Structural Consequences of Cardiac Troponin I Phosphorylation

Douglas G. Ward, Michael P. Cornes and Ian P. Trayer*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT. UK

*To whom correspondence should be addressed:
E-mail: I.P.trayer@bham.ac.uk
Telephone: +44 (0)121 4145402
Fax: +44 (0)121 414 2597
SUMMARY

β-Adrenergic stimulation of the heart results in bisphosphorylation of the N-terminal extension of cardiac troponin I (TnI). Bisphosphorylation of TnI reduces the affinity of the regulatory site on troponin C (TnC) for Ca$^{2+}$ by increasing the rate of Ca$^{2+}$ dissociation. What remains unclear is how the phosphorylation signal is transmitted from one subunit of troponin to another. We have produced a series of mutations in the N-terminal extension of TnI designed to further our understanding of the mechanisms involved. The ability of phosphorylation of the mutant TnIs to affect Ca$^{2+}$ sensitivity has been assessed. We find that the Pro residues found in a conserved (Xaa-Pro)$_4$ motif N-terminal to the phosphorylation sites are not required for the effect of the N-terminal extension on Ca$^{2+}$ binding in the presence or absence of phosphorylation. Our experiments also reveal that the full effects of phosphorylation are seen even when residues 1 to 15 of TnI are deleted. If further residues are removed, not only does the effect of phosphorylation diminish, but deletion of the N-terminal extension mimics phosphorylation. We propose that TnI residues 16 to 29 bind to TnC stabilising the ‘open’ Ca$^{2+}$ bound state. Phosphorylation (or deletion) prevents this binding, accelerating Ca$^{2+}$ release.
ABBREVIATIONS

TnC, troponin C; TnI, troponin I; TnT, troponin T; PKA, cAMP dependent protein kinase (EC 2.7.1.37); IAANS, 2-(4'-(iodoacetamido)anilino)napthalene-6-sulfonic acid.

INTRODUCTION

Troponin, working in conjunction with tropomyosin, acts as a molecular switch, regulating muscle contraction in response to changes in the intracellular Ca\(^{2+}\) concentration. Troponin is a complex of three proteins: the Ca\(^{2+}\) binding subunit, troponin C (TnC), the inhibitory subunit, troponin I (TnI), and the tropomyosin binding subunit, troponin T (TnT). Crystal and NMR structures (1;2) reveal that TnC is a dumbbell shaped molecule, consisting of a C-terminal domain possessing two Ca\(^{2+}/Mg\(^{2+}\) binding sites (sites III and IV) and a N-terminal domain possessing two Ca\(^{2+}\)-specific binding sites (sites I and II) in the skeletal isoform. The binding of Ca\(^{2+}\) at sites I and II produces a structural opening of the N-terminal domain exposing additional TnI binding sites (1;3). In cardiac TnC site I is inactive (4) and it is Ca\(^{2+}\) binding to site II that is the primary regulator of cardiac contractility. Binding of Ca\(^{2+}\) to the single ‘regulatory’ site of cardiac TnC alone is not sufficient to stabilise the open conformation of the N-terminal domain in the absence of TnI (2). However in the
presence of TnI and Ca\(^{2+}\) the N-terminal domain of cardiac TnC also adopts the open conformation (5).

Although we do not yet have atomic resolution structures of TnI and TnT much information has been afforded by studies with fragments and mutants of these proteins. TnI is an extended molecule that adopts an antiparallel orientation to TnC in the binary complex (6;7). Cardiac TnI (209 amino acids) has a 31 residue extension not present in other isoforms. Fig. 1 shows the aligned sequences of the N-terminal extensions of the mammalian cardiac TnIs sequenced to date (8-12). Within this extension are two residues, Ser 22 and 23, which are substrates for cAMP dependent protein kinase (PKA). C-terminal to the extension, residues 33-80 of cardiac TnI form an \(\alpha\)-helix that binds extremely tightly to the C-terminal domain of TnC (13;14)(Ward & Trayer, unpublished data). This is followed by a putative TnT binding site and then the ‘inhibitory’ (115-131) and ‘regulatory’ (147-163) regions (5;15). These latter regions bind to Ca\(^{2+}\) saturated TnC, but in the absence of Ca\(^{2+}\), bind to actin anchoring troponin and tropomyosin in the ‘blocked state’ of the McKillop and Geeves (16) model of thin filament regulation.

Ser 22 and 23 of cardiac TnI are phosphorylated by PKA in response to \(\beta\)-adrenergic stimulation of the heart. This bisphosphorylation desensitises the thin filaments to Ca\(^{2+}\) by 0.2 - 0.4 pCa units (17-19) due to an increase in the rate of Ca\(^{2+}\) dissociation from the
N-terminal regulatory site on TnC (19). The faster release of Ca$^{2+}$ produces faster relaxation of skinned fibres upon rapid Ca$^{2+}$ chelation (20). This enhanced relaxation, in conjunction with increased Ca$^{2+}$ re-uptake into the sarcoplasmic reticulum, may help maintain proper diastolic function at increased heart rates.

Despite intensive efforts, it remains unclear how the phosphorylation signal is transmitted from the N-terminal extension of TnI to the regulatory Ca$^{2+}$ binding site in the N-terminal domain of TnC, especially in view of the antiparallel orientation of the two proteins. $^1$H-NMR experiments on peptides corresponding to regions of the N-terminal extension show that the unphosphorylated extension is highly flexible but that bisphosphorylation increases the number of conformational restraints with residues 24 to 29 adopting a looped conformation (21;22). $^1$H and $^{31}$P-NMR on these peptides or whole TnI indicates that phosphoSer 23 interacts with Arg 21 whereas phosphoSer 22 behaves as if free in solution (22;23). In bisphosphorylated troponin the phosphate groups are in a more acidic environment than in isolated TnI which suggests that the phosphorylated extension may interact weakly with another component of troponin. However, the narrow $^{31}$P line widths observed were interpreted to indicate that the phosphate groups themselves are not involved in specific bond formation (23). Isolated TnI and the TnI-TnC complex both adopt a more compact shape upon phosphorylation (24) and FRET experiments have shown that the extreme N-terminal
region of a S5C mutant TnI labelled with 2-(4’-(iodoacetamido)anilino)napthalene-6-sulfonic acid (IAANS) moves 10-12 Å toward the rest of TnI (25). Binding experiments using peptides corresponding to the N-terminal extension have shown that the unphosphorylated form will bind to TnC whereas the phosphorylated form does not (21;26;27). The peptide work is supported by NMR and crosslinking data which indicate that the unphosphorylated N-terminal extension contacts the N-terminal domain of TnC in both TnI:TnC binary complex and in whole troponin (28-31). Bisphosphorylation results in a change in the net charge on the N-terminal extension from +2 to -2 and it is likely that this significantly alters the properties of the extension. Replacement of Ser 22 and 23 with negatively charged Asp residues mimics the effects of phosphorylation in skinned fibres (17) but does not produce the same conformational change in a TnI peptide (residues 19-29) as phosphorylation (21).

It seems likely that the phosphorylation signal is transmitted from TnI to TnC by alterations in a direct interaction between the N-terminal extension and the N-terminal domain of TnC. We now investigate the minimal structural requirements of the N-terminal extension required for successful transmission of the phosphorylation signal. It has been shown that (Xaa-Pro)n sequences favour an extended rigid conformation of the polypeptide chain (32;33). We initially set out to test whether the (Xaa-Pro)4 sequence found in the N-terminal extension acts as a rigid spacer arm (residues 10-17) conducting localised phosphorylation induced conformational changes
towards the N-terminus of TnI. This might alter any protein-protein interactions formed by the extreme N-terminus (residues 1-9) of TnI. Our results indicate that this is not the case as Pro→Ala mutations in this region do not alter the ability of bisphosphorylation to produce two of its characteristic effects: desensitisation of actomyosin ATPase activity toward Ca$^{2+}$ and acceleration of Ca$^{2+}$ release from troponin labelled at Cys 35 of TnC with IAANS. We have also progressively deleted residues from the N-terminus of TnI leading to three major conclusions: 1) residues 1-15 do not play a major role in transmitting the phosphorylation signal to TnC, 2) residues 16-18 are required for the full effect, and, 3) deletion of residues 16-29 mimics phosphorylation. The implications of these findings are discussed later.

**EXPERIMENTAL PROCEDURES**

*Materials* – Recombinant human cardiac TnC, TnI, TnT and α-tropomyosin were used in this study and purified as previously described (34;35). pET11c(TnC) and pET11c(TnI) constructs were provided by Dr. E. Al-Hillawi and pET11c(TnT) and pET11c(TM) constructs were provided by E. Austin and B. Wright respectively. Skeletal TnT, actin and myosin were purified as described previously (36;37). All protein
concentrations were determined using the bicinchoninic acid protein assay (Pierce) calibrated with bovine serum albumin. Oligonucleotide primers were produced by Alta Bioscience (University of Birmingham) and DNA sequenced at the Functional Genomics facility (University of Birmingham, BBSRC 6/JIF13209). Bovine PKA (catalytic subunit) was obtained from Sigma. IAANS was from Molecular Probes. \([\gamma^{33}\text{P}]\text{ATP}\) was from New England Nuclear. Platinum Pfx DNA polymerase was from Invitrogen. All other DNA modifying enzymes were from New England Biolabs and \(E.\text{coli}\) strains from Novagen.

**Mutagenesis, Expression and Purification** – Oligonucleotide directed mutagenesis by PCR using 5’-primers with an \(Nde\_1\) site and 3’-primers with a \(Bam\_H1\) site was as described previously (38). Briefly, base pair substitutions were achieved by mega-primer synthesis by PCR using Platinum Pfx DNA polymerase, followed by a second PCR to extend the DNA to the whole coding region. The N-terminal deletions of TnI were prepared via a single PCR reaction. Following \(Bam\_H1/Nde\_1\) digestion, the PCR products were ligated into pET11c and transformed into competent \(E.\text{coli}\) JM101 cells. Successful clones were screened for by restriction analysis and DNA sequencing. Proteins were overexpressed in \(E.\text{coli}\) Rosetta cells and purified by ion exchange chromatography in ‘urea buffer’ (8 M urea, 25 mM triethanolamine/HCl (pH 7.5), 1 mm EDTA, 1 MM dithiothreitol) as previously described (38). Protein purity (>95%) and identity were analysed by SDS-PAGE and electrospray mass
spectrometry (34).

**Actomyosin ATPase measurements** – The troponin was prepared by mixing TnC, TnI and TnT in a 1:1:1 ratio in urea buffer followed by extensive dialysis against ‘ATPase buffer’ (25 mM KCl, 20 mM MOPS/KOH (pH 7.0), 1 mM dithiothreitol). PKA was also dialysed against ATPase buffer. Assay conditions were: 4 µM actin, 1 µM tropomyosin, 1 µM troponin, 0.1 µM myosin, 3 mM MgCl₂, 2.5 mM ATP, 1 mM EGTA and CaCl₂ to give the required pCa. Prior to addition of the myosin to the reactions, the reaction mix was incubated at 30°C for 15 minutes with or without 50 units PKA/ml. In all experiments the paired ± PKA reaction mixes were prepared and assayed in parallel. Following addition of myosin, ATP hydrolysis was calculated from the P_i released during a 15 minute incubation at 30 °C. The P_i assay was adapted to 96 well plate format from Bilmen et al (39): the 50 µl ATPase reactions were stopped by the addition of 150 µl of chilled 0.2 M sodium acetate (pH 4.0), 0.25 % (w/v) copper sulphate. This was followed by 25 µl of 5 % (w/v) ammonium molybdate and 25 µl of 5% (w/v) sodium sulphite, 2 % (w/v) p-methylaminophenol sulphate. The absorbance at 850 nm was determined after 10 minutes at room temperature. Control experiments showed that the absorbance was linearly proportional to the P_i concentration, that P_i release was linear during our ATPase experiments and that the percentage of ATP hydrolysed did not exceed 25 %.
Ca$^{2+}$ release measurements – A C84S mutant TnC was labelled at Cys 35 with IAANS by dissolving in 6 M urea, 25 mM triethanolamine/HCl (pH 7.5), 1 mM EDTA and incubating at 4°C overnight with a five-fold molar excess of IAANS. Unincorporated IAANS was removed by dialysis. The IAANS labelled TnC was then incorporated into troponin by mixing with TnI and TnT in a 1:1:1 ratio in urea buffer. The complex was extensively dialysed against ‘stopped flow buffer’ (100 mM KCl, 20 mM MOPS/KOH (pH 7.0), 3 mM MgCl$_2$, 50 µM CaCl$_2$) and then diluted to 5 µM and 1 mM ATP added. PKA was also dialysed against stopped flow buffer. The troponin was incubated for 1 hour at 25°C with or without 50 units PKA/ml. Finally, the troponin was diluted to 1 µM in stopped flow buffer prior to use. The rate of Ca$^{2+}$ release was measured from the fluorescence enhancement of IAANS fluorescence upon emptying Ca$^{2+}$ from the regulatory site (40) by rapid mixing with an excess of EGTA. This was achieved by mixing 90 µl of 1 µM troponin with 10 µl of 100 mM EGTA/KOH (pH 7.0) at 25 °C using an Applied Photophysics SX-18MV stopped flow spectrofluorimeter equipped with a 400 nm filter (excitation at 325 nm). Rate constants were calculated by least squares fitting of the data to a single exponential.

RESULTS

Phosphorylation effects on wild type troponin – Fig. 2 shows a typical actomyosin ATPase activity versus pCa experiment. The mean (± SEM) rates of ATP hydrolysis
in our experiments were 2.5 ± 0.1 and 12.5 ± 0.4 nmol P_i released s\(^{-1}\) nmol\(^{-1}\) myosin at high and low pCa respectively. The curves represent least-squares fits to the Hill equation, from which we obtain the pCa giving half maximal activity (pCa\(_{50}\)). The mean pCa\(_{50}\)s from at least four repeats of the experiment were 6.35 for unphosphorylated troponin and 6.12 for bisphosphorylated troponin i.e. phosphorylation reduces the pCa\(_{50}\) by 0.23 pCa units (\(\Delta\text{pCa}_{50}\)). This \(\Delta\text{pCa}_{50}\) value is within the range reported in the literature (17-19). The minimum and maximum ATPase activities and the cooperativity of regulation were not significantly altered by phosphorylation or by any of the mutations tested in this study. Control experiments (data not shown) using \(\gamma^{33}\text{P}-\text{ATP}\) to phosphorylate the thin filaments followed by SDS-PAGE and autoradiography demonstrated that the TnI was maximally phosphorylated (no increase in phosphorylation with longer incubation times) and that no other thin filament proteins were phosphorylated.

The stopped flow measurement of Ca\(^{2+}\) release from unphosphorylated and phosphorylated troponin containing wild type TnI and TnT and C84S TnC-IAANS is shown in Fig. 3. In both cases the data is well fitted by a single exponential function. Ca\(^{2+}\) release is measured from the 2-3 fold increase in the fluorescence of the IAANS attached to inactive site I of TnC produced by emptying of site II. The mean rate constants obtained were 25.5 s\(^{-1}\) for unphosphorylated troponin and 33.0 s\(^{-1}\) for
phosphorylated troponin. This ~30% increase in the dissociation rate constant caused by TnI phosphorylation is somewhat less than the value of 45% reported by Robertson et al in 1982 (19) using bovine cardiac troponin, but is large enough to easily and reliably measure.

**Phosphorylation effects on troponin containing Pro→Ala TnI mutants** – We produced single (P15A), double (P15/17A) and quadruple (P11/13/15/17A) Pro→Ala mutants of TnI. The rationale being that if this region was acting as a rigid spacer arm, then replacement of the Pro residues with Ala would increase its flexibility and impair transmission of the phosphorylation signal. When treated with PKA all three mutants incorporated phosphate to the same extent as wild type TnI (data not shown). The results of the ATPase and stopped flow experiments are summarised in Tables 1a and 1b. All three mutants behave very much like wild type TnI with respect to the effects of phosphorylation: treatment with PKA produces a substantial shift to the right in the ATPase versus pCa plot and an increase in the Ca^{2+} dissociation rate constant. These results indicate that none of the four Pro residues is important *per se* and that the stiffness of the postulated spacer arm region is not required to transmit the phosphorylation signal to TnC.

**Phosphorylation effects on troponin containing N-terminal deletion mutants of TnI** – We constructed a series of TnI mutants with an increasing number of residues missing
from the N-terminus (-3, -6, -9, -13, -15, -16, -17, -18, -21, -29). The deletions up to –18 were designed to test the role of the region of TnI N-terminal to the phosphorylation motif. All of the mutants up to and including –18 were phosphorylated by PKA at the same rate and to the same extent as wild type TnI (Fig. 4). The –21 mutant is not phosphorylated by PKA because the Arg-Arg–Xaa–Ser/Thr recognition motif for PKA has been disrupted (see Fig. 1) and in the –29 mutant the phosphorylation sites are deleted. The –21 and –29 mutants were designed to: 1) act as a control that all the observed effects were actually due to phosphorylation of Ser 22 and 23, and, 2) test the effect of removing the whole of the N-terminal extension.

The pCa50 values obtained in the ATPase experiments with the N-terminal deletion mutants of TnI are listed in Table 2 and plotted against the number of residues deleted in Fig. 5. The ΔpCa50 upon phosphorylation is substantial (>0.12 pCa units) for all the deletion mutants up to and including –15. This shows that residues 1 to 15 play only a minor role if, any, in the transmission of the phosphorylation signal. Deletions C-terminal to Pro 15 reduce the ability of phosphorylation to produce a desensitisation to Ca^{2+} indicating an importance for residues 16, 17 and 18. As one would predict, PKA treatment has no effect on the –21 and -29 mutants. However, as we remove residues C-terminal to Pro 15 we see a desensitisation to Ca^{2+} in the unphosphorylated state that mimics the effect of phosphorylation. Thus, it would
appear that residues 16-29 of TnI are responsible for holding cardiac troponin in the high Ca\(^ {2+}\) affinity unphosphorylated conformation.

Stopped flow measurement of Ca\(^ {2+}\) release was used to confirm the effects of phosphorylation on the deletion mutants. The dissociation rate constants are presented in Table 3 and are plotted against the number of residues deleted in Fig. 6. The trends in the data mirror those seen in the ATPase experiments. Up to 15 residues can be removed from the N-terminus of TnI without inhibiting the ability of phosphorylation to accelerate Ca\(^ {2+}\) release. We find that larger deletions (-18) halve the effect of phosphorylation on the Ca\(^ {2+}\) off rate. The effect of deleting the N-terminal extension is to accelerate Ca\(^ {2+}\) release mimicking the effects of phosphorylation as seen in the ATPase experiments. In fact, deleting the N-terminal extension is rather more effective at destabilising Ca\(^ {2+}\) binding than phosphorylation. These results show that it is the unphosphorylated form of the N-terminal extension that actively stabilises Ca\(^ {2+}\) binding and not the phosphorylated form that destabilises Ca\(^ {2+}\) binding.

The effect of using skeletal isoforms of TnC and TnT — Table 4 shows the pCa\(_{50}\) values obtained in ATPase experiments where the troponin was reconstituted using skeletal TnC or TnT rather than all three cardiac subunits. Replacing the cardiac TnT
with skeletal TnT did not alter the ability of phosphorylation to produce a desensitisation toward Ca\textsuperscript{2+}. Skeletal TnT was also able to support the phosphorylation induced acceleration of Ca\textsuperscript{2+} release (data not shown). These results indicate that the sequence differences of cardiac TnT with respect to skeletal TnT are not adaptations relating to the phosphorylation effect. However, when the cardiac TnC was replaced with the skeletal isoform phosphorylation of the TnI was unable to alter Ca\textsuperscript{2+} sensitivity. This may be due to a specific effect e.g. skeletal TnC not presenting a binding site for the N-terminal extension, or simply because the very different energetics of the skeletal TnC N-terminal domain (involving the binding of two Ca\textsuperscript{2+} ions rather than one) override the effects of phosphorylation.
DISCUSSION

In our experiments we see clear effects of bisphosphorylation of cardiac TnI by PKA as previously reported (17-19). Bisphosphorylation increases the Ca\textsuperscript{2+} dissociation rate from troponin by ~30 % and desensitises actomyosin ATPase activity toward Ca\textsuperscript{2+} by ~0.2 pCa units. We have used these effects to monitor the ability of mutations in the N-terminal extension of TnI to disrupt the transmission of the phosphorylation signal to the regulatory Ca\textsuperscript{2+} site in the N-terminal domain of TnC.

The sequence of the N-terminal extension suggested to us that the conformational changes produced in the vicinity of the phosphoSer residues conformation (21;22) might be transmitted via a rigid spacer arm to a TnC binding site in the first 9 residues of TnI.
We find, however, that this is not the case. The four Pro residues in the putative rigid spacer arm can be mutated to Ala without disrupting the effects of phosphorylation. Equally, residues 1 to 15 of TnI can be removed with little reduction in the effects of phosphorylation, leading us to conclude that the extreme N-terminal region of TnI (residues 1-15) is not required to transmit the phosphorylation signal to TnC. Amino acids equivalent to human cardiac TnI residues 1-9 are present in all mammalian cardiac TnIs sequenced to date but are less conserved between species than the rest of TnI (Fig. 1) and may play a role other than other than transmitting the phosphorylation signal.

In 1991 Ikebe and Morita (41) found that removal of residues Arg_{13}-Pro-Gln-Arg_{16} of smooth muscle regulatory light chain abolished the effects of myosin light chain kinase mediated phosphorylation of serine 19 (without preventing phosphorylation). In TnI, however, removal of residues N-terminal to the phosphorylation sites only reduces but does not abolish the effect of phosphorylation: the effects of phosphorylation are severely reduced in the –16, –17 and –18 mutants. This suggests that Ala 16, Pro 17 and Ile 18 may be involved in the transmission mechanism, although we note that mutation of Pro 17 to Ala seems to be without effect. The phosphorylation of Ser 22 and 23 introduces 4 negative charges into a positively charged region of the N-terminal extension. We therefore expected altered ionic interactions to underlie the effects of phosphorylation and were rather surprised that
these relatively small hydrophobic residues attached to the N-terminal side of the
diphosphorylation motif were of much importance. They may, however, contribute
directly to TnC binding or play a structural role in either the unphosphorylated or
diphosphorylated conformation of the N-terminal extension of TnI.

An important observation in this study is that progressively deleting residues 16 to 29
very effectively mimics phosphorylation. This demonstrates that it is the
unphosphorylated form of the N-terminal extension that is ‘active’ in stabilising Ca\(^{2+}\)
binding at the regulatory site. This conclusion is the opposite of that reached by
Wattanapermpool et al (42) in a comparison of wild type rat cardiac TnI with mouse
cardiac TnI with residues 1-32 deleted. In skinned fibre experiments the –32 and full
length TnI gave the same ATPase and force versus pCa relationships. Their
conclusion was that the phosphorylated extension is the ‘active’ form that destabilises
the Ca\(^{2+}\) bound form of TnC. However, several lines of evidence argue against the
diphosphorylated species being the active form, including two studies on skinned
fibres and one with reconstituted regulated actomyosin ATPase assays in which the
–32 mutation shifts pCa relationships to the right by 0.1 to 0.3 pCa units, mimicking
phosphorylation (43-45). Secondly, binding studies show that peptides corresponding to the
N-terminal extension bind to TnC with an affinity of approximately 10^5 M\(^{-1}\) in the
unphosphorylated state and that phosphorylation weakens this binding 100-fold (26;27).
Two of these studies identify the TnC binding site(s) on the N-terminal extension as
between residues 16 and 29 (21) and 18 and 31 (26). These are exactly the residues that, when removed, mimic phosphorylation. Indeed, the fact that the affinity of whole TnI for TnC is reduced upon phosphorylation may result directly from the loss of the N-terminal extension’s binding contribution (46). A third line of evidence are the elegant NMR studies carried out by Rosevear and coworkers (28-30). These experiments show that a fragment of cardiac TnI (TnI 1-80) containing the major TnC C-terminal domain binding site (within residues 33-80) and the N-terminal extension, makes contacts with the N-terminal domain of TnC (primarily in the inactive Ca$^{2+}$ binding site I). These contacts are not seen if Ser 22 and 23 are mutated to Asp, if the TnI 1-80 is bisphosphorylated or if the TnI 1-80 is replaced with TnI 30-80 i.e. if the N-terminal extension is deleted. They also showed that, in intact troponin, phosphorylation of TnI influenced the exchange between the open and closed conformations of the N-terminal domain of TnC and that, again, deletion of residues 1-30 mimicked phosphorylation.

We propose that when TnI is unphosphorylated residues 16-28 of TnI bind to the N-terminal domain of TnC stabilising the ‘open’ Ca$^{2+}$ bound conformation. Upon phosphorylation of Ser 22 and 23, the introduction of negative charges and localised conformational changes within the N-terminal extension of TnI reduces its affinity for TnC relieving the stabilisation of the open conformation and enhancing Ca$^{2+}$ release. Residues 1-15 are not crucial in this mechanism but removal of the predicted
TnC binding region (residues 16-29) has exactly the same effect as phosphorylation.

Further work is required to exactly define the interactions between residues 16-29 of TnI and TnC. Replacement of Ser 22 and 23 with Ala has no effect on Ca$^{2+}$ sensitivity (17) indicating that the Ser side chains are not forming functionally important hydrogen bonds in unphosphorylated troponin. Our work suggests that residues in both regions 16-21 and 24-29 contribute to binding to TnC.

ACKNOWLEDGEMENTS

We would like to thank Jonathan Bilmen and Frank Michelangeli for their help with the stopped flow experiments and Sue Brewer and Nina Sewell for technical assistance. Thank you to Hylary Trayer and Giles Fairhead for helpful discussions and to the British Heart Foundation for funding.

REFERENCES


Biochemistry 40, 7334-7341


FIGURE LEGENDS

FIG. 1. Sequence alignment of mammalian cardiac TnI N-terminal extensions. The dark shaded region depicts the conserved bisphosphorylation motif. The paler shaded region represents the 3 or 4 Xaa-Pro repeats of the postulated spacer arm. The Glu at
position 31 (human numbering) is the C-terminal residue specific to the cardiac TnI isoform (from refs 8-12).

FIG. 2. The effect of phosphorylation of wild type TnI on the calcium dependence of regulated actomyosin ATPase activity. Assays were conducted as described in experimental procedures. Filled symbols represent actomyosin regulated by unphosphorylated troponin and hollow symbols actomyosin regulated by troponin bisphosphorylated with PKA. Data points represent the mean (± SEM) of quadruplicate determinations.

FIG. 3. The effect of phosphorylation of wild type TnI on the rate of calcium release from troponin. The rate of calcium release was measured by stopped flow utilising the fluorescent enhancement of IAANS tethered at Cys 35 of TnC as described in experimental procedures. The graph shows the raw data (mean of six repetitions) and best fit exponentials to calcium release from unphosphorylated (shallower curve) and phosphorylated (steeper curve) troponin.

FIG. 4. Phosphorylation of TnI mutants using $\gamma^{33}$P]ATP. Phosphorylation by PKA was as described in the actomyosin ATPase measurements section of experimental procedures. The samples were subjected to SDS PAGE on 12% slab gels. Panel A shows the coomassie stained gel of phosphorylated ATP mixes (without myosin) containing various deletion mutants of TnI. The autoradiogram of the gel is shown in Panel B. Panel C shows the autoradiogram from a second experiment in which troponin containing a 1:1 mixture of wt and –18 TnI was incubated with kinase and the time-course of phosphorylation measured by sampling and stopping the reaction at increasing times as indicated. The phosphorylation conditions in the experiment of Panel C were as described in the Ca$^{2+}$ release measurements section of experimental
procedures.

FIG. 5. The effect of N-terminal deletions on the ability of TnI phosphorylation to modulate the Ca^{2+} sensitivity of actomyosin ATPase activity. The pCa_{50}s of ATPase assays regulated by unphosphorylated (filled symbols) and phosphorylated (open symbols) TnI have been plotted against the number of residues deleted from the N-terminus. Error bars represent the SEM on a minimum of four repetitions.

FIG. 6. The effect of N-terminal deletions on the ability of TnI phosphorylation to accelerate Ca^{2+} release from troponin. The calcium dissociation rate constants for unphosphorylated (filled symbols) and phosphorylated (open symbols) troponin have been plotted against the number of residues deleted from the N-terminus. Each value represents the mean of at least four independent determinations (± SEM).

TABLE 1a

_ATPase results: Pro→Ala TnI mutants._
<table>
<thead>
<tr>
<th>TnI</th>
<th>pCa50  -PKA</th>
<th>pCa50  +PKA</th>
<th>ΔpCa50</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15A</td>
<td>6.28 ± 0.02</td>
<td>6.08 ± 0.01</td>
<td>-0.20</td>
</tr>
<tr>
<td>P15/17A</td>
<td>6.20 ± 0.02</td>
<td>6.00 ± 0.02</td>
<td>-0.20</td>
</tr>
<tr>
<td>P11/13/15/17A</td>
<td>6.19 ± 0.04</td>
<td>6.05 ± 0.03</td>
<td>-0.14</td>
</tr>
</tbody>
</table>

**TABLE 1b**

*Stopped flow results: Pro→Ala TnI mutants*

<table>
<thead>
<tr>
<th>TnI</th>
<th>k_{off} (s^{-1})  -PKA</th>
<th>k_{off} (s^{-1})  +PKA</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>P15A</td>
<td>25.8 ± 1.0</td>
<td>32.6 ± 0.8</td>
<td>26</td>
</tr>
<tr>
<td>P15/17A</td>
<td>25.3 ± 0.7</td>
<td>32.0 ± 1.0</td>
<td>26</td>
</tr>
<tr>
<td>P11/13/15/17A</td>
<td>28.7 ± 0.6</td>
<td>36.5 ± 1.3</td>
<td>27</td>
</tr>
</tbody>
</table>

The effects of the Pro→Ala mutations of TnI and phosphorylation on the Ca^{2+} sensitivity of actomyosin ATPase activity (1a) and Ca^{2+} dissociation rate (1b). In both cases the values shown are the mean (± SEM) of four independent determinations.
**TABLE 2**

ATPase results: N-terminal deletions of TnI

<table>
<thead>
<tr>
<th>TnI</th>
<th>pCa&lt;sub&gt;50&lt;/sub&gt; -PKA</th>
<th>pCa&lt;sub&gt;50&lt;/sub&gt; +PKA</th>
<th>ΔpCa&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>6.35 ± 0.03</td>
<td>6.12 ± 0.04</td>
<td>-0.23</td>
</tr>
<tr>
<td>-3</td>
<td>6.36 ± 0.04</td>
<td>6.16 ± 0.05</td>
<td>-0.20</td>
</tr>
<tr>
<td>-6</td>
<td>6.30 ± 0.05</td>
<td>6.15 ± 0.04</td>
<td>-0.15</td>
</tr>
<tr>
<td>-9</td>
<td>6.31 ± 0.05</td>
<td>6.17 ± 0.05</td>
<td>-0.14</td>
</tr>
<tr>
<td>-13</td>
<td>6.30 ± 0.03</td>
<td>6.18 ± 0.03</td>
<td>-0.12</td>
</tr>
<tr>
<td>-15</td>
<td>6.28 ± 0.07</td>
<td>6.15 ± 0.06</td>
<td>-0.13</td>
</tr>
<tr>
<td>-16</td>
<td>6.18 ± 0.06</td>
<td>6.12 ± 0.06</td>
<td>-0.06</td>
</tr>
<tr>
<td>-17</td>
<td>6.21 ± 0.04</td>
<td>6.15 ± 0.04</td>
<td>-0.06</td>
</tr>
<tr>
<td>-18</td>
<td>6.16 ± 0.03</td>
<td>6.10 ± 0.03</td>
<td>-0.06</td>
</tr>
<tr>
<td>-21</td>
<td>6.08 ± 0.02</td>
<td>6.07 ± 0.02</td>
<td>-0.01</td>
</tr>
<tr>
<td>-29</td>
<td>6.03 ± 0.03</td>
<td>6.03 ± 0.03</td>
<td>0</td>
</tr>
</tbody>
</table>

Actomyosin ATPase results obtained with N-terminal deletion mutants of TnI. The values shown are the mean (± SEM) of at least four independent determinations.
**TABLE 3**

*Stopped flow results: N-terminal deletions of TnI*

<table>
<thead>
<tr>
<th>TnI</th>
<th>pCa&lt;sub&gt;50&lt;/sub&gt; -PKA</th>
<th>pCa&lt;sub&gt;50&lt;/sub&gt; +PKA</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wt</td>
<td>25.5 ± 0.6</td>
<td>33.0 ± 0.3</td>
<td>29</td>
</tr>
<tr>
<td>-9</td>
<td>24.4 ± 0.3</td>
<td>32.4 ± 0.2</td>
<td>33</td>
</tr>
<tr>
<td>-15</td>
<td>25.5 ± 0.5</td>
<td>30.7 ± 0.6</td>
<td>20</td>
</tr>
<tr>
<td>-18</td>
<td>30.9 ± 1.0</td>
<td>35.8 ± 0.7</td>
<td>16</td>
</tr>
<tr>
<td>-21</td>
<td>32.8 ± 0.5</td>
<td>33.6 ± 0.4</td>
<td>2</td>
</tr>
<tr>
<td>-29</td>
<td>41.4 ± 0.6</td>
<td>41.2 ± 0.3</td>
<td>0</td>
</tr>
</tbody>
</table>

Ca<sup>2+</sup> dissociation rates from troponin reconstituted with TnI deletion mutants. The values are the mean (± SEM) of at least four independent determinations.
TABLE 4  
_Skeletal isoform switching_

<table>
<thead>
<tr>
<th>Isoform switch</th>
<th>pCa50 -PKA</th>
<th>pCa50 +PKA</th>
<th>Δ pCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>skeletal TnT</td>
<td>6.21 ± 0.04</td>
<td>6.04 ± 0.05</td>
<td>-0.17</td>
</tr>
<tr>
<td>skeletal TnC</td>
<td>5.94 ± 0.02</td>
<td>5.95 ± 0.02</td>
<td>+0.01</td>
</tr>
</tbody>
</table>

Actomyosin ATPase results obtained with troponin in which the cardiac TnT or TnC have been replaced with the skeletal isoforms. The values shown are the mean (± SEM) of four independent determinations.
FIG 3

% Calcium Release

Time (s)

+ PKA

- PKA
FIG 4

A

B

C

0 1 2 4 8 15 30 45 60 90 min

← Actin
← TM
← TnT

} TnI
← TnC

} TnI

← wt TnI
← -18
Structural consequences of cardiac troponin I phosphorylation
Douglas G. Ward, Michael P. Cornes and Ian P. Trayer

J. Biol. Chem. published online August 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206744200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2002/08/30/jbc.M206744200.citation.full.html#ref-list-1