Ca^{++} INDUCED CONFORMATIONAL CHANGES IN THE Ca^{++} BINDING COMPONENT OF TROPONIN

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Summary. The Ca^{++}binding component of troponin undergoes reversible conformational changes in the presence or absence of micromolar amounts of Ca^{++}. The presence of Ca^{++} causes an increase in the fluorescence intensity of the tyrosine residues as well as an increase of the helix content. The binding constant is $5.10^{6}M^{-1}$. Fluorescence polarisation shows that the motion of the tyrosine residues is much smaller in the presence than in the absence of Ca^{++}. Thus the Ca^{++} binding component of troponin appears more rigid in the presence than in the absence of Ca^{++}.

Muscle contraction is regulated by small amounts of Ca^{++}, which released from the sarcoplasmic reticulum on excitation of the nerve, bind to troponin located on the thin filaments[1]. It is generally assumed that Ca^{++} induces a conformational change in troponin, which in turn induces a change in tropomyosin and ultimately in actin, resulting in the binding of actin to myosin. Accordingly interest has arisen recently in the substructure of troponin. In three laboratories troponin has been fractionated into 2 (Hartshorne and Mueller[2]), 3 (Ebashi et al.[3]), and 4 subunits (Greaser and Gergely[4]), only one of which appears to bind Ca^{++}. This component can be readily identified as it has an unusually low E_{280}/E_{260} ratio because of the absence of tryptophan and the high Phe/Tyr ratio[5].

We set out to test whether the conformation of this Ca^{++} binding component (CBC) changes on the presence or absence of Ca^{++}. We have found that indeed Ca^{++} induces a striking change in the conformation of this component.

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MATERIALS AND METHODS.

Preparation of troponin and CBC. Troponin was prepared from rabbit skeletal muscle according to the procedure of Ebashi et al.[6]. Troponin was checked for the absence of tropomyosin by its inability to regulate the superprecipitation of synthetic actomyosin in the absence of any added tropomyosin. CBC was prepared from troponin by chromatography on DEAE Sephadex in 6M ura as described by Greaser and Gergely[4]. CBC showed a single band on gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)[7] and showed very little tryptophan fluorescence on excitation at 290 nm.

Ultraviolet absorption. The UV spectrum of CBC was measured with a Zeiss model PMQ 2 spectrophotometer.

Fluorescence spectroscopy. An Hitachi model MPF-2A fluorescence spectrophotometer was used for fluorescence measurements. The sample compartment was water-jacketed and connected with a Haake thermoregulator. Tyrosine fluorescence was observed at 306 nm., after excitation at 276 nm. The OD_{276} value of the sample was always less than 0.10. Polacoat ultraviolet polarising filters (105 UV) were used for measuring the degree of fluorescence polarisation. The rotary molecular motion was calculated using Perrin's equation[8], from observations of the degree of fluorescence polarisation P, at a number of temperatures and viscosities. Glycerol was used to change the viscosity at constant temperature.

Optical Rotation Dispersion measurements. The apparatus used was a Jasco Autospectrometer UV 5. The helix content was calculated from the specific rotations at 233 nm. For 100% helix a $[m]'_{233}$ value of 14,700, and for 0% helix a $[m]'_{233}$ value of 2200 was taken[9].

Ca^{++} concentration. Ca^{++} concentrations were adjusted using

a Ca-buffer of Ca^{++} and 0.5 mM EGTA (ethylene glycol bis (β-amino-ethyl ether)-N,N' tetraacetic acid) in 10 mM cacodylate-HCl buffer at pH 6.8 [10].

Protein concentration. Protein concentrations were determined using the method of Lowry[11].

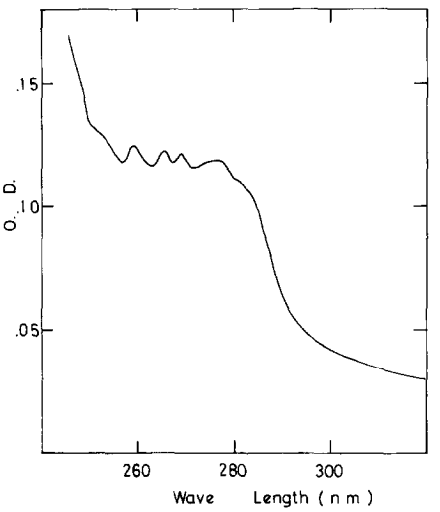

Figure 1. Absorption spectrum of the Ca^{++} binding component of troponin. The protein concentration is 0.26 mg/ml in 0.3 mM $NaHCO_3$. The spectrum is identical in the presence and absence of Ca^{++}.

RESULTS AND DISCUSSION.

Ultraviolet spectrum. The UV spectrum of CBC is shown in Figure 1. The maximum at 275 nm. corresponds to the maximum of the tyrosine absorption. The maxima at 259, 266 and 269 nm. correspond to maxima of the phenylalanine absorption. In most proteins the finestructure of the phenylalanine absorption cannot be observed because the molar extinction of phenylalanine is much lower than those of tyrosine and tryptophan. In CBC however, no tryptophan is present and the tyrosine content is low[5], so that the finestructure of the phenylalanine absorption easily can be detected.

Fluorescence intensity and helix content. The helix content

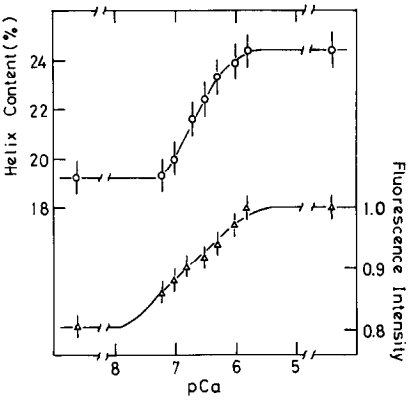

Figure 2. The helix content and change in fluorescence intensity of CBC versus Ca^{++} concentration at 25°C. o-o ; helix content, Δ-Δ ; fluorescence intensity. Vertical bars represent possible errors due to line width. Protein concentrations are o.45 and 0.12 mg/ml for the helix content and the fluorescence measurements respectively. The binding constant calculated from these curves is $5.10^{6}M^{-1}$.

and the tyrosine fluorescence intensity of CBC as a function of the Ca^{++} concentration are plotted in Figure 2. There is a very distinct increase in helix content, which is paralleled by an increase in the fluorescence intensity of the tyrosine residues. The binding constant for the Ca^{++}-CBC complex, estimated from these curves is $5x10^{6}M^{-1}$. This is in good agreement with the value of $5.4x10^{6}M^{-1}$ obtained by Hartshorne and Pyun[12] using the Chelex 100 method. The increase in helix content and the increase in fluorescence intensity could be reversed by adding EGTA to bind any excess Ca^{++}. The increase in fluorescence intensity could not have been caused by aggregation of CBC molecules but only by an intramolecular change because the UV spectrum was identical in the presence and absence of Ca^{++}. The conformational change in CBC occurs at Ca^{++} concentrations in the micromolar range, i.e. at the physiological concentration where muscle contraction is regulated.

Fluorescence polarisation. With changing temperature shows the Perrin's plot a straight line in the presence of Ca^{++} (see

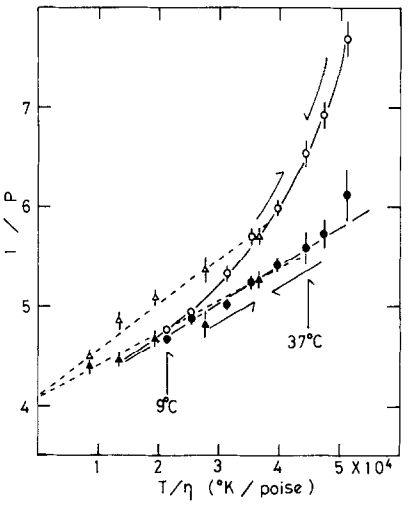

Figure 3. The effect of temperature and viscosity on the fluorescence polarisation of CBC. o-o ; heating curve at pCa>8 ●-● ; heating curve at pCa=5.7. Δ-Δ ; isothermal curve at 30°c and pCa>8. ▲-▲ ; isothermal curve at 30°C and pCa=5.7. Vertical bars represent the standard deviation of 3 observations. T is the absolute temperature and η is the viscosity of the solvent.

Figure 3). On the other hand there is a large deviation from a straight line in the absence of Ca^{++}. This suggests that in the presence of Ca^{++} the tyrosine residues occupy sites within the protein molecule which allow only very limited motion. However, in the absence of Ca^{++}, the tyrosine residues easily can undergo thermal activation of motion independent of the overall motion of the CBC molecule. More accurate rotational relaxation times of the tyrosine residues in CBC can be obtained from a Perrin's plot with changing viscosities at a constant temperature (isothermal Perrin's plot, see Figure 3), because in that case the thermal activation of motion is constant. For the fluorescence lifetimes at 30°c we used the values of 1.8 and 2.2 nsec in the absence and presence of Ca^{++} respectively. These values were derived from a value of 2.6 nsec for the fluorescence lifetime of free tyrosine in water at 23°C [13] and corrected for fluorescence intensity changes (Fi-

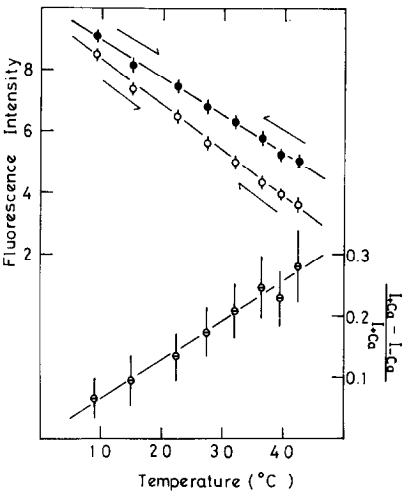

Figure 4. The temperature effect on the fluorescence intensity of CBC. o-o ; at pCa>8, ●-● ; at pCa=5.7, ⊖-⊖ ; the relative difference in fluorescence intensity between the 2 conditions. Vertical bars represent possible errors due to line width.

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gure 4). Using these data we obtained a rotational relaxation time of 14.5 and 29.2 nsec in the presence and absence of Ca^{++} respectively at 30°C. These values support the results of the temperature variation that CBC is flexible in the absence of Ca^{++} and becomes rather rigid on the addition of Ca^{++}.

The temperature effect on the fluorescence intensity is shown in Figure 4. Strong thermal quenching of the fluorescence can be observed, while the difference in intensity of Ca^{++}-bound CBC and Ca^{++}-free CBC is increased with raising temperature. The conformational change in CBC is therefore much more pronounced at physiological temperatures.

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