A ^{43}Ca NMR AND ^{25}Mg NMR STUDY OF RABBIT SKELETAL MUSCLE TROPONIN C

Exchange rates and binding constants

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1. Introduction

The regulation of vertebrate skeletal muscle contraction involves the binding of Ca^{2+} to a protein complex, troponin–tropomyosin, on the actin thin filament. Troponin consists of three subunits, troponin C (TnC), troponin I and troponin T. The initial event in the contraction is generally considered to be the binding of Ca^{2+} to TnC, which then undergoes a conformational change. In a series of events that are less well documented this conformational change results in the sliding of the thin and thick muscle filaments past each other [1].

TnC has been reported to possess 4 Ca^{2+} binding sites, 2 with K_a 2 × 10^7 M^{-1} (the high affinity sites), and 2 with K_a 3 × 10^5 M^{-1} (the regulatory sites) [2]. Mg^{2+} is also reported to bind to the high affinity sites, with K_a 5 × 10^3 M^{-1} [2]. In addition to the 2 classes of sites described above, there is evidence for an additional class, the weak sites, that bind Mg^{2+} as well as Ca^{2+} with $K_a \sim 10^3$ M^{-1} [2,3].

The contraction and relaxation of skeletal muscles are dynamic processes which are enacted on a millisecond time scale. In the assessment of the roles played in these processes by the different cation binding sites on TnC, the rates of binding and release of Ca^{2+} and Mg^{2+} to and from the different sites are significant parameters. Here, we present a ^{25}Mg and ^{43}Ca NMR study of the binding of Mg^{2+} and Ca^{2+} to TnC, as well as the first direct measurements of the rate of exchange of these ions from the cation–TnC complexes.

Abbreviations: TnC, rabbit skeletal muscle troponin C; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulphate

2. Materials and methods

2.1. *Materials*

TnC was prepared from rabbit skeletal muscles according to a slight modification of the procedure in [4]. The purity of the protein was checked on agarose and polyacrylamide gel electrophoreses. In the SDS disc gel electrophoresis one band, corresponding to ~19 000 M_r, was obtained. The absorption ratio A_{280}/A_{260} for the protein was 0.89. Ca^{2+}- and Mg^{2+}-free TnC was prepared by slowly passing the protein solution through a Chelex-100 column. The Ca^{2+} content after this treatment was found by atomic absorption spectrophotometry to be <0.1 mol Ca^{2+}/mol TnC. Determinations of the protein concentration were made spectrophotometrically using an absorption coefficient $E^{1\,cm}$=0.23 mg/ml at 277 nm [5].

A 0.086 M $CaCl_2$ solution was prepared by dissolving $CaCO_3$ (60% isotopically enriched in ^{43}Ca, Oak Ridge Natl. Lab., USA) in 0.1 M HCl. A 0.512 M $MgCl_2$ solution was made by dissolving MgO (98% isotopically enriched in ^{25}Mg, Oak Ridge Natl. Lab., USA) in 1 M HCl. Both solutions were neutralized to a final pH of 7.0.

2.2. *Methods and data treatment*

The ^{43}Ca and ^{25}Mg NMR spectra were obtained at 17.16 and 15.61 MHz, respectively, using a home-made Fourier transform spectrometer [6]. The relaxation of both ^{25}Mg and ^{43}Ca can safely be assumed to be exclusively quadrupolar. For correlation times sufficiently short, such that $\omega\tau_c \lesssim 1$, the relaxation can be approximated by a single exponential, corresponding to a Lorentzian line in the Fourier-transformed spectrum. When the observed signal is due to metal ions exchanging between two sites, the line shape is

no longer Lorentzian, except for very slow and very fast exchange rates. When the intermediate exchange condition applies the full width at half height of the NMR signal, $\Delta\nu$, is not only influenced by the transverse relaxation rates in the two sites and their populations, but also by the exchange rate (i.e., $k_{off} = 1/\tau_b$), cf. the Swift-Connick equation:

$$R_{2,obs} = (1-p_b)R_{2,f} + p_b/(R_{2,b}^{-1}+\tau_b)$$

which is valid for p_b values $\ll 1$ [7]. When the Swift-Connick approximation is not fulfilled, a total bandshape analysis is needed to obtain values of k_{off} and $R_{2,b}$. The details of the bandshape analysis will be reported elsewhere. To visualize the agreement between calculated and observed signals, the full width at half height ($\Delta\nu = R_{2,obs}/\pi$) will be used in the same way as when the Swift-Connick equation is valid.

3. Results

3.1. *^{43}Ca NMR*

The dependence of the ^{43}Ca NMR linewidth on the $[Ca^{2+}]$ at a constant [TnC] is shown in fig.1. At high $[Ca^{2+}]$ this dependence can be simulated assuming that only the regulatory sites have a calcium exchange sufficiently fast to affect the linewidth of the observed signal. Using the assumption of two independent sites with the same binding constant we find $K_a^{Ca} > 10^4$ M^{-1} and $R_{2,b} = 2 \pm 0.2 \times 10^3$ s^{-1} when spectra for $[Ca^{2+}]/[TnC]$ ratios from 3–60 are used. The signal seen at a $[Ca^{2+}]/[TnC]$ ratio of 1.8 (fig.2) is presumably due to the ^{43}Ca ions bound to the high affinity sites on TnC. The linewidth corresponds to $R_{2,b} = 2 \times 10^3$ s^{-1}, which, assuming a correlation time of 1×10^{-8} s, gives a quadrupole coupling constant, $\chi = 0.9$ MHz. The temperature dependence of the ^{43}Ca linewidth is shown in fig.3 at two different $[Ca^{2+}]/[TnC]$ ratios. Assuming the observed signal to be caused by Ca^{2+} exchanging between the regulatory

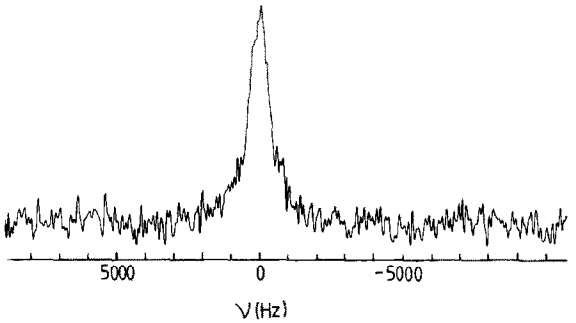

Fig.2. The ^{43}Ca NMR spectrum of a 1.72 mM TnC solution (pH 7.1) containing 3.16 mM Ca^{2+} at 23°C. The spectrum was recorded using a pulse interval of 20 ms and ~2 × 10^6 transients where recorded over ~12 h accumulation. The signal is ~800 Hz broad and is shifted 10 ppm downfield from the signal of free Ca^{2+}.

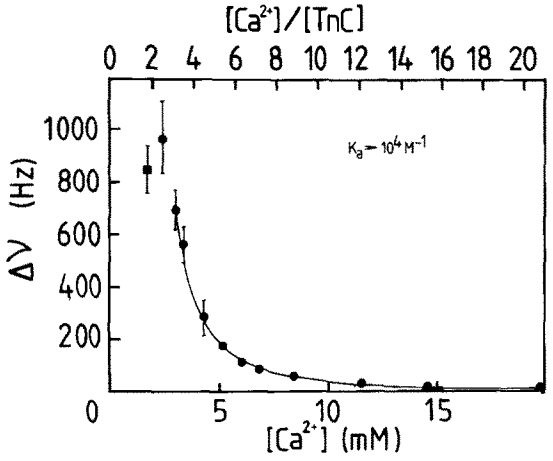

Fig.1. The linewidth of the ^{43}Ca NMR signal as a function of $[Ca^{2+}]$ for a: (•) 0.94 mM TnC solution at pH 6.6; (▪) 1.72 mM TnC solution at pH 7.1, $[Ca^{2+}]$ = 3.16 mM. The spectra were recorded at 23°C. The solid line is a theoretical curve using the parameters obtained from the computer fit described in the text.

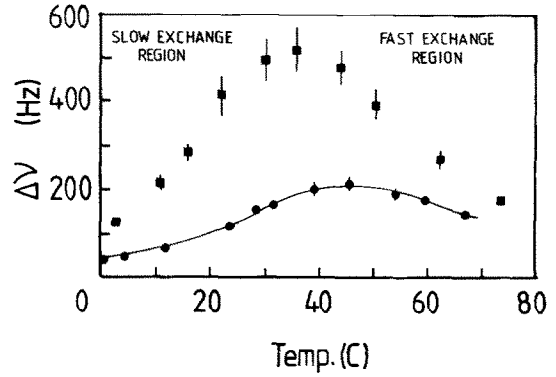

Fig.3. The temperature dependence of the ^{43}Ca NMR linewidth for a: (▪) 0.86 mM TnC solution containing 3.67 mM Ca^{2+} at pH 7.0; (•) 0.75 mM TnC solution containing 5.92 mM Ca^{2+} at pH 7.1. The solid line is the theoretical curve drawn using $k_{off} = 1.0 \times 10^3$ s^{-1} and $R_{2,b} = 2 \times 10^3$ s^{-1}.

sites and the bulk solution, with no effect from the high affinity sites, a bandshape analysis results in $k_{off} = 1.0 \pm 0.1 \times 10^3\ s^{-1}$ at 23°C. It is also assumed, based on ^1H NMR measurements [8], that there is no large conformational change in the temperature interval used.

When Mg^{2+} is added to a 0.75 mM TnC solution containing 3.0 mM Ca^{2+}, the observed ^{43}Ca NMR linewidth decreases ~60% (20 mM Mg^{2+}). This result may be interpreted in several ways. One explanation is that Mg^{2+} is competing for the same sites as Ca^{2+}. If we assume that the observed ^{43}Ca broadening mainly is due to binding to the regulatory sites this is not a likely explanation since Mg^{2+} is known to bind weakly to these sites [2]. If we assume that the observed ^{43}Ca broadening partly is due to Ca^{2+} binding to the weak sites, what we are observing could be a Mg^{2+}–Ca^{2+} competition for these sites. We consider however, that this a less likely situation since the fraction of ions bound to this class of site(s) is low under the experimental conditions. A remaining explanation is that the decrease in the observed ^{43}Ca NMR linewidth is due to a decrease in the exchange rate of Ca^{2+} to the regulatory sites caused by the binding of Mg^{2+} to the weak sites of TnC. In order to test this the temperature dependence of the ^{43}Ca linewidth was studied (on a 3.0 mM Ca^{2+} solution containing 0.75 mM TnC) in the presence of excess Mg^{2+} (20 mM). This study showed the Ca^{2+} exchange rate to be considerably reduced and that a simple two site exchange model no longer was sufficient to account for the data. A model with two protein sites with exchange rates differing by about a factor of 10 was however found satisfactory.

The pH dependence of the ^{43}Ca NMR linewidth can be described using two pK_a values, 4.6 and 6.0.

3.2. *^{25}Mg NMR*

The linewidth of the ^{25}Mg NMR signal as a function of the $[Mg^{2+}]$ at a constant [TnC] is shown in fig.4. A bandshape analysis, assuming two sites, results in $K_a^{Mg} = 5 \pm 1 \times 10^2\ M^{-1}$ at 23°C.

A bandshape analysis of the temperature dependence of the ^{25}Mg NMR signal gives $k_{off} = 8 \pm 5\ 10^3 \times s^{-1}$ and $R_{2,b} = 5 \pm 3\ 10^4 \times s^{-1}$ at 23°C.

The pH dependence of the 25 Mg NMR linewidth can be described using a single pK_a value of 5.4.

Fig.5 shows the effect of added Ca^{2+} on the ^{25}Mg NMR signal. An 80% reduction in the linewidth is observed after the addition of 2 mol Ca^{2+}/mol TnC and after the addition of 4 mol Ca^{2+}/mol TnC ~15% of the initial broadening still remains.

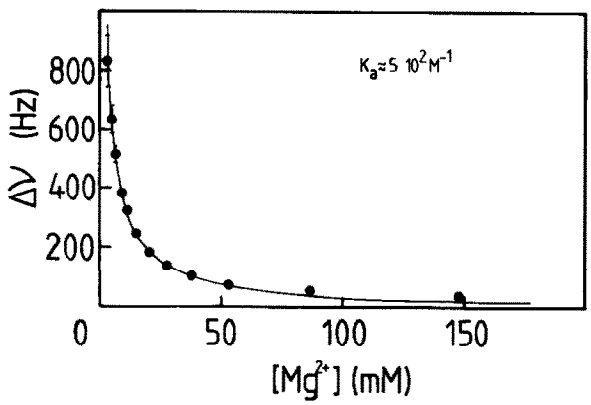

Fig.4. The dependence of the ^{25}Mg NMR linewidth upon addition of Mg^{2+} to apoTnC. The measurements were done at 23°C using a 0.93 mM TnC solution (pH 6.8). The curve represents the results of fitting the data as described in the text.

4. Discussion

It has often been stressed that it would be very difficult, if not impossible, to directly observe the NMR signal from a quadrupolar nucleus bound to a protein. We now believe that this was too pessimistic a view, and wish to report what is probably the first NMR signal from a quadrupolar ion bound to a protein:

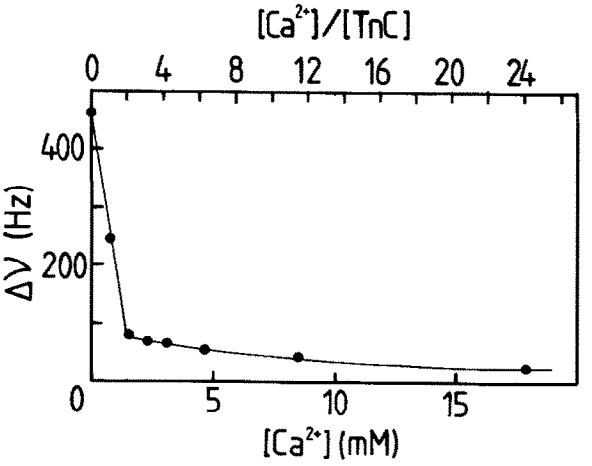

Fig. 5. The ^{25}Mg NMR linewidth as a function of $[Ca^{2+}]$ for a 2.9 mM Mg^{2+} solution (pH 7.1) in the presence of 0.74 mM TnC. The data were obtained at 24°C.

The ^{43}Ca NMR signal from Ca^{2+} bound to the high affinity sites in TnC (fig.2).

We believe that this signal is due to the protein-bound Ca^{2+}, since the free $[Ca^{2+}]$ is exceedingly small under the conditions used.

For ^{43}Ca and ^{25}Mg NMR the lower practical concentration limit presently is ~1 mM, which sets an upper limit of $\sim 10^4\ M^{-1}$ on determinable association constants, K_a. We can therefore not expect a good estimate of K_a for Ca^{2+} to the regulatory sites of TnC, and even less so for the high affinity sites. It is however, encouraging that our analysis of the Ca^{2+} concentration dependence results in a $K_{Ca} > 10^4\ M^{-1}$, in agreement with equilibrium dialysis data [2]. For Mg^{2+} binding to the high affinity sites of TnC the conditions are more favourable, with a reported K_a at 4°C of $5 \times 10^3\ M^{-1}$ [2]. The corresponding ^{25}Mg data, assuming two independent but identical sites, results in $K_a\ 5 \pm 1 \times 10^2\ M^{-1}$ at 23°C. This value is an average of all binding sites and at the high concentrations we employed, the weak binding sites can also contribute to the observed line broadening. Fig.5 shows, however, clearly that most of the broadening (80%) is due to the high affinity sites, with a non-negligible broadening also from other weak sites. The shape of the curve at higher $[Ca^{2+}]$ indicates that Mg^{2+} and Ca^{2+} have approximately the same affinity to these sites.

The kinetics of the metal binding to TnC and the conformational changes caused by the metal binding can be approximated by the following scheme:

$$2\,M + TnC \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M_2TnC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M_2TnC^*$$

$$2\,M + M_2TnC^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M_4TnC^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} M_4TnC^{**}$$

The rate of the conformational changes in the protein, steps 2 and 4, have been measured using a stopped-flow technique with a fluorescence probe attached to the protein [9]†. They found $k_{-2} = 1\ s^{-1}$ and $k_{-4} = 300\ s^{-1}$ for Ca^{2+} and $k_{-2} = 8\ s^{-1}$ and $k_2 = 100\ s^{-1}$ for Mg^{2+}. The forward reactions of the calcium protein were too fast to be measured. In [9] it was concluded that the observed structural change (step 4) is the same as the calcium off-rate whereas for step 2 the conformational change is slower than the removal of Ca^{2+}. Under the experimental conditions we have used, the only calcium exchange that can be observed is that involving the regulatory sites. Furthermore, the concentration of the complex Ca_4TnC^* is most probably negligibly small (we are measuring under equilibrium conditions), which means that what we are observing is the exchange rate of calcium in the $TnC(Ca_4)$ complex. Our value $k_{off} = 1 \pm 0.1 \times 10^3\ s^{-1}$, should be compared to $k_{-4} = 3 \times 10^2\ s^{-1}$ in [9]. It therefore appears possible that calcium removal from the regulatory sites could be faster than the conformational change.

According to [9] the conformational change following the removal of magnesium from the high affinity sites is slightly faster than that following the removal of calcium, which indicates that the $TnC(Ca_2)$ protein conformation is more structured than that of $TnC(Mg_2)$, in agreement with the protein NMR study in [8]. However, our data indicate the off-rate of the Mg^{2+} to be 3 orders of magnitude faster than the conformational change. In [2,9] it has been concluded that it is only the Ca^{2+} binding to and release from the regulatory sites that is responsible for the regulation of the muscle activity. While it seems to be quite well demonstrated that the regulatory sites are involved in the regulation of the muscle activity, it seems far less obvious whether or not the high affinity sites are involved.

The temperature dependence of the ^{43}Ca linewidth in the presence of excess Ca^{2+} shows that the Ca^{2+} exchange rate of the two regulatory sites are of equal magnitude (at least within a factor of two). However, in the presence of excess amounts of Mg^{2+}, the Ca^{2+} exchange rates of the two regulatory sites become different (at least by a factor of 10). The Ca^{2+} competition experiments (fig.5) indicate that Mg^{2+} and Ca^{2+} are bound to the same class of low affinity sites, the weak sites, with an equal affinity. Mg^{2+} binding, in contrast to Ca^{2+} binding, to these sites appears to influence the kinetics of Ca^{2+} exchange to at least one of the regulatory sites. The present NMR data thus point to a possible role of Mg^{2+} in regulating Ca^{2+} exchange rates from TnC.

† The temperature of the kinetic measurements in [9] is not explicitly given in that article. We will however assume that they were performed at ambient temperature (23–25°C)

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