PROTON MAGNETIC RESONANCE STUDY OF TROPONIN-C

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Received 15 October 1975

1. Introduction

The contraction of vertebrate skeletal muscle in vivo is triggered by an increase in the calcium concentration in the region of the actin filaments. This sensitivity to calcium is mediated by the troponin complex which is bound to the actin filaments [1]. One component of troponin, troponin-C (TN-C) binds Ca^{2+} [2] and in so doing undergoes a conformational change [3,4]. Such a reaction may be followed by spectroscopic methods and by virtue of its low mol. wt. (approx. 18 000) TN-C is particularly suitable for study by proton magnetic resonance (pmr). We wish to investigate the structure of troponin-C in solution and to examine the role of TN-C in the complete troponin complex.

The binding of H^+ of Ca^{2+} to troponin-C induces a large conformational change as detected by pmr spectroscopy. The binding of Ca^{2+} shows site specificity resulting from different Ca^{2+} affinities for the four binding sites which can be spectroscopically identified. In order to differentiate structural changes from pH dependencies and other alterations in the spectra it is necessary to assign resonances to particular amino acids. We have applied a Carr-Purcell pulse sequence [5] to distinguish the histidine, *N*-acetyl and some methionine singlets from the rest of the protein spectrum. The transitions between the conformational states adopted by TN-C under varying solution conditions are discussed.

2. Materials and methods

Rabbit skeletal troponin-C was prepared and characterized as previously described [6,7]. The Ca^{2+}-free form was obtained by treatment of TN-C with EGTA followed by desalting on a G75 Sephadex column. All glassware had been washed in 10 mM EGTA/EDTA. The monomer protein peak fractions were concentrated using an Amicon ultrafiltration system, model 8MC, with a 5 UM membrane. Typically <7% of the 4 binding sites [2] remained filled, as determined by atomic absorption analyses using a Pye-Unicam SP90A spectrophotometer. The samples were lyophilized and redissolved in 0.2 M KCl in D_2O for pmr measurements. Protein concentrations were measured on a Unicam SP800 u.v. spectrometer, $\epsilon_{280} - \epsilon_{300} = 2700$ [8].

The pH values quoted are direct meter readings.

Pmr spectra were recorded at 22°C on a 270 MHz Brucker spectrometer operating in the Fourier Transform mode. 2,2-dimethyl-2-silapentane-5-sulphonate was used as internal standard, the nominal resolution being ±0.008 parts per million (ppm).

3. Results

3.1. *Calcium bound protein*

In fig.1 the conventional and the convolution difference [9] spectra of TN-C, saturated with calcium (⩾ 4 moles per mole protein) pH 7.63, are shown. The convolution difference spectrum shows improved resolution although multiplet structure is still not easily resolved at this temperature (22°C). The spectrum shows several ring-shifted methyls ($\delta <$ 0.9 ppm) and the aromatic region is well spread out proving that this protein is folded. Several sharp resonances are seen, including two singlets in the

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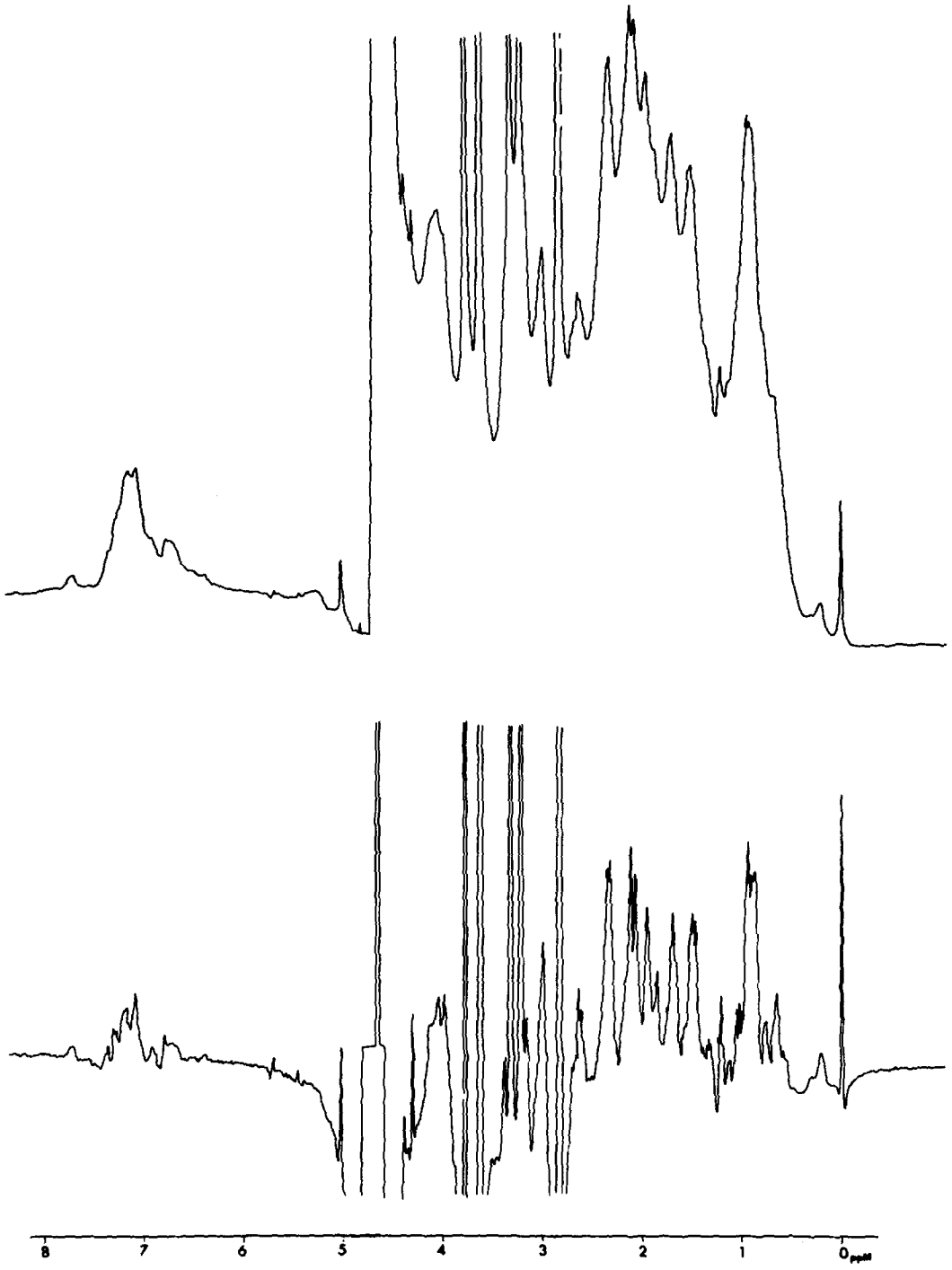

Fig.1. Normal and convolution difference pmr spectra of Ca^{2+}-saturated TN-C, pH 7.63. Sharp EGTA resonances are observed just upfield of the HOD signal.

aromatic region of the spectrum. Since there are no tryptophan residues in TN-C [10], these two sharp singlets, at δ = 7.86 ppm and 7.02 ppm, which both titrate with a normal, simple p*K* value of 6.6 ± 0.2 are therefore assigned to the C-2 and C-4 protons respectively of the histidine residue at position 125. It may be noted that a less well resolved spectrum was obtained at a TN-C concentration greater than approx. 2.5×10^{-3} M, suggestive of dimerisation.

Further assignment of resonances to particular types of amino acids has been accomplished through the use of pulse sequences [5] which allow resonances to be observed selectively due to intrinsic differences in relaxation time or multiplet structure. Fig.2 shows the Ca^{2+}-saturated TN-C spectrum obtained using a Carr-Purcell pulse sequence, 90–t–180–t, with non-selective 180° and 90° pulses [5] and a t value of 60 sec. This pulse sequence distinguishes singlets and triplets from doublets. Several sharp peaks due to singlets are clearly seen. The only peaks in the aromatic region correspond to the C-2 and C-4 protons of His-125. The sharp positive resonances in the aliphatic region arise from singlet methyls. The two resonances, at δ = 2.16 ppm and 2.08 ppm arise therefore from the *N*-acetyl group and methyl protons of methionine residues in agreement with their chemical shift positions.

Upon raising the pH through the range 5–7.5 the resonance at 2.08 ppm is observed to broaden. Other changes in the spectrum of the Ca^{2+}-bound form are slight implying little overall change in the protein conformation with pH.

It is of interest to note that no other well resolved resonances of the Ca^{2+}-bound form are discernible in the spectrum of fig.2. This is a result of T_2^{-1} being greater than J, the spin–spin coupling constant, for many resonances. Spin decoupling cannot therefore be used as an assignment aid at this temperature and pH.

3.2. *Calcium-free protein*

Fig.3 shows the normal and convolution difference spectra of the Ca^{2+}-free form of TN-C ($\leqslant$ 0.4 moles per mole protein) at pH 7.61. This is again a sharp spectrum. Notable differences between this spectrum and that of the Ca^{2+}-saturated form are clearly seen. The ring shifted methyl region ($\delta < 0.9$ ppm) of the Ca^{2+}-saturated convolution difference spectrum shows several resonances (e.g. δ = 0.767, 0.658, 0.593 ppm) which are absent from the spectrum of fig.3. The ring shifted methyl at δ = 0.210 ppm in the Ca^{2+}-bound spectrum appears at 0.174 ppm in the Ca^{2+}-free form (as discerned upon addition of Ca^{2+}) while the resonance corresponding to δ = 0.145 ppm (fig.3) is not observed in the Ca^{2+}-bound spectrum. The aromatic region of the spectrum appears much narrower in the absence of Ca^{2+}, and the main aromatic peak (δ =

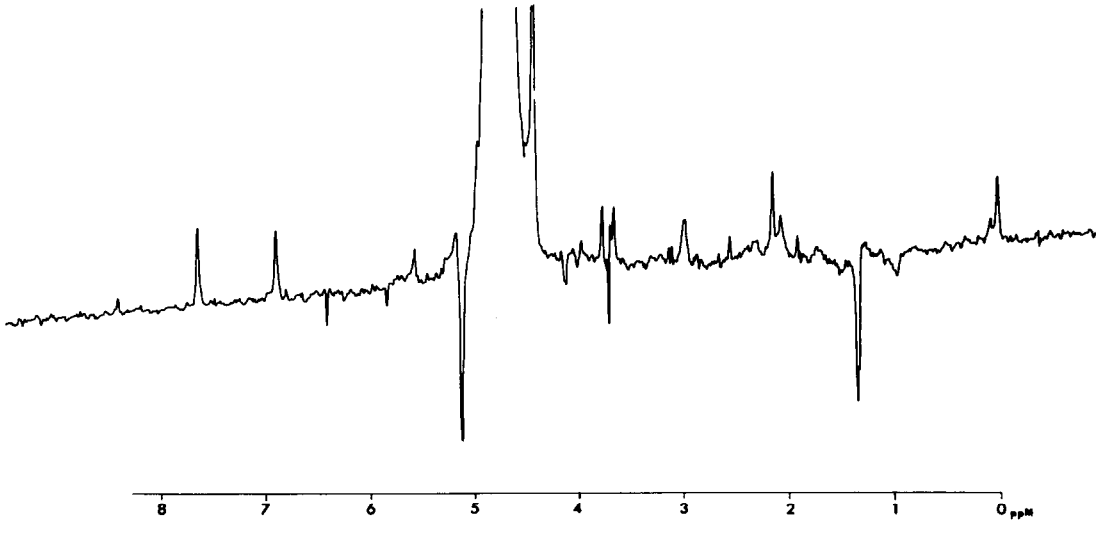

Fig.2. Carr-Purcell 90–t–180–t sequence. Spectrum at t = 60 msec.

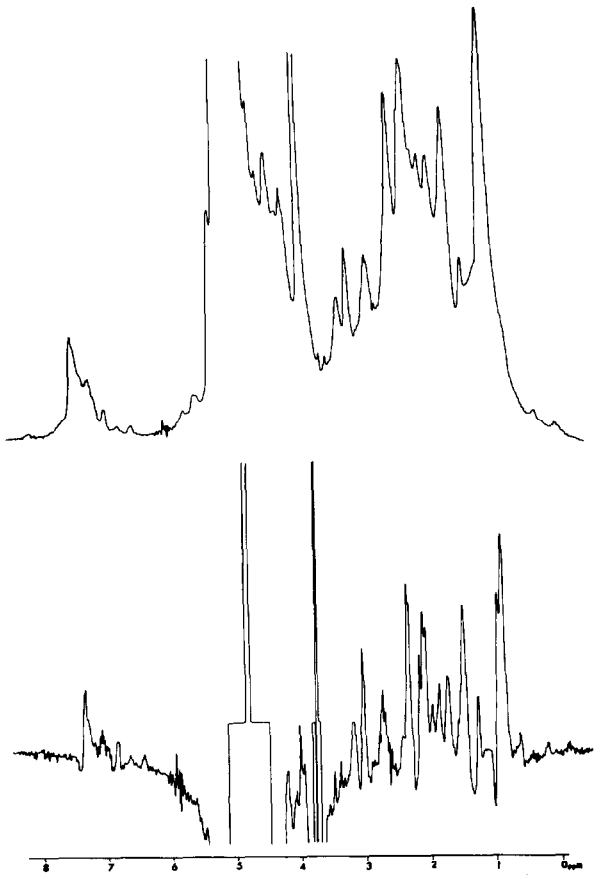

Fig.3. Normal and convolution difference pmr spectra of Ca^{2+}-free TN-C, pH 7.61 in the presence of Tris buffer.

7.32 ppm in fig.3) does not give rise to many resolvable resonances. There is no correspondence between resonance positions found in this region of the two spectra.

A pH titration of Ca^{2+}-free TN-C shows gross changes in linewidth and chemical shift in the pH range 5–8. The Ca^{2+}-saturated form did not show such marked changes with pH. The histidine C-2 resonance exhibited peak doubling as the pH was varied and, unlike the simple pK observed in the Ca^{2+}-saturated form, was found to titrate over a narrower pH range than would be the case for the binding of one proton at a single site. At least a two proton cooperativity must be involved. The mid point pH for the titration was 7.2 ± 0.2.

3.3. *Calcium titration*

A Ca^{2+} titration of the Ca^{2+}-free form of TN-C showed particular resonances shifting at different stages of increase of metal ion concentration. For example, at pH 5.1 the methyl resonance at δ = 0.145 ppm and aromatic resonances at δ = 6.424 and 6.641 ppm, were broadened and shifted downfield upon addition of Ca^{2+} sufficient to fill one site. Increasing metal concentration further led to other specific changes in the spectrum, such as the downfield shift of the resonance at 0.174 ppm. These changes will be fully detailed elsewhere but it is possible to separate spectroscopically different forms at different calcium to protein concentration ratios [11].

4. Discussion

The proton magnetic resonance spectra thus show that TN-C adopts different conformational states under different conditions of pH or Ca^{2+} ion concentration. These states and the transitions between them are summarised in fig.4.

The broad resonances observed in both the aromatic and ring shifted region of the spectrum of Ca^{2+}-bound TN-C correlate with the lack of resolution obtained during the Carr-Purcell pulse-sequence experiments. These observations are indicative of a tightly folded protein conformation (P'') for the Ca^{2+}-bound form of TN-C.

The titration of the lone histidine C-2 and C-4 with a simple pK value of 6.6 ± 0.2 for the Ca^{2+}-saturated form shows that this is a non-cooperative fast-binding transition ($P'' \rightleftharpoons P'' H^+$). Little conformational change occurs upon increasing the pH as seen from the overall spectrum. In contrast, the titration of the Ca^{2+}-free form of TN-C appears to involve cooperative interactions since the effective histidine C-2 titration curve shows a transition which is not that for one H^+ binding at a single site, (or independent binding at several sites) ($P \rightleftharpoons P'' (H^+)_n$). An overall conformational change to a more compact form of the protein occurs in going from acid to alkaline pH as witnessed by an increase in the linewidth of some of the aromatic and methyl resonances. This conformation ($P'(H^+)_n$) however differs from that of the Ca^{2+}-bound protein ($P''H^+$) where several ring shifted and aromatic resonances are clearly discernable.

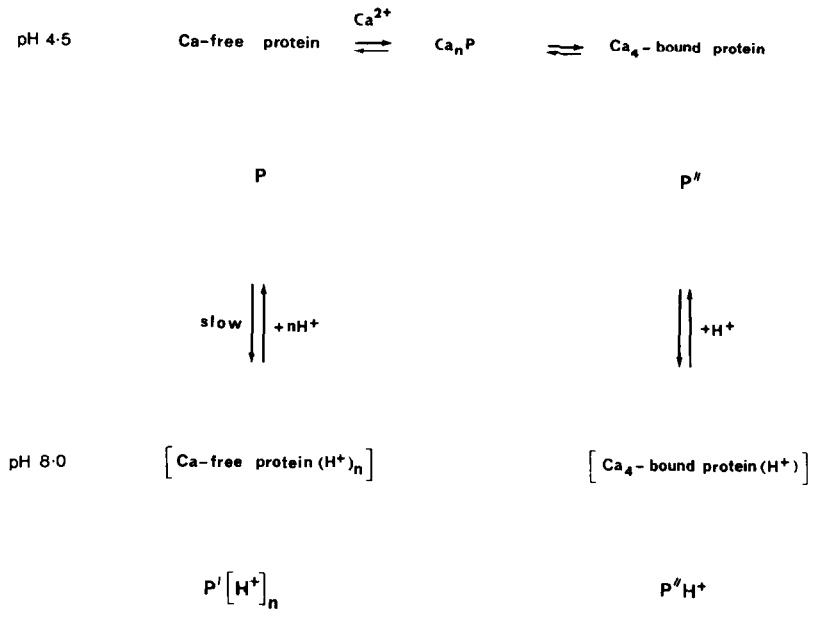

Fig.4. Diagramatic representation of the various conformational changes of TN-C induced by H^+ and Ca^{2+} binding.

The Ca^{2+}-titration shows evidence for site specificity resulting from different Ca^{2+} affinities for the different sites. Once further resonance assignments have been made, it should be possible to identify the four binding sites and thereby elucidate the nature of the conformational change which triggers muscle contraction.

Acknowledgements

We wish to thank Professor R. J. P. Williams, Miss B. Bullard and Professor D. C. Phillips for their advice, criticism and encouragement. This work is supported by the Medical Research Council.

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