# Comparative studies on thermostability of calmodulin, skeletal muscle troponin C and their tryptic fragments

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## 1. INTRODUCTION

Troponin C and calmodulin belong to the family of homologous intracellular calcium binding proteins. Each is composed of 4 calcium-binding domains. In the case of skeletal muscle troponin C, 2 of calcium-binding sites are high-affinity sites which also bind $Mg^{2+}$ and 2 others are low-affinity, so-called '$Ca^{2+}$-specific' sites [1]. In calmodulin molecule the affinity constants for all 4 calcium sites are similar to each other, having values roughly between those of the low and high affinity sites of troponin C [2,3]. $Mg^{2+}$ competes with $Ca^{2+}$ for all binding sites in calmodulin [2,3]. Binding of $Ca^{2+}$ to troponin C and calmodulin causes large conformational changes in the secondary structure of the molecules.

Tryptic cleavage of troponin C and calmodulin [4–6] has enabled us to analyse $Ca^{2+}$- and $Mg^{2+}$-induced conformational changes in the particular parts of these 2 proteins separately. The increase of $\alpha$-helix content caused by the binding of $Ca^{2+}$ to troponin C is much higher for the C-terminal part of molecule, containing high affinity binding sites, than for the N-terminal part, containing low affinity binding sites [7]. The difference between the extent of $Ca^{2+}$-dependent increase of $\alpha$-helix in the N- and C-terminal halves of calmodulin is much smaller than that in troponin C [8,9].

To obtain additional information about the effect of $Ca^{2+}$ and $Mg^{2+}$ on the secondary structure of $Ca^{2+}$-binding proteins we have studied thermostability of skeletal muscle troponin C and calmodulin using circular dichroism technique and the influence of $Ca^{2+}$- and $Mg^{2+}$-binding on thermal unfolding. The use of the tryptic fragments of both calcium-binding proteins enabled to localise the structural changes caused by temperature changes in the particular parts of both molecules.

## 2. MATERIALS AND METHODS

Bovine brain calmodulin [10] and rabbit skeletal troponin C [11] were prepared as described. The fragments were prepared as in [4–6]. The purity of proteins and their fragments were checked by SDS– and urea–polyacrylamide gel electrophoresis. Measurement of the dependence of ellipticity at 222 nm ($\theta_{222}$) on temperature was performed in 0.93 nm rectangular cell using J-41A Jasco Spectropolarimeter. The rate of heating was 0.9°C/min and the temperature changes were monitored by a thermistor inserted in the thermostated cell holder. The highest available temperature using a water thermostat was 92°C.

The melting curves were plotted in an $XY$ recorder in which the $X$ coordinate was the linear temperature scale driven by the thermistor voltage and the $Y$ coordinate was driven by the ellipticity signal from J-41A instrument. The first derivatives of the curves showing the changes of $\theta_{222}$ depending on the temperature ($d\theta_{222}/dt$) were obtained with the HP 9825 A computer equipped with digitizer and plotter. The positions of maxima of the circular dichroism thermal unfolding profiles

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so obtained ($T_{1/2}$) were corrected for the difference between the temperature in the cell holder and that of the measured solution in the cell. Since only values $\theta$ (ellipticity) not $[\theta]$ (mean residual ellipticity) were measured so heights of maxima are not comparable and all curves in the figures are normalized. Protein was 0.2–0.5 mg/ml. All measurements were performed in 20 mM Hepes buffer, pH 7.0 (whose thermal pH dependency is $pK_a/1°C = -0.014$) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA and 5 mM $Mg^{2+}$ or 2 mM EDTA.

## 3. RESULTS

The size of the fragments used and their position in the original protein molecule are given in table 1. The TR-C fragments (i.e., fragments obtained by digestion of the proteins in the presence of $Ca^{2+}$) correspond in both cases to the two halves of each protein studied (i.e., $TR_1$-C fragment to the N-terminal and $TR_2$-C to the C-terminal half). In case of calmodulin the TR-E fragments (i.e., the fragments obtained upon cleavage in the presence of EDTA) were also analysed. The $TR_1$-E fragment contains in addition to two N-terminal domains the third loop as well and a part of $\alpha$-helical fragment following the loop. $TR_3$-E contains only one entire domain (the fourth one) and a piece of $\alpha$-helical fragment from the third domain.

Upon binding $Ca^{2+}$ not only calmodulin and troponin C [12,13], but also both TR-C as well as $TR_1$-E fragments are extremely thermally stable. At $\leq 90°C$, the derivatives show no maxima and only a slow increase due to a slight change in the ellipticity with temperature. An example of this type of the curve is given for $TR_1$-E fragment in fig.3. The ratio of $\theta_{90°}:\theta_{12°}$ was in all cases in the range of 0.58–0.84 (table 1).

Fig.1 shows the first derivatives of the circular dichroism thermal unfolding profiles of calmodulin, troponin C and their TR-C fragments measured in the presence of EDTA. The calmodulin thermal unfolding profile has a maximum at 55°C with a shoulder at ~30°C. The $T_{1/2}$-values of $TR_1$-C and $TR_2$-C of calmodulin are somewhat lower than those of intact calmodulin, and show no shoulder. $TR_2$-C has a higher halfwidth of transition than $TR_1$-C and, in addition, the shape of its thermal unfolding profile is much more asymmetric. The $T_{1/2}$ of intact troponin C in the presence of EDTA is the same as that of calmodulin (55°C), but for $TR_1$-C the $T_{1/2}$ is higher (60°C) than that for the homologous calmodulin fragment. On the other hand, the $TR_2$-C fragment of troponin C has very low $T_{1/2}$ (20°C), much lower than the homologous calmodulin fragment. The ratio of $\theta_{90°}:\theta_{12°}$ for proteins and all TR-C fragments measured in the presence of EDTA is 0.24–0.38.

Table 1

Parameters of thermal unfolding for calmodulin, troponin C and their fragments

| Protein | Fragment | Amino acid residues | $T_{1/2}$ (°C) in the presence of | | | $\theta_{90°}:\theta_{12°}$ ratio in the presence of | | |
|---|---|---|---|---|---|---|---|---|
| | | | $Ca^{2+}$ | $Mg^{2+}$ | EDTA | $Ca^{2+}$ | $Mg^{2+}$ | EDTA |
| Calmodulin | | 1–148 | >90 | >90 | 55 | 0.72 | 0.49 | 0.32 |
| | $TR_1$-C | 1– 77 | >90 | 80 | 49 | 0.75 | 0.35 | 0.25 |
| | $TR_2$-C | 78–148 | >90 | 76 | 46 | 0.84 | 0.51 | 0.33 |
| | $TR_1$-E | 1–106 | >90 | 88 | 58 | 0.83 | 0.60 | 0.24 |
| | $TR_3$-E | 107–148 | 45 | – | – | 0.45 | – | – |
| Troponin C | | 1–159 | >90 | 72 | 55 | 0.58 | 0.43 | 0.38 |
| | $TR_1$-C | 9– 84 | >90 | 68 | 60 | 0.67 | 0.36 | 0.33 |
| | $TR_2$-C | 89–159 | >90 | 83 | ~20 | 0.72 | 0.45 | 0.34 |

Measurements were performed in 20 mM Hepes buffer (pH 7.0) and in the presence of 1 mM $Ca^{2+}$ (in case of $TR_3$-E fragment 5 mM $Ca^{2+}$) or 1 mM EGTA and 5 mM $Mg^{2+}$, or 2 mM EDTA

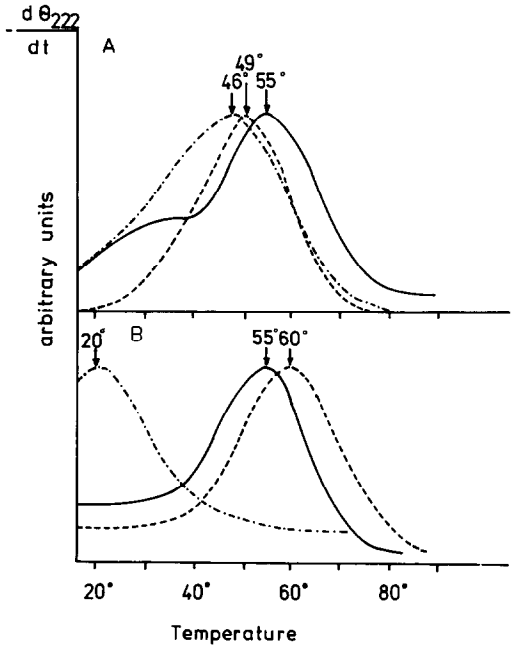


Fig.1. Circular dichroism thermal unfolding profiles of calmodulin (A) and troponin C (B) and their TR-C fragments in EDTA. Measurements were performed in 20 mM Hepes buffer (pH 7.0) with addition of 2 mM EDTA: intact proteins (——); $TR_1$-C fragments (– – –); $TR_2$-C fragments (-----).

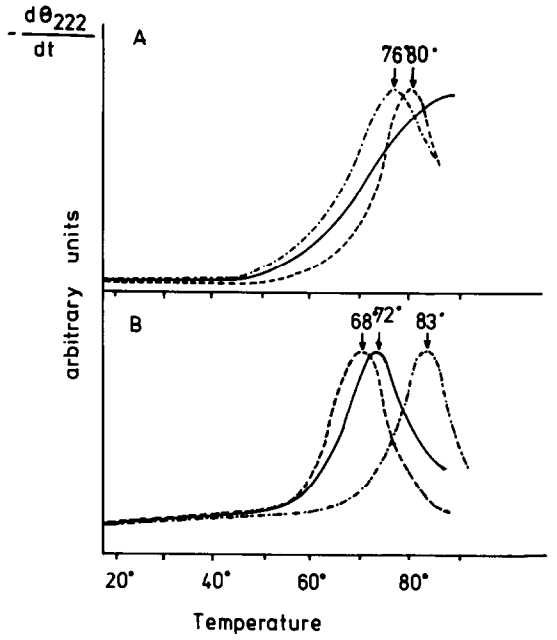


Fig.2. Circular dichroism thermal unfolding profiles of calmodulin (A), troponin C (B) and their TR-C fragments in $Mg^{2+}$. Measurements were performed in 20 mM Hepes buffer (pH 7.0) with addition of 1 mM EGTA and 5 mM $Mg^{2+}$: intact proteins (——); $TR_1$-C fragments (– – –); $TR_2$-C fragments (-----).

Fig.2 shows that $Mg^{2+}$ partially stabilizes the structure of both proteins as well as their TR-C fragments. The stabilization caused by $Mg^{2+}$ is higher for intact calmodulin than for troponin C. Both $TR_1$-C and $TR_2$-C fragments of calmodulin are similarly affected by $Mg^{2+}$. The effect of $Mg^{2+}$ on the TR-C fragments of troponin C is different (fig.2). $TR_1$-C is stabilized by $Mg^{2+}$ only to a very small degree; i.e., its $T_{1/2}$-value in the presence of $Mg^{2+}$ is only few degrees higher than that in the absence of bivalent cations. In contrast the effect of $Mg^{2+}$ on $TR_2$-C is very pronounced. This fragment, which in the absence of divalent cations is unusually unstable, becomes in the presence of $Mg^{2+}$ the most stable among all TR-C fragments (table 1). The ratio of $\theta_{90°}:\theta_{120}$ for the samples measured in the presence of $Mg^{2+}$ is between that for EDTA and for $Ca^{2+}$.

Fig.3 shows the CD thermal unfolding profiles of the TR-E fragments of calmodulin. The $TR_1$-E fragment behaves similarly to the $TR_1$-C fragment.

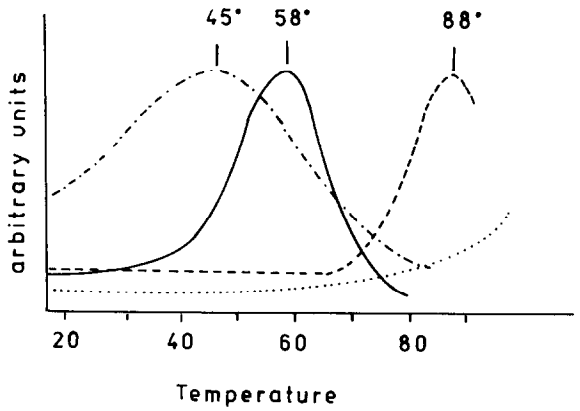


Fig.3. Circular dichroism thermal unfolding profiles of TR-E calmodulin fragments. Measurements were performed in the 20 mM Hepes (pH 7.0). $TR_1$-E fragment in the presence of: 2 mM EDTA (——); 1 mM EGTA and 5 mM $Mg^{2+}$ (– – –); or 1 mM $Ca^{2+}$ (· · · ·); $TR_3$-E fragment in the presence of 5 mM $Ca^{2+}$ (-----).

The $TR_3$-E fragment composed of only one $Ca^{2+}$-binding domain is in the presence of EDTA and/or $Mg^{2+}$ almost completely unfolded at 11°C [9] and, hence, does not show any increase of ellipticity with the increase of temperature. In the presence of $Ca^{2+}$ a very broad transition with the $T_{1/2}$ near 45°C has been observed for this fragment.

All changes of the ellipticity of the samples due to heating, except those of $TR_3$-E fragment of calmodulin, are fully reversible when $Ca^{2+}$ or $Mg^{2+}$ were present. When heating was performed in the presence of EDTA, the differences between values of ellipticity before and after heating and subsequent recooling were $\leq 8\%$ for all fragments. The changes of the ellipticity of $TR_3$-E were only partially reversible even when heating was performed in the presence of $Ca^{2+}$.

## 4. DISCUSSION

The circular dichroism spectra and values of $[\theta]_{222}$ for both proteins studied and their tryptic fragments measured at constant temperature at various ionic conditions appeared in [7,9].

Application of the ratio $\theta_{90°}:\theta_{12°}$ given in table 1 to those values of ellipticity enables us to calculate the ellipticity at 90°C and hence, to obtain information about the remaining secondary structure upon heating. Thus, at 90°C in the presence of EDTA both proteins and all fragments are virtually completely unfolded. In the presence of $Mg^{2+}$, however, a large part of helical structures remains at 90°C.

Previous results have indicated that cleavage of calmodulin and troponin C in half does not lead to any significant conformational changes and the secondary structure of the fragments is similar to that of the corresponding parts in the intact molecules [7,9]. Since, however, TR-C fragments of both proteins have values of $T_{1/2}$ in the presence of $Ca^{2+}$ above 90°C as the intact molecules, comparison of the structure can be performed only for the thermal stability in the presence of $Mg^{2+}$ and in the absence of bivalent cations.

In the presence of EDTA the $T_{1/2}$ values are the same for both proteins except that the thermal unfolding profile of calmodulin shows a shoulder at 30°C, the origin of which is unclear at this moment. A similar shoulder was found in cardiac troponin C [12] without explanation. The $T_{1/2}$ value of skeletal muscle troponin C in the presence of EDTA found in this work is somewhat lower than that in [12]. This difference is probably caused by different methods of the measurement of temperature of the sample.

In the presence of EDTA the N-terminal fragments of both proteins are more stable than the C-terminal fragments. This difference is very small in case of calmodulin but unusually high in case of troponin C. These results provide additional information to the previous studies indicating that N-terminal halves of both proteins are more folded than their C-terminal halves in the presence of EDTA and that this is more pronounced in troponin C [7,9]. That two halves of troponin C differ considerably in their melting temperature can be also predicted from measurements of the melting profiles of intact troponin C using scanning calorimetry [13].

The binding of $Mg^{2+}$ causes the increase of thermostability of both proteins and their fragments to a lower degree than the binding of $Ca^{2+}$. The $TR_2$-C fragment of troponin C, containing the so-called $Ca^{2+}$–$Mg^{2+}$-binding sites, shows the highest effect of $Mg^{2+}$ whereas the lowest effect is observed for $TR_1$-C fragment of troponin C containing low affinity sites. In the calmodulin molecule the effect of $Mg^{2+}$ is similar for both N- and C-terminal halves. These results are in agreement with those of the fluorescence properties and secondary structure changes obtained for both proteins [7,9] and with the data obtained by NMR [2] showing that there is competition in all 4 calcium-binding sites between $Ca^{2+}$ and $Mg^{2+}$.

The thermal denaturation profile of $TR_3$-E of calmodulin, composed of only one complete $Ca^{2+}$-binding domain, differs considerably from those of all other fragments. Under the best conditions (i.e., in the presence of $Ca^{2+}$) the $T_{1/2}$ is only 45°C. This observation supports all other results [9] which have stressed the importance of the interaction between domain III and IV and have indicated that a single $Ca^{2+}$-binding domain is stabilised by $Ca^{2+}$ to a much lower degree than a segment containing two domains.

Independently of the transition midpoints of the melting curves of the proteins studied, there is a slow constant increase of ellipticity with the increase of temperature (e.g., the thermal profile of

$TR_3$-E fragments in fig.3). This phenomenon may be one of the reasons for the differences in ellipticity values observed for calcium-binding proteins, since measurements were made over 10–25°C.

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