Single Mutation (A162H) in Human Cardiac Troponin I Corrects Acid pH Sensitivity of Ca$^{2+}$-regulated Actomyosin S1 ATPase*

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In contrast to skeletal muscle, the efficiency of the contractile apparatus of cardiac tissue has long been known to be severely compromised by acid pH as in the ischemia of myocardial infarction and other cardiac myopathies. Recent reports (Westfall, M. V., and Metzger, J. M. (2001) News Physiol. Sci. 16, 278–281; Li, G., Martin, A. F., and Sokoloff, R. J. (2001) J. Mol. Cell. Cardiol. 35, 1309–1320) have indicated that the reduced Ca$^{2+}$ sensitivity of cardiac contractility at low pH (≤pH 6.5) is attributable to structural difference(s) in the cardiac and skeletal inhibitory components (TnIs) of their troponins. Here, using a reconstituted Ca$^{2+}$-regulated human cardiac troponin-tropomyosin actomyosin S1 ATPase assay, we report that a single TnI mutation, A162H, restores Ca$^{2+}$ sensitivity at pH 6.5 to that at pH 7.0. Levels of inhibition (pCa 7.0), activation (pCa 4.0), and cooperativity of ATPase activity were minimally affected. Two other mutations (Q155R and E164V) also previously suggested by us (Pearlstone, J. R., Sykes, B. D., and Smillie, L. B. (1997) Biochemistry 36, 7601–7606) and involving charged residues showed no such effects. With fast skeletal muscle troponin, a single TnI H130A mutation reduced Ca$^{2+}$ sensitivity at pH 6.5 to levels approaching the cardiac system at pH 6.5. These observations provide structural insight into long-standing physiological and clinical phenomena and are of potential relevance to therapeutic treatments of heart disease by gene transfer, stem cell, and cell transplantation approaches.

In both cardiac and skeletal muscles, the generation of force through the interaction of thick and thin filaments of the sarcomere is under the control of Ca$^{2+}$ concentration in the sarcoplasm. The heterotrimetric troponin (Tn)$^3$ complex through its interaction with the thin filament proteins F-actin (A) and tropomyosin (TM) is the dominant player in this control. Conformational changes associated with Ca$^{2+}$ binding to the regulatory N domain of troponin C (TnC) alter its interactions with both of the other Tn subunits, troponin I (TnI, inhibitory subunit) and troponin T (TnT, TM binding subunit), and of TnI and TnT with TM and A. In consequence, TM moves from its steric blocking position, facilitating interaction of thick filament myosin heads with the thin filament and generation of ATPase activity and contraction. Sequestration of Ca$^{2+}$ by the sarcoplasmic reticulum reverses the process with ensuing relaxation (for reviews, see Refs. 1 and 2).

As key components of this system, the functional and structural relationships of TnC and TnI and their interaction in the presence and absence of Ca$^{2+}$ have been the subject of intensive investigation over several decades (for reviews, see Refs. 2–5). While high resolution structures of the binary or ternary (with TnT) complexes by x-ray crystallographic or NMR approaches have remained elusive, considerable progress has been made in the mapping of interaction sites of the TnC and TnI components. Two such TnI regions, first described by S. V. Perry and colleagues (for review, see Ref. 5) (see Fig. 1), involve the NH2-terminal segment of fast skeletal TnI (fsTnI) (residues –1–30) and the so-called inhibitory peptide or Ip region (residues 96–116). While the high affinity interaction involving residues –1–30 likely persists throughout the contraction-relaxation cycle, the weaker Ca$^{2+}$-dependent interaction involving Ip permits toggling between TnC (+Ca$^{2+}$) and TMA (–Ca$^{2+}$). As such it accounts for a significant proportion of the inhibitory capacity of TnI and the Tn complex (–Ca$^{2+}$). More recently several studies have shown that for full functional competence additional TnI segments that are COOH-terminal to the Ip region are operative. Thus, for example, Tripet et al. (6) identified an additional Ca$^{2+}$-sensitive TnC binding region (fsTnI-(116–131)) and a second TMA binding segment (fsTnI-(140–148)). In a report from our laboratory (7), we described a sequence motif repeated 3-fold, once in the Ip region (residues –101–114, designated α) and twice more in the region of residues –121–132 (β) and –135–146 (γ). The latter two correspond approximately to those described by Tripet et al. (6). Based on binding studies to TnC and its isolated domains we concluded that while residues 96–116 (Ip) largely interact with the structural C domain, residues 117–148 (β and γ) bind to the regulatory N domain (7).

In contrast to skeletal muscle, the efficiency of the contractile apparatus of cardiac tissue has long been known to be compromised by acid pH (≤pH 6.5) as in the ischemia of myocardial infarction and other cardiac myopathies (8–10). Recent reports (11–14) have indicated that this differential effect can be ascribed to differences in their TnI components and in particular to the COOH-terminal regions distal to their Ip segments. Consistent with these observations and from a comparison of the cardiac and skeletal TnI α, β, and γ sequence motifs, we previously identified several highly conserved charge differences, especially in the β motif, and suggested (7) these as

TnI, inhibitory component of Tn; TnT, TM binding component of Tn; fsTnI, chicken fast skeletal isoform of TnI; ssTnI, slow skeletal isoform of TnI; cTnI, human cardiac isoform of TnI; cTnC, human cardiac isoform of TnC; fsTnC, chicken fast skeletal isoform of TnC; cTnT, human cardiac isoform of TnT; fsTnT, chicken fast skeletal isoform of TnT; Ip, inhibitory peptide.
potential candidates responsible for the differential acid pH effect on the Ca\(^{2+}\) sensitivities of the two systems (see Fig. 1).

Here we describe experiments to test this proposal.

**EXPERIMENTAL PROCEDURES**

*Preparation of cTnC*\(^{WT}\) *and fsTnC—*The pET3a/cTnC and pET3a/fsTnC constructs have been described previously (15, 16). For the present study, the Cys-34 and Cys-83 codons (TGC) of pET3a/cTnC were mutated to Ser codons (AGC) using paired 31- and 37-mer oligonucleotides, respectively (Stratagene). The 37-mer oligonucleotide also incorporated a base change for the Arg-84 codon from CGG (rare in *Escherichia coli*) to that for Ala (GCC) was by the PCR chain extension method to produce pET3a/cTnC. Proteins for transformation, expression in *E. coli* BL21 (DE3) pLysS cells, and purification were as described previously (17). It was engineered into the *NdeI BamHI* sites of pET3a using standard PCR technology. These processes also involved the mutation of Ala-1 and Gly-3 codons to GCC and GGT, respectively, as recommended (18) for high yield expression. Using this construct (pET3acTnC) as template, the Cys-79 and Cys-96 codons were mutated to Ser (AGC) using paired 24- and 27-mer oligonucleotides. Codon changes were to CGT for Arg, GTG for Val, and CAT for His. The C79S and C96S mutants were then incorporated into these constructs as for pET3a/cTnC. The pET3a/cTnC was converted to pET3a/cTnC\(^{WT}\). The pET3acCnTnC(Q155R/A162H/E164V), pET3acTnC(Q155R/E164V), and pET3acTnC(A162H) were initially constructed using pET3acTnC as template for sequential rounds of mutagenesis using paired 25-, 27-, or 28-mer oligonucleotides. Codon changes were to CGT for Arg, GTG for Val, and CAT for His. The C79S and C96S mutants were then introduced into these constructs as for pET3acTnC\(^{WT}\). The fsTnI construct in pET3a was a gift from F. C. Reinach (19). Sequence analysis of this construct, which expressed poorly in our experiments, showed that the codons for Arg-13 and Arg-14 were both CGG (rare in *E. coli*). In the case of the Cys-96 to Ser mutation the oligonucleotides used also introduced a codon mutation for Arg-97 from CGT to GCC (common in *E. coli*). The final construct is designated pET3acfsTnI\(^{WT}\). The pET3acCnTnC(Q155R/A162H/E164V), pET3acTnC(Q155R/E164V), and pET3acTnC(A162H) were initially constructed using pET3acTnC as template for sequential rounds of mutagenesis using paired 25-, 27-, or 28-mer oligonucleotides. Codon changes were to CGT for Arg, GTG for Val, and CAT for His. The C79S and C96S mutants were then introduced into these constructs as for pET3acTnC\(^{WT}\). The fsTnI construct in pET3a was a gift from F. C. Reinach (19). Sequence analysis of this construct, which expressed poorly in our experiments, showed that the codons for Arg-13 and Arg-14 were both CGG (rare in *E. coli*). In the case of the Cys-96 to Ser mutation the oligonucleotides used also introduced a codon mutation for Arg-97 from CGT to GCC (common in *E. coli*). The final construct is designated pET3acfsTnI\(^{WT}\).

*Preparation of Human Cardiac TnT (cTnT) and fsTnT—*A clone from rabbit skeletal muscle was prepared as described previously (20). Final dialysis against pH 7.0 or 6.5 assay buffer (plus 1 mM ATP). Final stock concentration was 90–115 \(\mu\)g/ml. Assays were carried out within 2 weeks. Rabbit skeletal myosin subfragment 1 (S1) with associated light chains (A1 and A2) was prepared as described previously (23) and dialyzed against pH 7.0 or 6.5 assay buffer. Final stock concentration was 22–26 \(\mu\)g/ml. Rabbit skeletal TM, prepared as freeze-dried powder (24) without fractionation into \(\alpha\) and \(\beta\) chains, was dissolved and dialyzed against pH 7.0 or 6.5 assay buffer to give a 12–14 \(\mu\)g/ml stock solution. Precise concentrations of all three stock solutions were determined by amino acid analyses.

*Reconstitution of Tn—*The stock solution of TnC (−5 mg/ml) was in assay buffer; TnI (−5 mg/ml) was in \(\text{H}_2\text{O} \) at pH 4.0–4.5, and TnT (−5 mg/ml) was in 4 M guanidine-HCl, 1 M KCl, 100 mM imidazole, 4.5 mM MgCl\(_2\), 1 mM dithiothreitol, pH 7.0. Concentrations of each stock solution were determined by amino acid analyses, and then all three were combined to give a molar ratio of TnI:TnT:TnC of 1:1:2. Following dialysis against pH 7.0 or 6.5 assay buffer (−EGTA, +50 \(\mu\)M Ca\(^{2+}\)) the precise Tn stock concentration (16–24 \(\mu\)M) expressed as [TnI], was determined by amino acid analyses. This simplified reconstitution protocol gave equivalent ATPase assay data as native Tn in control experiments.

*ATPase Assays—*At pH 7.0 the assay buffer was 30 mM KCl, 20 mM imidazole, 4.5 mM MgCl\(_2\), 0.15 mM NaH\(_2\)PO\(_4\), 0.5 mM EGTA, 1 mM dithiothreitol. At pH 6.5 the [KCl] was reduced to 26 mM. The protein molar ratios for A:S1 assays were 6:1, for TM:A:S1 assays were 1:6:1, and for Tn:TM:A:S1 assays were 2:1:6:1. Final [A] was 7.5 \(\mu\)g/ml in all assays. The order of addition to each assay tube was pH 7.0 or 6.5 assay buffer, S1, A, TM, varying amounts of 2 mM Ca\(^{2+}\) in pH 7.0 or 6.5 assay buffer, and Tn. Reaction at 25 °C was initiated by final addition of 12.5 \(\mu\)l of 20 mM ATP in pH 7.0 or 6.5 assay buffer to a final total volume of 200 \(\mu\)l. Final free [Ca\(^{2+}\)] was computed with an in-house program and expressed as pCa = −log[Ca\(^{2+}\)]. Reactions were terminated, and inorganic phosphate was determined by the method of Heinonen and Lahlbi (25).

**RESULTS AND DISCUSSION**

Initially as controls we compared human cardiac and chicken fast skeletal Tns reconstituted from their respective isolated recombinant components. In all experiments the two Cys res-
residues on each of cTnI and fsTnI had been previously mutated to Ser. These residues in cTnC were also mutated to Ser. These changes have been shown not to alter ATPase activities or Ca$^{2+}$ sensitivities in reconstituted TnC. We first mutated all three residues of cTnI and fsTnI, and in our experiments had increased assay sensitivity in reconstituted TnA and TnT actomyosin ATPase reconstituted with cardiac or skeletal "WT" and mutant TnI at pH 7.0 and 6.5 were measured simultaneously with the same F-actin preparation.

Parameters of Ca$^{2+}$-regulated troponin-tropomyosin actomyosin S1 ATPase reconstituted with cardiac or skeletal "WT" or mutant TnI at pH 7.0 and 6.5 are shown in Table I. About the corresponding mutant activities at pH 7.0 and 6.5 were measured simultaneously with the same F-actin preparation.

WT

![Image](image.png)

**Table I**

| TnI | pH | ATPase Activity (% of A) | pCa$_{0.5}^{a,b}$ | Hill Coefficient (n) | No. of determinations |
|-----|----|--------------------------|------------------|---------------------|----------------------|
| cTnI | 7.0 | 57.2 ± 1.8 | 121.8 ± 8.6 | 5.89 ± 0.05 | 1.9 | 12 |
| "WT" | 6.5 | 79.1 ± 2.8 | 130.2 ± 14.9 | 5.40 ± 0.06 | 1.5 | 10 |
| cTnI | 7.0 | 57.6 ± 1.1 | 120.3 ± 2.3 | 6.03 ± 0.04 | 2.1 | 3 |
| Q155R/A162H/E164V | 6.5 | 69.9 ± 1.8 | 123.2 ± 5.2 | 5.88 ± 0.02 | 1.9 | 3 |
| cTnI | 7.0 | 54.2 ± 0.5 | 118.0 ± 0.3 | 5.90 ± 0.07 | 1.9 | 3 |
| Q155R/E164V | 6.5 | 71.9 ± 1.7 | 126.3 ± 5.9 | 5.38 ± 0.03 | 1.4 | 3 |
| cTnI | 7.0 | 58.4 ± 2.4 | 117.4 ± 1.8 | 5.89 ± 0.01 | 2.0 | 3 |
| A162H | 6.5 | 76.4 ± 0.9 | 126.1 ± 0.5 | 5.79 ± 0.01 | 1.7 | 3 |
| fsTnI | 7.0 | 56.7 ± 2.2 | 176.8 ± 6.0 | 6.18 ± 0.04 | 2.0 | 3 |
| "WT" | 6.5 | 81.2 ± 1.4 | 189.2 ± 11.9 | 6.08 ± 0.04 | 1.7 | 3 |
| fsTnI | 7.0 | 56.7 ± 2.2 | 104.1 ± 0.7 | 5.96 ± 0.01 | 2.4 | 3 |
| H130A | 6.5 | 81.2 ± 1.4 | 116.0 ± 2.0 | 5.65 ± 0.04 | 2.0 | 3 |

*a* A S1 ATPase activities in various preparations of F-actin varied from 0.70 to 0.94 s$^{-1}$ at pH 7.0 and from 0.62 to 0.85 s$^{-1}$ at pH 6.5 with the A S1 ratio (6:1) and buffer conditions used. All values reported are from paired experiments in which "WT" and corresponding mutant activities at pH 7.0 and 6.5 were measured simultaneously with the same F-actin preparation.

*b* pCa$_{0.5}$ is defined as pCa at half-maximal ATPase activity.

![Image](image.png)

**Fig. 3.** Comparison of Ca$^{2+}$ regulation of cTnI"WT" and cTnI(Q155R/A162H/E164V) (A) and cTnI"WT" and cTnI(Q155R/E164V) (B). A, cTnI"WT"; ○, pH 7.0; ●, pH 6.5; cTnI(Q155R/A162H/E164V); □, pH 7.0; ■, pH 6.5. B, cTnI"WT"; ○, pH 7.0; ●, pH 6.5; cTnI(Q155R/E164V); +, pH 7.0; ×, pH 6.5.

![Image](image.png)

**Fig. 4.** Comparison of Ca$^{2+}$ regulation of cTnI"WT" and cTnI(A162H) (A) and fsTnI"WT" and fsTnI(H130A) (B). A, cTnI"WT"; ○, pH 7.0; ●, pH 6.5; cTnI(A162H); +, pH 7.0; ×, pH 6.5. B, fsTnI"WT"; ■, pH 7.0; □, pH 6.5; fsTnI(H130A); ▲, pH 7.0; △, pH 6.5.

To test the effects of the β motif charge differences between the cardiac and skeletal TnIs (see Fig. 1) we first mutated all three residues in human cTnI to the corresponding residues in the β motif of fsTnI to produce cTnI(Q155R/A162H/E164V). Following reconstitution with human cardiac TnC and TnT and measurement of ATPase activity as a function of pCa (where pCa = $-\log([Ca^{2+}])$) at pH 7.0 and 6.5 (see Fig. 3A and Table I) it was evident that the mutation of all three residues had markedly decreased the acid sensitivity of the human cardiac TnC. We next examined the effects of mutating only two of the residues to produce cTnI(Q155R/E164V). Similar ATPase assays (Fig. 3B) at pH 7.0 and 6.5 as a function of pCa showed that this double mutant had a Ca$^{2+}$ sensitivity virtually identical to cTnC"WT"; i.e. these mutations had no effect on the acid sensitivity of the cardiac system. Since this result clearly indicated the primary importance of the A162H mutation in this phenomenon we next carried out assays on the single mutant cTnI(A162H). In comparison with cTnI"WT" (Fig. 4A and Table I) it was clear that this single mutation had largely eliminated the acid pH sensitivity of the system. Thus while the pCa$_{0.5}$ for the cTnI"WT" was reduced...
from 5.89 ± 0.05 at pH 7.0 to 5.40 ± 0.06 at pH 6.5, the corresponding values for cTnI(A162H) were 5.89 ± 0.01 and 5.79 ± 0.01, respectively (see Table I). This latter decrease in pCa0.5 (ΔpCa0.5 = −0.10) is similar to that observed with the fsTnI"WT (ΔpCa0.5 = −0.08) as shown in Fig. 2 and Table I.

We have also examined the effects of the corresponding single reverse mutation (fsTnI(H130A)) in the reconstituted fast skeletal muscle Tn system (Fig. 4B and Table I). The data show that in contrast to fsTnI"WT, this single mutation significantly increased the acid pH sensitivity of the Ca2+-regulated complex. Thus the ΔpCa0.5 values in going from pH 7.0 to 6.5 are −0.08 for fsTnI"WT and −0.31 for fsTnI(H130A). The data clearly indicate an important role for His-130 in the maintenance of Ca2+ sensitivity against the effects of decreased pH in the fast skeletal muscle system.

The ATPase data of Table I provide additional insights into the roles of the cardiac and skeletal Tn components and of their Tn mutants. Under +Ca2+ conditions the ATPases of both the cardiac and skeletal "WT" Tn systems showed enzymic activity levels significantly above that of A51 alone (taken as 100%). Previous studies (Ref. 28 and references therein) have shown that this potentiation of activity occurs only with the complete Tn complex and not with TnI lacking TnT. The present direct comparison of the cardiac and fast skeletal "WT" Tns under identical experimental conditions shows a large difference in their potentiating effects (−125 and −180%, respectively), an observation to our knowledge not previously reported. The data thus imply distinct functional differences of the two TnTs and their interactions with their respective TnI and TnC isoforms.

The data for the effects of the Ala to His and His to Ala TnI mutations on their respective reconstituted Ca2+-activated Tn-TM-A51 activities also show major differences. Thus the cTnI(A162H) mutation showed little effect either on the level of inhibition (−Ca2+) or on the potentiating effect (+Ca2+) in comparison with cTnI"WT." Based on the present data we may conclude that this mutation has a singular effect in blunting the effects of acid pH on the Ca2+ sensitivity of the cardiac system without materially affecting its minimal (−Ca2+) and maximal (+Ca2+) ATPase activities. This is not the case with the fast skeletal system in which the H130A mutation led to virtual elimination of this potentiation effect reducing ATPase activity from −180% to levels close to 100%. Thus, the fast skeletal TnI His-130 residue appears to play a critical role, either directly or indirectly, in interactions among the Tn components, not only in conferring resistance to acid pH but also in facilitating significant increases in ATPase levels over those observed with A51 alone.

The present data are of direct relevance to studies on slow skeletal muscle TnI (ssTnI) (see Refs. 11–14 and references therein). As the major TnI isoform in fetal and newborn hearts, ssTnI is replaced during development by the adult protein. Consistent with the glucose-dependent metabolism and high lactate levels of fetal/neonatal cardiac tissue (29), its contractile properties have a resistance to acid pH comparable to that of fast skeletal muscle. Recent evidence has implicated the COOH-terminal region of ssTnI as responsible for this phenomenon (11–13). Sequence comparisons of the several TnI isoforms (Fig. 1) reveal that as with fsTnI the acid resistance of ssTnI can be attributed to the equivalent His.

The acid base properties of the imidazole side chain of His (pKa = 6.5) are, of course, uniquely suited to the role of His in blunting the effects of decreased pH on the Tn system. As pH is decreased from 7.0, the increased positive charge of His can be postulated to lead to stronger interaction with one or more Glu or Asp side chains in the regulatory N domain of TnC, thereby stabilizing this region. Identification of such acidic residue(s), currently not possible based on present structural information (30, 31) of the TnC interface, will require further investigation.

In conclusion we consider the observations reported here to be of potential relevance to therapeutic treatments of heart disease by gene transfer, stem cell, and cell transplantation approaches. In addition to restoring Ca2+ sensitivity at pH 6.5 to that at pH 7.0, the data indicate that neither maximal ATPase (pCa 4.0) nor inhibition (pCa 7.0) is significantly compromised by this TnI A162H mutation as compared with wild type. This singular property would of course be an important consideration in the development of remedial protocols.

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Note Added in Proof—A recent communication (Westfall, M., Burton, A. R., Albayya, F. P., Forfa, S., and Metzger, J. M. (2002) Biophys. J. 82, 388a) reports that for the slow skeletal isoform of TnI, expressed in adult cardiomyocytes, the mutations H132A and V134E increase the acid pH sensitivity of tension development to levels similar to that observed with cardiac TnI. Since these are equivalent to two of the reverse mutations observed in the present study we predict that only their H132A mutation will be found responsible for the observed effects.

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