgement. This work was supported in part by a grant from the National Heart, Lung, and Blood Institute (HL-16678). We wish to thank David H.-C Lee for his participation in the early phase of this work.

REFERENCES

1. Ebashi, S., Endo, M., and Ohtsuki, I. (1969). Quart. Rev. Biophys. 2:351-397.
2. Huxley, H. E. (1972) Cold Spring Harbor Symp. Quant. Biol. 37:361-376
3. Wakabayashi, T., Huxley, H. E., Amos, L. A., and Klug, A. (1975) J. Mol. Biol. 93:477-497.
4. Briskey, E.J. and Fukazawa, T. (1971) Adv. in Food Res. 19:333-343.
5. Lin, T.-I (1982) Biophys. Chem. 15:277-288.
6. Lin, T.-I and Dowben, R. M. (1982) Biophys. Chem. 15:289-298.
7. Lin, T.-I and Dowben, R. M. (1983) J. Biol. Chem. 258:5142-5150.
8. Lin, T.-I, Lambert, P., and Dowben, R. M. (1983) Biophys. J. 47:297a.