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Proton-magnetic-resonance studies on the interaction of rabbit skeletal-muscle troponin I with troponin C and actin

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1. The p.m.r. spectra of the larger CNBr-cleavage peptides of troponin I from rabbit fast-twitch skeletal muscle corresponded largely to those of fairly flexible solution structures. 2. On addition of troponin C to each of the CNBr-cleavage peptides in turn, perturbations of side chains were noted only for peptides CN5 (residues 1–21) and CN4 (residues 96–116). 3. In the presence of Ca^{2+}, troponin C induced perturbations of the side chains of threonine-11, alanine, isoleucine and arginine residues of peptide CN5. 4. In the presence of Ca^{2+}, troponin C induced perturbations of the side chains of phenylalanine, lysine and leucine residues of peptide CN4. 5. Irrespective of the presence or absence of Ca^{2+}, specific interaction with actin was observed only with peptide CN4. In this case the side chains of arginine residues were perturbed. 6. It is concluded that actin interacts with the *C*-terminal region of peptide CN4, whereas troponin C interacts with the *N*-terminal region of peptide CN4 and with peptide CN5.

The binding of Ca^{2+} to troponin C causes contraction to take place in skeletal muscle by release of the inhibition of the Mg^{2+}-stimulated actomyosin ATPase. Although *in vitro* troponin I inhibits the actomyosin ATPase, it is unlikely that in living muscle the protein acts as a simple inhibitor of the interaction of actin with myosin. This is evident from the fact that there is only one molecule of the troponin complex for every seven actin monomers in the I filaments of skeletal muscle (Ebashi *et al.*, 1969). To understand fully the mechanism of action of the troponin complex, more precise information is required about the surfaces of contact between its components and the other I-filament proteins. A study of the biological properties of the peptides obtained by specific cleavage of the components of the troponin complex has allowed certain interaction sites to be assigned to defined parts of the primary sequences (for review see Perry, 1979). In particular, it has been shown that two regions of the primary sequence of troponin I are of special significance for its interaction with troponin C and actin.

Evidence for the interaction of troponin C with the *N*-terminal region of troponin I has been obtained from affinity-chromatographic studies on the peptides formed by CNBr digestion (Syska *et al.*, 1976) and from the inhibition of phosphorylation at threonine-11 (Moir *et al.*, 1974) by phosphorylase kinase (Perry & Cole, 1974). Similar investigations and enzymic studies have also shown that a region extending from residue 96 to residue 116 is implicated in interaction with troponin C and with actin (Syska *et al.*, 1976). The significance of this region for interaction with troponin C is also implied from the evidence of inhibition of phosphorylation at serine-117 by protein kinase (Perry & Cole, 1974; Moir *et al.*, 1974).

Two sites of interaction with troponin I have been identified by similar studies on troponin C (Weeks & Perry, 1978; Leavis *et al.*, 1978), and some of the residues involved in these regions have been identified by p.m.r. studies (Evans & Levine, 1980).

In the present investigation p.m.r. spectroscopy was used to identify the residues in the sequence of troponin I that are involved in interaction with troponin C and actin in rabbit fast-twitch skeletal muscle. The results confirm the special significance of the regions of the sequence of troponin I consisting of residues 1–21 and residues 96–116, and indicate that specific side-chain groups are involved in interaction between the two proteins. Interaction of actin involves residues in the *C*-terminal half of the peptide extending from residues 96 to 116, whereas interaction with troponin C is probably restricted to residues in the *N*-terminal part of this peptide.

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A preliminary report of some aspects of this work has been published (Grand *et al.*, 1980).

Materials and methods

Preparation of proteins

Troponin was prepared from rabbit skeletal muscle by the method of Ebashi *et al.* (1971). Troponin C and troponin I were prepared either from whole troponin by the method of Perry & Cole (1974) or from troponin A and troponin B respectively by the method of Wilkinson (1974*a*).

G-actin was prepared from an acetone-dried powder of rabbit skeletal muscle by the method of Spudich & Watt (1971). The protein was either stored in solution at 4°C and used within 1 week or freeze-dried for longer storage. Solutions of G-actin were prepared for use by dissolving the freeze-dried protein in 5mM-Tris (adjusted to pH 8.0 with 1M-HCl)/0.2mM-ATP/0.5mM-dithiothreitol/0.2mM-$CaCl_2$, dialysing against this solution and centrifuging at 100000*g* for 0.5h to remove polymerized and denatured material. All pH measurements were made at 20°C.

Preparation of troponin-I peptides

Peptides were prepared by cleavage of [^{14}C]-carboxymethylated troponin I from rabbit skeletal muscle with CNBr as described by Wilkinson (1974*b*), with the modification that the initial digest was chromatographed on a column (2.2cm × 140cm) of Sephadex G-50 equilibrated and eluted with 10mM-HCl rather than with 6M-urea/0.2M-sodium formate. This modification simplified the preparative procedure, since it was not necessary to desalt the fractions from the columns containing the partially purified peptides before freeze-drying. Peptides were then chromatographed on phosphocellulose as described by Wilkinson (1974*b*). No significant difference in fractionation could be observed between gel filtration in 6M-urea/0.2M-sodium formate or 10mM-HCl. As the troponin I was alkylated with iodo[^{14}C]acetic acid before digestion, peptides CN1, CN3 and CN7 were radioactive.

Characterization of CNBr-cleavage peptides from troponin I

Peptides were characterized by amino acid analysis and *N*-terminal analysis by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method of Hartley (1970). The results obtained agreed well in all cases with those of earlier studies (Wilkinson, 1974*b*; Wilkinson & Grand, 1975), and virtually no cross-contamination of one CNBr-cleavage peptide with another could be detected.

Peptide concentrations were determined by amino acid analysis as described by Wilkinson *et al.* (1972).

Recovery of CNBr-cleavage peptides after p.m.r. studies

In some cases peptides that had been used for interaction studies with actin or troponin C were recovered for re-use by gel filtration. The mixture of peptide and protein from the p.m.r. sample tube was dissolved in 6M-urea/0.2M-sodium formate buffer, pH 3.8 (3ml), and chromatographed on a column (2.2cm × 150cm) of Sephadex G-50 equilibrated and eluted with the same buffer. Fractions (5ml) were collected and peptides detected as follows: peptide CN1 by measuring radioactivity, peptide CN2 by absorbance at 280nm and peptides CN4 and CN5 by electrophoresing samples (0.1ml) of fractions on polyacrylamide gels run at pH 3.5 in the presence of 6M-urea (Panyim & Chalkley, 1969). The repurified peptides were desalted on a column (2.2cm × 110cm) of Sephadex G-25 equilibrated and eluted with 10mM-HCl, freeze-dried and subjected to amino acid analysis to assess the purity and concentration of the recovered material.

P.m.r. methods

Spectra were recorded at both 300 and 270MHz on Brucker instruments operating in the Fourier-transform mode. Two pulse spin-echo methods [$(90\text{–}t\text{–}180\text{–}t)_n$] were used in order to resolve signals on the basis of relaxation time (T_2) and coupling constants (Levine *et al.*, 1979). An inter-pulse overall delay time of 3s was used to allow for full relaxation. Difference spectroscopy was used as a routine to monitor spectral changes in the resonances of the different peptides with trimethylsilanesulphonate as internal standard.

Preparation of samples

Freeze-dried peptide samples were dissolved in ^2H_2O to a final concentration of approx. 1mM. The pH of the solution in the p.m.r. tube was adjusted with 0.1M- or 1M-^{2}HCl or 0.1M- or 1M-NaO^2H solutions, by using a micro-electrode (3mm diameter). The pH values quoted are uncorrected for isotope effects.

Troponin C (30mg/ml) dissolved in 0.2M-KCl/5mM-dithiothreitol in ^2H_2O, pH 7.2, was added to the p.m.r. tube with a micro-pipette. The extent of Ca(II) binding to troponin C was changed by addition of 0.2M-EGTA (1–5μl) and the pH was maintained constant with 0.1M-NaO^2H. Troponin C with four or two calcium atoms bound per molecule was identified from observed signals in its p.m.r. spectrum corresponding to the two different Ca(II)-induced conformational states of the protein, e.g. the upfield-shifted methyl-group resonances at $\delta = 0.16$ and −0.1p.p.m. (Levine *et al.*, 1978; Evans *et al.*,

1980). Addition of solutions of troponin C or EGTA usually led to negligible dilution of the original 0.4ml of peptide solution used for each experiment. In all cases dilution was monitored by observing the signal intensity of the internal standard, trimethylsilanesulphonate.

G-actin was prepared as described above to a concentration of 8mg/ml. Samples of this solution were then added by micro-pipette by the same procedure as for troponin C. Some polymerization of the added actin may have occurred in the p.m.r. sample tube, in view of the presence of NaCl from the neutralization of the peptide solutions, the pH of which was adjusted from approx. 1.3 during preparation of the sample, and the traces of bivalent cations no doubt present.

For peptide CN4, therefore, titration with G-actin was also performed after dialysis of a solution of actin (5mg/ml) in 5mM-triethanolamine adjusted to pH8.0 with 1M-HCl containing 0.2mM-ATP, 0.2mM-$CaCl_2$ and 0.5mM-dithiothreitol against the same buffer for 12h. The solution of peptide CN4 was also dialysed against the same buffer, pH8.0. After dialysis both solutions of peptide (0.5ml) were further dialysed for 3h with two changes against 25ml of the buffer in ^2H_2O solution to obtain a sufficient proportion of ^2H required for the operation of the spectrometers, as well as to decrease the size of the signal deriving from $^1H^2HO$.

Results

When rabbit skeletal-muscle troponin I is digested completely with CNBr, ten peptides are obtained (Wilkinson, 1974*b*). In the present study only peptides containing 14 or more residues, namely peptides CN1, CN2, CN3, CN4, CN5, CN6 and CN7 (nomenclature of Wilkinson, 1974*b*), were examined. The remaining peptides (CN8, CN9 and CN10) were derived from a five-residue sequence in the centre of the protein and an 11-residue sequence at the *C*-terminus, in all representing 9% of the molecule (Fig. 1).

The p.m.r. spectra of the seven large CNBr-cleavage peptides corresponded largely to those expected for fairly flexible solution structures and yielded signal intensities consistent with their individual amino acid compositions. The spectra obtained for peptides CN1, CN4 and CN5 are shown in Fig. 2. The chemical-shift position of the various signals enabled assignment to corresponding chemical groups and residue type. The correlation of these signals with specific residues in the sequence was based on individual amino acid compositions of the peptides. The signals of particular residues could thus be readily monitored and served as intrinsic probes for the environment of side chains at different points along each peptide sequence. Complex-formation involving any peptide was thus reflected in the chemical shift and/or linewidth (relaxation-time) changes that resulted from the environment experienced by the corresponding side chain in the complex as well as any change in segmental mobility that may occur upon interaction.

Interactions of troponin I peptides with troponin C

Possible complex-formation between the different peptides and troponin C in the presence of excess Ca^{2+} was investigated by addition of troponin C in turn to solutions of each of the peptides CN1, CN2, CN3, CN4, CN5, CN6 and CN7. Difference spectroscopy and two pulse spin-echo methods (Levine *et al.*, 1979) were used during titration to detect any spectral alterations that may have occurred. In the latter technique a decrease in relaxation time resulting from interaction manifests itself as a decrease in signal intensity of the group(s) influenced by binding. Specific interactions with troponin C were observed only with two of the CNBr-cleavage fragments, peptide CN5 (residues 1–21) and peptide CN4 (residues 96–116), the amino acid sequences of which are shown in Fig. 3.

Addition of troponin C to a solution of peptide CN5 led to a notable decrease in intensity of signals corresponding to the γ-CH_3 group of threonine-11, the β-CH_3 group of alanine (residues 9 and 12), the δ-CH_3 group of isoleucine-10 and δ-CH_2 groups of arginine (residues 6, 8, 13 and 14) (Fig. 4). Relatively little perturbation occurred in signals derived from the side chains of histidine-16, lysine-5 and -18, glutamine-3 and -4 or the *N*-acetyl terminus of this peptide. These findings implicate the central portion of the peptide (residues 6–14) in the surface

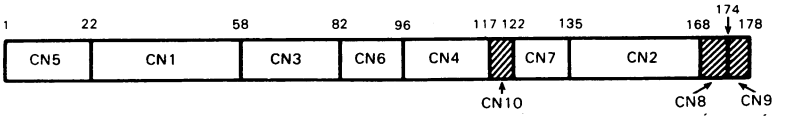

Fig. 1. *Schematic representation of the primary structure of troponin I from rabbit fast-twitch skeletal muscle*
Numbers above the line are the positions of the methionine residues in the amino acid sequence at which cleavage occurs with CNBr. Hatched regions indicate peptides CN8 (residues 168–173), CN9 (residues 174–178) and CN10 (residues 117–121), which were not studied in the present investigation (Wilkinson & Grand, 1975).

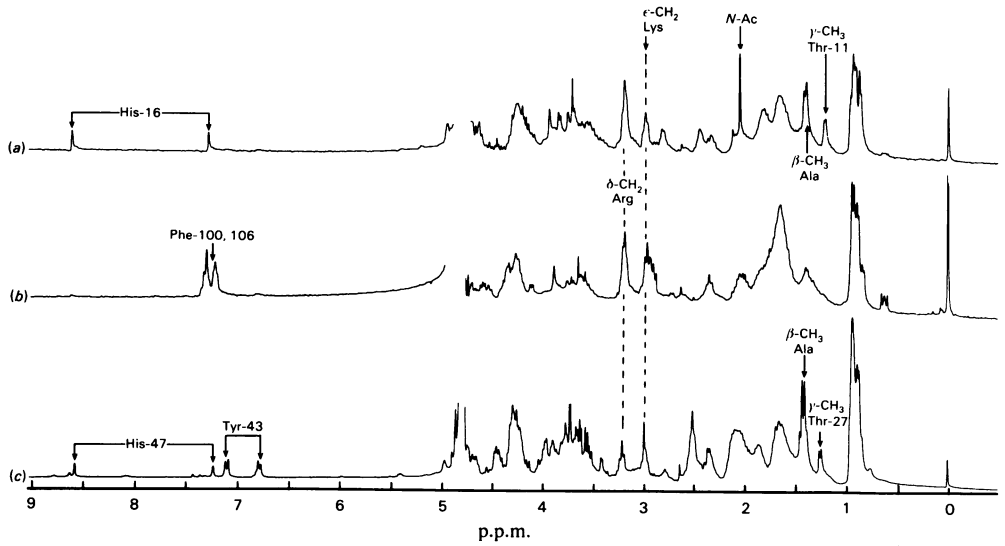

Fig. 2. *P.m.r. spectra of three of the CNBr-cleavage peptides of troponin I at pH 7.1 at 25°C*
Certain signals described in the text are labelled. (*a*) Peptide CN5 (residues 1–21); (*b*) peptide CN4 (residues 96–116); (*c*) peptide CN1 (residues 22–57). In all cases, the H^2HO resonance was suppressed as described in the Materials and methods section.

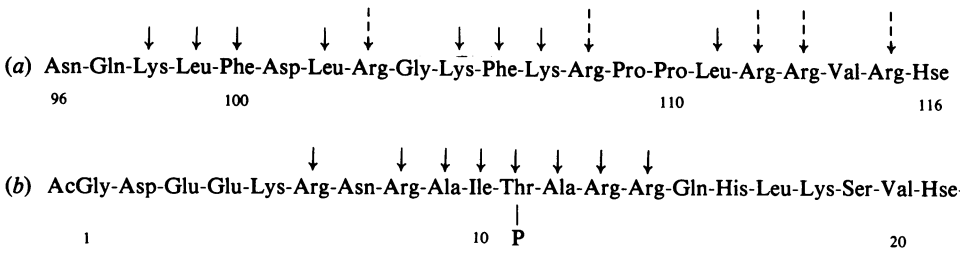

Fig. 3. *Residues in peptides CN4 and CN5 perturbed on interaction with troponin C and actin*
(*a*) Sequence of peptide CN4 and (*b*) sequence of peptide CN5 (Wilkinson & Grand, 1975). Full arrows indicate all the residues that may be perturbed in the presence of troponin C, and broken arrows all those that may be perturbed in the presence of actin.

matching between the troponin I peptide and the troponin C. Release of Ca^{2+} from troponin C, effected by addition of 5 mM-EGTA to the solution, resulted in the loss of these spectral perturbations, indicating that Ca^{2+} was in some way involved in the interaction.

Titration of troponin C into a solution of peptide CN4 in the presence of Ca(II) led to the upfield shift and broadening of the composite aromatic side-chain signal deriving from phenylalanine-100 and phenylalanine-106, as well as the broadening of signals corresponding to ε-CH_2 groups of lysine (residues 98, 105 and 107) and the δ-CH_3 groups of leucine (residues 99, 102 and 111) (Fig. 5). In marked contrast with the spectral effects observed on complex-formation between troponin C and peptide CN5, little perturbation of arginine signals was observed when troponin C was added to peptide CN5. The results thus implicated the *N*-terminal segment of peptide CN4 as contributing to the surface of contact with troponin C. On removal of Ca^{2+} by the addition of 5 mM-EGTA, perturbation of the signals disappeared, suggesting dissociation of the complex between troponin C and

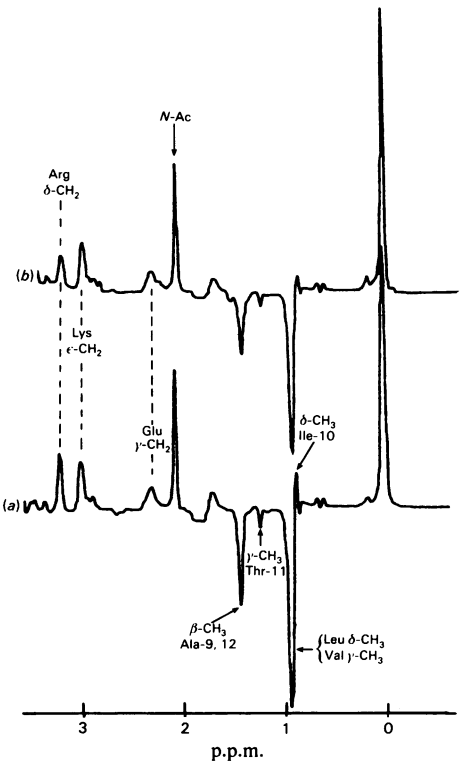

Fig. 4. *Two pulse spin-echo spectra of peptide CN5 in the absence and presence of Ca^{2+}-saturated troponin C (5:1 molar ratio peptide/protein)*

An inter-pulse delay time, *t*, of 60ms was used. Ambient probe temperature was 25°C. Traces: (*a*) 0.8mM-peptide CN5, pH7.2; (*b*) 0.8mM-peptide CN5 + troponin C, pH7.2. Pulse techniques were used to distinguish overlapping signals of peptide CN5 on the basis of spin–spin coupling (*J*) and relaxation time (T_2). The contribution of signals of troponin C to the spectrum obtained in this fashion has previously been shown to be negligible at the temperature and protein concentration used, owing to the relatively shorter relaxation times (T_2) of the protein resonances (Levine *et al.*, 1978).

peptide CN4. Similar changes in the perturbations suggested that the interaction was markedly weakened at higher ionic strengths (>0.1M-KCl). The same perturbations were observed whether peptide CN4 was titrated with troponin C alone or with the complex of troponin C and peptide CN5.

The signal obtained from peptide CN4 complexed with troponin C was not modified if peptide CN5 was titrated into the solution. This suggests that the two peptides interact with different regions on troponin C.

Interaction of troponin-I peptides with actin

One of the properties of troponin I that is clearly of functional significance is its inhibitory action on the Mg^{2+}-stimulated ATPase of actomyosin (Perry *et al.*, 1972; Perry, 1979), an effect that is much increased by tropomyosin. After digestion with CNBr the property survives, but is restricted to peptide CN4, suggesting that this region of the molecule has a special role in the interaction of troponin I with actin. When each of the seven CNBr-cleavage peptides was titrated with rabbit skeletal-muscle actin, spectral perturbation was observed only for peptide CN4 (Fig. 6). In contrast with the effects observed on binding by troponin C, association between peptide CN4 and actin influenced the charged side chains of arginine (δ-CH_2 and γ-CH_2; Fig. 6), whereas the signals deriving from residues of the *N*-terminal segment of the peptide (e.g. phenylalanine-100 and -106, lysine-98, -105 and -107) were relatively unperturbed. This suggests that different regions of the 21-residue peptide CN4 are involved in interaction with troponin C and actin.

Interaction of troponin I with F-actin

During titration of troponin I with F-actin (see the Materials and methods section) up to a final molar ratio of actin to troponin I of 7:1, several signals originating from side chains of troponin I were observed to broaden. These signals derived primarily from side chains of arginine (δ- and γ-CH_2), leucine and/or valine, lysine (ε-CH_2) and glutamate. The observed reaction was not Ca^{2+}-dependent. Although these data do not correspond directly to the results obtained with peptides of troponin I, the differences observed may be rationalized in terms of a conformational constraint on troponin I on interaction with actin.

Discussion

The p.m.r. studies of troponin-I CNBr-cleavage peptides clearly demonstrate that there are two sites of interaction on the molecule for troponin C, confirming earlier results obtained by Syska *et al.* (1976). These authors also suggested, on the basis of affinity-chromatographic and polyacrylamide-gel-electrophoretic evidence, that peptides CN4 and CN5 could form complexes with troponin C, under certain conditions. It is reasonable to extrapolate from the results obtained with isolated peptides of troponin I to the whole molecule, since the p.m.r. spectra of troponin I and peptides CN4 and CN5 indicate that similar residues are perturbed on interaction with troponin C. The demonstration that the sites phosphorylated on fast-twitch skeletal-muscle troponin I by cyclic-AMP-dependent protein kinase and phosphorylase kinase, serine-117 and

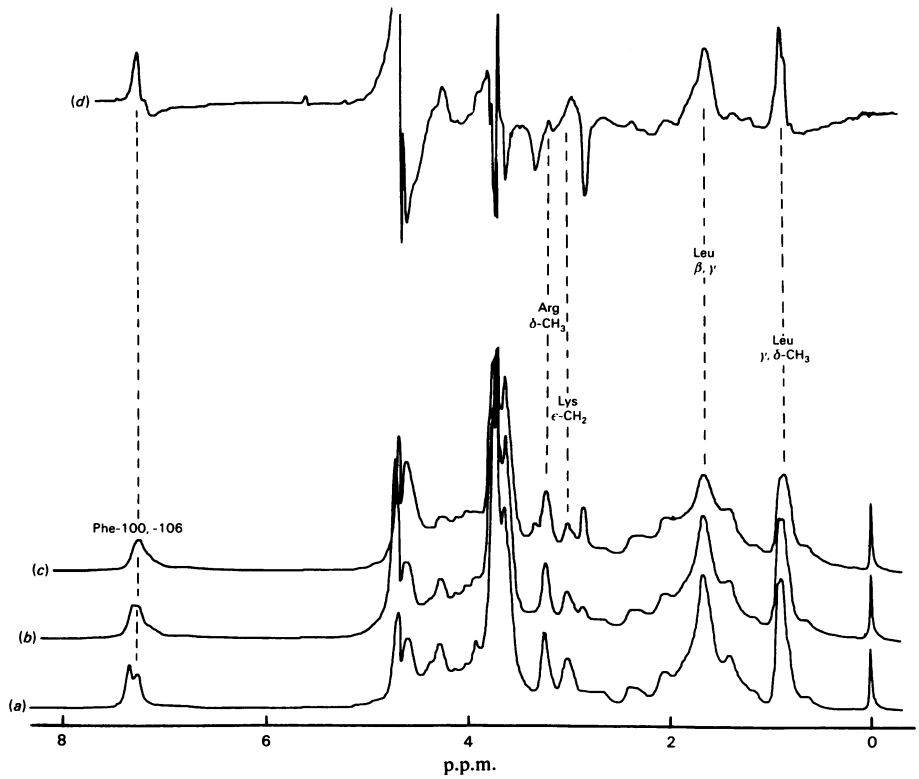

Fig. 5. *Spectral perturbations of peptide CN4 induced by interaction with Ca^{2+}-bound troponin C at pH 7.2*
Traces: (*a*) 0.8 mM-peptide CN4; (*b*) and (*c*) increasing troponin C concentrations (7:1 and 4:1 molar ratios peptide CN4/troponin C) added. (*d*) Difference spectrum (*a*)–(*c*), revealing signals of peptide CN4 perturbed by the interaction.

threonine-11 respectively, are both blocked directly by troponin C (Perry & Cole, 1974; Cole & Perry, 1975) also supports the concept of two sites of interaction on troponin I.

Although p.m.r. spectroscopy offers a very powerful tool for fixing the sites of protein–protein or protein–peptide interactions, at the resolution used in the present study the technique does not differentiate between resonances of groups of the same amino acid at different points in the sequence. For example, when peptide CN4 interacts with troponin C it is not possible to decide whether one or more of the leucine residues in positions 99, 102 or 111 (Fig. 3) are involved. However, since changes can be seen in that part of the spectrum due to phenylalanine residues (100 and 106), but not to arginine residues (located towards the *C*-terminus of the peptide), it can be concluded that changes in the environment of leucine-99 and/or -102, rather than that of leucine-111, are more likely to contribute to the observed spectral changes. If, however, the residues that are conserved in all three forms of troponin I from skeletal muscle of the rabbit (Wilkinson & Grand, 1978) are responsible for the effects observed, then lysine-98, -105 and -107, leucine-102, phenylalanine-106 and arginine-108, -112, -113 and -115 would be implicated in the interaction of peptide CN4 with troponin C and actin. Similar reasoning applied to peptide CN5 would implicate isoleucine-10, alanine-12 and arginine-13.

Both of the interaction sites for troponin C on troponin I are very close in the amino acid sequence to the phosphorylation sites (Moir *et al.*, 1974; Huang *et al.*, 1974). Threonine-11, the site principally phosphorylated by phosphorylase kinase, lies virtually at the centre of the troponin C interaction site on peptide CN5. Serine-117, the main site phosphorylated by cyclic AMP-dependent protein kinase, is adjacent to the actin-interaction site on peptide CN4, as indicated by the p.m.r. studies. The significance of these observations is not apparent at

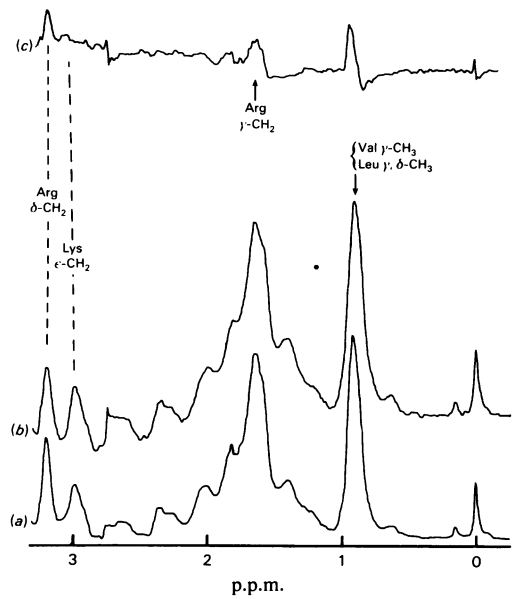

Fig. 6. *Spectral perturbations of peptide CN4 on interaction with actin at pH 7.8*

Traces: (*a*) 0.8 mM-peptide CN4; (*b*) 0.8 mM-peptide CN4 + 0.1 mM-actin (molar ratio 8:1 peptide CN4/actin). (*c*) Difference spectrum (*a*)–(*b*).

present, but phosphorylation at either site could considerably change the binding constant for the protein–protein interaction.

The present studies also confirm the original finding by Syska *et al.* (1976) that the site of interaction on troponin I for actin is contained in peptide CN4. P.m.r. spectroscopy has, however, allowed the region to be defined more specifically, in that it has been shown to occupy the *C*-terminal portion of the peptide. Ca^{2+} ions had no effect on this reaction, although they have been shown to be essential for the interaction of peptides CN4 and CN5 with troponin C. In this property the peptide may differ from the intact molecule, for there is some evidence for the interaction of the intact troponin I molecule with troponin C in the absence of Ca^{2+}. For example, troponin C neutralizes the inhibitory activity of troponin I on the Mg^{2+}-stimulated ATPase of desensitized actomyosin in the presence of EGTA (Perry *et al.*, 1972; Amphlett *et al.*, 1976).

Earlier studies by p.m.r. spectroscopy of skeletal-muscle troponin I and its interaction with troponin C (Evans & Levine, 1980; Levine & Mercola, 1980) have provided evidence for the involvememt of charged (arginine) and hydrophobic (alanine, leucine) residues of troponin I in complex-formation with troponin C. Also in keeping with the results given in the present paper on the interaction between troponin I peptides and troponin C, the studies on the intact molecule demonstrated that complex-formation led to a decrease in segmental mobility of one or two of the three threonine γ-CH_3 groups of troponin I (cf. effect observed with peptide CN5), which was an effect dependent on Ca^{2+} binding to the lower-affinity sites (I and II) in the *N*-terminal half of the troponin sequence. The agreement between these data and those reported in the present paper thus lends support to the notion that troponin C-reactive segments of troponin I are localized in short sequences along the rod-like structure of troponin I observed by p.m.r. spectroscopy. The notion that interaction between the proteins involves the matching of short segments in the sequence of each of the troponin components does not exclude the possible contribution to their surface(s) of contact of other side chains not necessarily adjacent in the primary sequence. It is likely that these short reactive segments act as relay sites that serve to modulate the conformation, and hence transmit information through the configurational constraints imposed by their interaction.

Insofar as these results can be extrapolated to the troponin complex, they indicate a possible mechanism for the Ca^{2+} regulation of the actomyosin ATPase. It is clear from the p.m.r. studies that both actin and troponin C interact with different portions of a very restricted region (residues 96–116) of troponin I. This suggests that there may be a mechanism by which the strengthening of the binding of troponin C to troponin I could displace or decrease the binding of actin to the latter protein. Any explanation of the mechanism by which interaction of Ca^{2+} with troponin controls the actin-activated ATPase must be reconciled with these observations.

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