Elucidation of Isoform-dependent pH Sensitivity of Troponin I by NMR Spectroscopy

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Background: pH sensitivity differences between skeletal and cardiac muscle originate from distinct troponin I isoforms.
Results: Histidine 130 in skeletal troponin I, absent in the cardiac isoform, makes an electrostatic interaction with cardiac troponin C at low pH.
Conclusion: This interaction compensates for decreased calcium affinity under acidic conditions.
Significance: This understanding may aid in the development of therapies that reverse the negative inotropic effects of acidosis.

Myocardial ischemia is characterized by reduced blood flow to cardiomyocytes, which can lead to acidosis. Acidosis decreases the calcium sensitivity and contractile efficiency of cardiac muscle. By contrast, skeletal and neonatal muscles are much less sensitive to changes in pH. The pH sensitivity of cardiac muscle can be reduced by replacing cardiac troponin I with its skeletal or neonatal counterparts. The isoform-specific response of troponin I is dictated by a single histidine, which is replaced by an alanine in cardiac troponin I. The decreased pH sensitivity may stem from the protonation of this histidine at low pH, which would promote the formation of electrostatic interactions with negatively charged residues on troponin C. In this study, we measured acid dissociation constants of glutamate residues on troponin C and of histidine on skeletal troponin I (His-130). The results indicate that Glu-19 comes in close contact with an ionizable group that has a pKₐ of ~6.7 when it is in complex with skeletal troponin I but not when it is bound to cardiac troponin I. The pKₐ of Glu-19 is decreased when troponin C is bound to skeletal troponin I and the pKₐ of His-130 is shifted upward. These results strongly suggest that these residues form an electrostatic interaction. Furthermore, we found that skeletal troponin I bound to troponin C tighter at pH 6.1 than at pH 7.5. The data presented here provide insights into the molecular mechanism for the pH sensitivity of different muscle types.

In myocardial ischemia, cardiomyocytes do not receive adequate oxygen supply, which culminates in a significant drop in intracellular pH (~6.5). This acidosis is coupled with a dramatic reduction in the Ca²⁺ sensitivity and force of muscle contraction (1, 2). Ca²⁺ entering the cytosol following muscle cell excitation triggers a series of thin and thick filament protein-protein interactions that lead to the force-generating actomyosin ATPase activity. The thin filament is composed of three proteins, actin, tropomyosin, and the Ca²⁺-binding molecule troponin. Troponin is a heterotrimeric protein complex with three subunits as follows: cardiac troponin C (cTnC), responsible for binding Ca²⁺; cardiac troponin I (cTnI), the inhibitory subunit; and cardiac troponin T, the subunit that attaches troponin to the thin filament via interactions with troponin I and tropomyosin. During muscle relaxation (diastole), the “inhibitory” and C-terminal regions of cTnT interact with actin preventing contraction. The association of Ca²⁺ with the N-terminal domain of cTnC (cNTnC) leads to the binding of the “switch” region of cTnI (cTnI(147–163)) to cNTnC. This interaction prompts the dissociation of the inhibitory and C-terminal regions of cTnI from actin, resulting in contraction (for reviews see Refs. 3–5).

The negative inotropic effect (decrease in contractility) of acidosis is due, in part, to a decrease in the Ca²⁺ (6–8) and cTnI (9) affinity for cTnC. Cardiomyocytes of neonatal rats are less sensitive to low pH than adult heart cells (10), and Westfall et al. (11) noticed that the pH sensitivity of cardiac muscle cells is dramatically reduced by the substitution of cTnI with the neonatal troponin I (also termed slow skeletal TnI (ssTnI)). Likewise, the fast skeletal isoform of TnI (sTnI) has been shown to make the myofilament less sensitive to acidic conditions (8, 12, 13). The regions of ssTnI and sTnI that are responsible for the isoform-specific response to pH were initially localized to the C-terminal region (12, 14). It was later determined that the difference in pH sensitivity between TnI isoforms largely arises from a single histidine in the switch region of sTnI and ssTnI, which is replaced by an alanine in cTnI (13, 15). For a comparison of the sequences of the switch regions of cTnI, sTnI, and ssTnI, see Fig. 1. Smillie and co-workers (13) found that when
Electrostatic Interaction between Troponin C and Troponin I

This alanine of cTnI (Ala-162) was replaced by a histidine, a dramatic reduction in pH sensitivity was observed. Furthermore, when His-130 of sTnI was replaced by an alanine (the numbering is different between the isoforms, because cTnI has an extra 32 residues at its N terminus not present in sTnI and ssTnI), the pH sensitivity of muscle containing sTnI was similar to cTnI. Recently, this alanine to histidine substitution (dubbed the “histidine button”) has been shown to partially blunt the adverse effects of acidosis in intact myocytes, isolated hearts, and whole mice (15).

The enhanced pH resistance of sTnI has been suggested to stem from interactions with the side chain of histidine; it is the only amino acid that can be either neutral or positively charged in the physiological pH range. Thus, if the imidazole group of histidine is protonated during acidosis, its positive charge may interact with the abundant negatively charged side chains of cNTnC. This may increase the affinity of sTnI for cNTnC and could explain how replacing cTnI with sTnI partially restores myocardial sensitivity at low pH (13). In the skeletal x-ray crystal structure, His-130 of sTnI forms a salt bridge with Glu-20 of sNTnC (4.1 Å between Nδ1 of His-130 and Cβ of Glu-20), amino acid numbering is taken from the deposited x-ray crystal structure (16). Solution NMR relaxation (17) and chemical shift (18) data of sTnI in complex with sTnC confirm that this region of sTnI is rigid, consistent with the formation of this salt bridge in solution. The x-ray and NMR structures of cardiac troponin indicate that the corresponding glutamate in cTnC, Glu-19, does not make a homologous interaction with cTnI (19, 20).

The focus of this study was to use NMR spectroscopy to define the molecular basis for the reduced depression of myofilament Ca2+ sensitivity at low pH caused by this single histidine residue of sTnI. The nuclear Overhauser enhancement (NOE) is a fundamental measurement utilized by NMR spectroscopists to determine the atomic resolution structure of proteins. NOE spectroscopy (NOESY) provides structural information of a molecule by relying on short proton-proton distances (≤ 5 Å) (21). Because the closest distances between the imidazole protons of His-130 and the side chain protons of Glu-20 are 5.4 and 5.6 Å (Hβ1 and Hβ2) in the skeletal x-ray structure (16), NOEs between these residues would be too weak to observe. However, if these groups make an electrostatic interaction, knowledge of their acid dissociation constants could provide clues as to whether the same interaction occurs when sTnI is bound to cNTnC. Palpant et al. (22) used computational methods to predict that the pKd of His-130 in sTnI would be shifted upward when in complex with cNTnC, an indication of the formation of a salt bridge with a negatively charged moiety (23). We used NMR spectroscopy to investigate the role of electrostatic interactions between sTnI and cNTnC because it is a particularly well suited technique to monitor amino acid pKd values of individual residues in a protein (24–26).

The pKd values of glutamate residues on cNTnC were determined for four different states of cNTnC as follows: Ca2+-free (apo), Ca2+-bound, Ca2+ - and cNTnC(147–163)-bound, and the complex of cNTnC bound to Ca2+ and the switch region of sTnI (sNTnC(115–131)). The pKd of His-130 of sNTnC(115–131) was also monitored in both free and bound states. The pKd of Glu-19 was depressed when in complex with sNTnC(115–131), and a second ionization event was observed (from the protonation of the imidazole of His-130). The pKd of His-130 increased when sNTnC(115–131) was bound to cNTnC. Both the increase in His-130 pKd and the decrease in the pKd of Glu-19 are consistent with the formation of an electrostatic interaction between these two residues. The affinity of cNTnC for sNTnC(115–131) was also measured at two pH values (6.1 and 7.5), and it was found that sNTnC(115–131) bound with a tighter affinity at pH 6.1. The pH-dependent differences in the affinity of sNTnC(115–131) for cNTnC revealed that the ionization state of His-130 fine-tunes the affinity of sNTnC(115–131) for cNTnC. These results provide structural insights into the mechanism by which a single histidine on troponin I can reverse the decrease in myofilament Ca2+ sensitivity during acidosis.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Recombinant human cNTnC (residues 1–89, C84S/C35S, a-Cys form). The engineering of the vector and the expression of 15N- and 13C,15N-labeled proteins in *Escherichia coli* were as described previously (27). GL Biochem Ltd. (Shanghai, China) synthesized cTnI(147–163) (acetyl-RISADAMMQALLGAR-amide) and Alberta Peptide Institute (API) synthesized sTnI(115–131) (acetyl-RMS-ADAMLKALLGSKHK-amide). HPLC and mass spectrometry (ESI and MALDI) were employed to verify peptide quality and purity. All NMR samples were prepared in 5-mm NMR tubes and had a volume of ~500 µl. Protein samples were prepared in an NMR buffer composed of 90% H2O, 10% D2O, 100 mM KCl, 10 mM imidazole, 5–10 mM CaCl2 (Fluka), and 0.5 mM sodium salt (DSS) (Chenomx Inc.). The NMR sample prepared for measuring the pKd value of residues on cNTnC contained excess sTnI ([cNTnC] = 0.25 mM and [sTnI(115–131)] = 0.83 mM). The sample prepared for measuring the pKd of His-130 on sTnI(115–131) contained excess cNTnC ([cNTnC] = 0.41 mM and [sTnI(115–131)] = 0.07 mM).

**NMR Spectroscopy**—All NMR experiments were run on either a Varian Inova 500 MHz or Unity 600 MHz NMR spectrometers at 30 °C. Prior to each multidimensional experiment, one-dimensional 1H and two-dimensional 1H,15N HSQC NMR spectra were acquired. The three-dimensional CBCA(CO)NNH, three-dimensional HNCACB, and three-dimensional HCCONH NMR experiments were acquired for backbone and side chain assignments (Fig. 2) of...
Electrostatic Interaction between Troponin C and Troponin I

\[ f_t = \frac{K_d + [sTnI] + [cNTnC] - \sqrt{(K_d + [sTnI] + [cNTnC])^2 - 4[sTnI][cNTnC]}}{2[sTnI]} \]

(Eq. 4)

[\text{[sTnI]}\text{ and [cNTnC]}\text{ are the concentrations of sTnI}(115–131) and cNTnC, respectively.} \]

\[ \delta_{\text{obs}} = \delta_{\text{HA}} + \frac{\Delta \delta}{1 + 10^{n_pK_a - \text{pH}}} \]  

(Eq. 1)

where \( \delta_{\text{obs}} \) is the observed chemical shift at a given pH; \( \delta_{\text{HA}} \) is the chemical shift for the protonated form; \( \Delta \delta \) is the total shift from deprotonated to protonated forms, and \( n \) is the Hill parameter (held constant at 1 unless otherwise indicated). The biphasic \( pK_a \) datasets were fitted in xcrvfit and Wolfram Mathematica to Equation 2 (24),

\[ \delta_{\text{obs}} = \delta_{\text{HA}} + \frac{\Delta \delta_1}{1 + 10^{n_pK_{a1} - \text{pH}}} + \frac{\Delta \delta_2}{1 + 10^{n_pK_{a2} - \text{pH}}} \]  

(Eq. 2)

where \( \delta_{\text{obs}} \) is the observed chemical shift at a given pH; \( \delta_{\text{HA}} \) is the chemical shift for the protonated form, and \( \Delta \delta_1 \) and \( \Delta \delta_2 \) are the total shifts from deprotonated to protonated forms, and \( n \) is the Hill parameter (held constant at 1 unless otherwise indicated).

The fraction sTnI(115–131) bound (\( f_t \)) to cNTnC and fraction cNTnC bound (\( f_t \)) with sTnI(115–131) were determined using Equations 3 and 4,

\[ f_t = \frac{K_d + [sTnI] + [cNTnC] - \sqrt{(K_d + [sTnI] + [cNTnC])^2 - 4[sTnI][cNTnC]}}{2[sTnI]} \]  

(Eq. 3)

where P is free protein and L is free ligand, and PL is the protein-ligand complex. Concentrations of sTnI(115–131) and cNTnC were corrected for dilution during the titration. The dissociation constants were determined using the global fitting protocol in xcrvfit (37). Rather than fitting NMR chemical shift data to each individual residue and then averaging the individual dissociation constants, the global fitting method works by fitting all chemical shift data to one dissociation constant that best represents the ensemble of titration curves (i.e., had the lowest sum of squared error). Residues used in the global fit were those that underwent large chemical shift perturbations and did not significantly overlap with other signals.
RESULTS

cNTnC Glutamate pKa Values as a Function of Structure—
The acid dissociation constants for ionizable residues can pro-
vide insight into the molecular interactions within a protein
complex. The shift in pKa of an ionizable residue can signify the
formation of an electrostatic interaction or hydrogen bond (23).
To investigate the electrostatic forces between cNTnC and
cTnI or sTnI, we measured the pKa values of the glutamates of
cNTnC for a variety of troponin complexes. Two-dimensional
1H,15N HSQC and two-dimensional 1H,13C HCBCGCO NMR
spectra were acquired over a range of pH values for four differ-
ent complexes of cNTnC as follows: cNTnC(apo), cNTnC-
Ca2+/H11001, cNTnC-cTnI(147–163), and cNTnC-sTnI(115–131).
To ensure that cNTnC was mostly in their complexed states,
excess Ca2+/H11001, cTnI(147–163), and sTnI(115–131) were used. The
1H,15N HSQC NMR experiment correlates backbone amide pro-
tons with 15N nuclei so that each signal in the 1H,15N HSQC spec-
trum belongs to a single residue in the 15N-labeled protein. The

FIGURE 2. Assignment of glutamate carboxyl carbons of cNTnC(apo) (a) and cNTnC-sTnI(115–131) (b). The glutamate region of the two-dimensional
1H,13C HCBCGCO spectrum of cNTnC is shown on the right. Slices from the three-dimensional HCCONH NMR experiment are shown on the left. The carboxyl
carbon chemical shifts were assigned by matching the γ proton chemical shifts.

4 Unless otherwise stated, cNTnC will be assumed to be Ca2+-saturated, and
therefore the Ca2+ will be omitted when discussing the different states of
cNTnC.

two-dimensional 1H,13C HCBCGCO NMR experiment (28) cor-
relates the aliphatic protons of a residue with its side chain 13C-
carboxyl (or 13C-carbonyl) nucleus. The glutamates of cNT-
nC(apo) and cNTnC-sTnI(115–131) were assigned in this work
the assignments of cNTnC and cNTnC-cTnI(147–163) have
been previously published) (20, 38, 39). The assignments of
the two-dimensional 1H,13C HCBCGCO NMR spectra of
cNTnC(apo) and cNTnC-sTnI(115–131) are shown in Fig. 2; some glutamates could not be resolved in the two-dimen-
sional 1H, 13C HCBCGCO spectrum due to signal overlap.
Surprisingly, the pattern of chemical shifts in the two-di-
mensional 1H,13C HCBCGCO spectrum were consistent in
the four states of cNTnC (Figs. 2 and 3). The only exception
was Glu-76, which had its carboxyl 13C shifted from 182.7
ppm in cNTnC(apo) to 188.8 ppm in the other states of
cNTnC (Fig. 2). This large downfield shift is expected for a
bidentate ligand of Ca2+/H11001 (40, 41), such as Glu-76.

At low pH, the cNTnC solutions were not stable. In the case
of the Ca2+–bound complexes, precipitation was observed
below pH 4.25, and the NMR spectra resembled the apo-form,
suggesting Ca2+ had dissociated. Because it was not possible to
measure the glutamate chemical shifts at pH values below 4.25, in most instances the \( pK_a \) values reported are approximate. Furthermore, given that the \( pK_a \) of aspartates are typically even lower than that of glutamates (42), it was not possible to determine their acid dissociation constants with confidence. Many of the \( pK_a \) values obtained from the \( ^1\text{H},^{15}\text{N} \) HSQC spectra were difficult to interpret because the change in amide chemical shifts can stem from several phenomena, such as intraresidue ionization, pH-dependent conformational changes, or the ionization of nearby residues (24). An expanded region of the \( ^1\text{H},^{13}\text{C} \) HCBCGCO NMR spectrum acquired during each pH titration is shown for all four states of cNTnC insupplemental Fig. S1.

Given the difficulty associated with interpreting \( ^1\text{H},^{15}\text{N} \) HSQC NMR chemical shifts, we turned to the two-dimensional \( ^1\text{H},^{13}\text{C} \) HCBCGCO NMR experiment to track the pH-dependent chemical shift perturbations of the carboxyl carbons and the aliphatic \( \gamma \) protons of the glutamate residues (Fig. 3). Because the two-dimensional \( ^1\text{H},^{13}\text{C} \) HCBCGCO NMR experiment directly monitors the chemical shift of each carboxyl group, the \( pK_a \) values obtained from this experiment will likely arise primarily from intraresidue ionization. As the pH was reduced, the carboxylate \( ^{13}\text{C} \) nuclei shifted to a lower value, and the methylene \( \gamma \) protons shifted toward higher chemical shifts. These chemical shift trends are consistent with the protonation of a carboxylate moiety (43, 44). Titration data for three residues, Glu-55 (a residue removed from the binding site of TnI), Glu-19 (a residue possibly interacting with His-130, based on the crystal structure of the skeletal complex), and Glu-15 (a residue in close proximity to His-130 in the skeletal crystal structure, and may interact with His-130), are highlighted in Fig. 3.

The results for the \( pK_a \) values calculated using the \( ^1\text{H},^{15}\text{N} \) HSQC and \( ^1\text{H},^{13}\text{C} \) HCBCGCO NMR data for all cNTnC glutamates are listed insupplemental Tables S1 and S2, respectively. Residues with missing \( pK_a \) values either had overlapping signals or did not titrate over the pH range examined. The \( pK_a \) curve fitting data for Glu-55, Glu-15, and Glu-19 from the two-dimensional \( ^1\text{H},^{13}\text{C} \) HCBCGCO and two-dimensional \( ^1\text{H},^{15}\text{N} \) HSQC experiments are shown in Fig. 4 for the cTnI(147–163)- and sTnI(115–131)-bound complexes. The \( pK_a \) values for all three residues in the four different states of cNTnC monitored
FIGURE 4. pH dependence of the $^{1}H$, $^{15}N$, $^{13}C$, and $^{1}H$ resonances of Glu-15, Glu-19, and Glu-55 of cNTnC when bound to cTnI(147–163) (a) and sTnI(115–131) (b). The $pK_a$ curves of the amide resonances from the two-dimensional $^{1}H$, $^{15}N$ HSQC experiment are shown on the left, and the aliphatic proton and carboxylate carbon resonances from the two-dimensional $^{1}H$, $^{13}C$ HCBCGCO experiment are shown on the right. Peaks that were overlapped with other signals or that did not titrate were omitted. All five $pK_a$ curves of Glu-19 in the sTnI(115–131) bound state were fitted to Equation 2. c, second inflection of the $^{13}C_a$ chemical shift of Glu-19 is expanded. For the $pK_a$ values measured refer to Table 1 and supplemental Tables 1 and 2.
TABLE 1

| pKₐ values determined for Glu-15, Glu-19, and Glu-55 as determined by the pKₐ values determined from the C₀ chemical shift two-dimensional ¹H,¹³C HBCGCOS NMR experiments |

Data were fitted using Equations 1 or 2, setting the Hill coefficient to 1. Ave means average.

|            | Glu-15 | Glu-19 | Glu-55 |
|------------|--------|--------|--------|
| cNTnC      |        |        |        |
| Apo        | 5.09   | 5.06   | 6.77   |
| Ca²⁺       | 5.03   | 5.00   | 6.73   |
| cTnI(147–163) | 5.00 | 4.83   | 6.00   |
| sTnI(115–131) | 5.00 | 5.03   | 6.77   |

The results above suggest that Glu-19 interacts with His-130 in the presence of cNTnC. The stacked one-dimensional ¹H,¹³C HCBCGOCOS NMR experiments for free sTnI(115–131) are bound to 4.70 pKₐ when sTnI(115–131) is the presence of a histidine in sTnI(115–131), probably representing an average from the five nuclei monitored. The large ¹³C perturbation corresponds to ionization of the carboxyl of Glu-19 (pKₐ 4.70 ± 0.01), and the smaller chemical shift change was assigned to a neighboring residue.

Because the major difference between cTnI(147–163) and sTnI(115–131) is the presence of a histidine in sTnI(115–131) (Fig. 1), it is likely that the pKₐ of 6.73 ± 0.17 is from His-130 of sTnI(115–131). In addition, it has been shown that the direction of the ¹³C-carbonyl chemical shift is correlated to hydrogen bonding (46, 47). Hydrogen bonding to the carbonyl results in an increase in the polarity of the carbonyl bond, which causes a decrease in the shielding of the ¹³C-carbonyl nucleus and an increase in its chemical shift. Close inspection of Fig. 3d reveals an initial increase in the ¹³C chemical shift of Glu-19 as the pH was decreased from 8.5 to 6.5, consistent with the formation of an electrostatic interaction with a neighboring residue that is becoming protonated. The pKₐ of Glu-19 decreases from 5.06 ± 0.02 when Ca²⁺ is bound to 4.70 ± 0.01 when sTnI(115–131) is bound (a difference of 0.36 ± 0.03). The reduction in the pKₐ of Glu-19 is more evidence that Glu-19 is involved in the formation of an electrostatic interaction.

sTnI(115–131) Histidine pKₐ Values as a Function of Structure—The results above suggest that Glu-19 interacts with His-130 on sTnI(115–131); to further investigate this possibility, the pKₐ of His-130 was measured for sTnI(115–131) in the absence and presence of cNTnC. The stacked one-dimensional ¹H NMR spectra for the pH titrations are shown in Fig. 5. The pKₐ of His-130 in free sTnI(115–131), using the histidine aromatic protons H2 and H5, was determined to be 6.11 ± 0.01 and 6.14 ± 0.03, respectively (Fig. 5a). Because fitting the pKₐ curves for His-130 in the presence of cNTnC required fitting the Hill parameter (see below), this coefficient was also fitted for free sTnI(115–131). As expected, the Hill coefficient was close to unity for both H2 and H5 (0.96 and 1.07, respectively).

The pKₐ of His-130 was then measured in the presence of cNTnC. The pH titration of sTnI(115–131) with excess cNTnC (ratio ~6:1) is shown in Fig. 5b. Because cNTnC signals overlapped H2 and H5, we employed a ¹³C,¹⁵N-filtered ¹H,¹H-NOESY NMR experiment (29–31) to monitor the sTnI(115–131) signals (Fig. 5b). The pKₐ derived from proton H2 was 6.33 ± 0.02 (the chemical shift of the protonated state was fixed at 8.70 ppm), and the pKₐ shift of H5 was 6.39 ± 0.04. The pKₐ values were determined by also fitting the Hill coefficient, because the pKₐ curves did not fit the simple pKₐ model. Low Hill coefficients fitted for H2 (n = 0.63) and H5 (n = 0.58) indicated that His-130 is most likely interacting with other ionizable group(s). For example, as a nearby residue is deprotonated, the protonated form of His-130 is stabilized; this will result in a flattened pKₐ curve (n < 1). For a summary of the pKₐ values measured for His-130 see Table 2. The pKₐ curves of H2 and H5 are overlaid in Fig. 6, and the rightward shifts in the pKₐ curves clearly illustrate an increase in the stability of the positively charged species of His-130 when in the presence of cNTnC.

There were minor peaks next to H2 and H5 noted in samples containing excess sTnI(115–131). The pKₐ values of these secondary peaks were near the values for free sTnI(115–131) and probably represent a second bound conformation of sTnI(115–131) in slow exchange with the major conformation (48). Similar observations have been made for histidine residues in neurophysin II (49) and staphylococcal nuclease (50). In line with this conclusion, sTnI has been shown by NMR experiments to be in two conformations when bound to sNTnC (18). These peaks are presumably not from impurities because HPLC and mass spectrometry indicated that peptide was pure. Moreover, these minor peaks were not witnessed in the free sTnI(115–131) experiments. To ensure the second minor peak was indeed from histidine on sTnI(115–131), the ¹³C,¹⁵N-filtered ¹H,¹H-TOCYS NMR experiment was run (29–32). The TOCYS spectrum shows the presence of signals attributable to two separate histidine conformations (supplemental Fig. S2).

The pKₐ values of H2 and H5 from His-130 are shifted upward but is not shifted to the value measured from the Glu-19 pKₐ curves (pKₐ = 6.73 ± 0.17). This can be explained by the fact that even though the concentration of cNTnC is ~6:1 sTnI(115–131), the fraction-bound sTnI(115–131) may not be one. As mentioned earlier, a Hill coefficient different from unity may indicate an interaction with another titrating...
Electrostatic Interaction between Troponin C and Troponin I

species. Another potential cause of the altered Hill coefficient may be that the fraction of sTnI(115–131)-bound changes as a function of pH, for example if sTnI(115–131) binds with different affinity at low and high pH values. The dissociation constant was determined at two pH values to assess this possible pH dependence.

FIGURE 5. pH titrations of His-130 of sTnI(115–131) when free (a) and in 6-fold excess cNTnC (b). One-dimensional 1H NMR spectra are shown on the left with histidine aromatic protons assigned. The pKₐ curves for H2 and H5 in the free and bound states are shown on the right.

TABLE 2 pKₐ values determined for His-130 using one-dimensional NMR experiments
Data were fitted using Equations 1 or 2; errors are shown in parentheses.

| Fraction bound | pKₐ | n     | pKₐ | n     | pKₐ | n     |
|----------------|-----|-------|-----|-------|-----|-------|
| 0              | 6.11 (0.01) | 0.96 (0.02) | 6.14 (0.03) | 1.07 (0.08) | 6.13 (0.02) | 1.02 (0.08) |
| 0.51–0.78      | 6.33 (0.02) | 0.63 (0.02) | 6.39 (0.04) | 0.58 (0.04) | 6.36 (0.04) | 0.61 (0.04) |

* Fraction bound was calculated using Equation 3; the lower value was calculated using the K_D determined at pH 7.5 (360 μM), and the higher value was calculated using the K_D determined at pH 6.1 (100 μM).
Electrostatic Interaction between Troponin C and Troponin I

**FIGURE 6.** pH dependence of the pKₐ values of His-130 (H2 and H5) as a function of fraction sTnI(115–131) bound. I, curve for free sTnI(115–131); ○, the curve for excess cNTnC (mostly bound).

**pKₐ-dependent Dissociation Constants of sTnI(115–131)—**
sTnI(115–131) was titrated into cNTnC at two pH values (6.1 and 7.5). At each aliquot of sTnI(115–131), two-dimensional ¹H,¹⁵N HSQC spectra were acquired. The global dissociation constant (Kₑ) was determined to be 100 μM (sum of squared error² = 0.066) at pH 6.1 and Kₑ = 360 μM (sum of squared error = 0.076) at pH 7.5 (for an overlay of fits at the two pH values, residue Thr-71 is shown in Fig. 7a, and the global fits are shown in supplemental Figs. S3 and S4).

The change in affinity as a consequence of pH indicates that the fraction bound will change over the course of the pH titrations. Using Equation 3, the fraction bound goes from 0.51 to 0.78 as the pH goes from 7.5 to 6.1. Typically, one could calculate fraction bound and extrapolate to the bound species to calculate the pKₐ value of the bound species; however, with the varying Kₑ, values as a function of pH, this was difficult. We did two extrapolations using averaged pKₐ values of H2 and H5, one assuming fraction bound of 0.78 and another assuming fraction bound of 0.52; the extrapolated pKₐ values for the bound species ranged from 6.43 to 6.58. The pKₐ of 6.58 is close to the pKₐ monitored by Glu-19 (6.73 ± 0.17).

If the pH-dependent change in the affinity of sTnI(115–131) for cNTnC is solely related to the protonation state of His-130, then this should be reflected in the acid dissociation constant of His-130. We fitted the dissociation data to the simple model shown in the scheme in Fig. 7b to investigate whether the pKₐ shifts of His-130 are directly correlated to the modulation of the Kₑ value of sTnI(115–131). Protonation constants are represented by Kₐ (free) and Kₐ⁺ (bound), and the peptide dissociation constants are indicated by Kₑ (deprotonated peptide) and Kₑ⁺ (protonated peptide). We used the pKₐ value for His-130 monitored from Glu-19 due to the uncertainty of the bound pKₐ of His-130 by monitoring His-130 directly as already mentioned. The same model has been previously fitted to characterize the pH dependence of a peptide binding to calmodulin (51).

For His-130, Kₑ = 7.4 × 10⁻⁷ M (pKₑ = 6.13, an average of H2 and H5) and Kₑ⁺ = 1.9 × 10⁻⁷ M (pKₑ⁺ = 6.73). Kₑ = 3.9 × 10⁻⁴ M and Kₑ⁺ = 1.0 × 10⁻⁴ M. Kₑ/Kₑ⁺ (3.9) is equal to Kₑ/Kₑ⁺ (3.9), which is consistent with the scheme in Fig. 7b. This result implies that the protonation state of His-130 directly tunes the pH-dependent affinity of sTnI(115–131).

**DISCUSSION**

Impairment of coronary blood flow results in ischemia, which is characterized by a decline of oxygen and substrate supply to the cardiomyocytes. The decrease in blood supply leads to a buildup of ions and metabolic products, whose accumulation culminates with intracellular acidosis and a concomitant reduction in the force and Ca²⁺ sensitivity of muscle contraction (1, 2). This decrease in contractility occurs despite an elevation of the Ca²⁺ transient during acidosis (52). Instead, the increase of adenosine diphosphate, inorganic phosphate, and proton concentrations may inhibit actomyosin-ATPase activity (53, 54), and the increased [H⁺] may decrease Ca²⁺ sensitivity by lowering the affinity of troponin for Ca²⁺ (6–8).

Interestingly, slow and fast skeletal muscles are less sensitive to acidic conditions than cardiac muscle (10, 12). These differences in pH sensitivity have been localized to the C-terminal region of TnI (12, 14), specifically in its switch region (13). The sequence comparison of the switch regions of cTnI, sTnI, and ssTnI (Fig. 1) reveals that both sTnI and ssTnI have a histidine in place of the alanine present in cTnI. *In vitro* (13) and *in vivo* (15) functional studies have verified that this histidine is the chief source of the reduced pH sensitivity of sTnI and ssTnI. The data presented here provide a structural understanding for how the sTnI isoform is less sensitive to the decline in pH associated with acidosis.

In the crystal structure of the skeletal complex (16), an electrostatic interaction between Glu-20 of sTnC and His-130 of sTnI is observed, and the data presented herein suggest that an analogous interface is formed between Glu-19 and His-130 in the cNTnC-sTnI(115–131) complex. In the crystal structure, the imidazole N3 of His-130 is only 4.1 Å away from the carboxylate carbon of Glu-20, and assuming this interaction also occurs in the cNTnC-sTnI(115–131) complex, a change in the pKₐ values of both Glu-19 and His-130 should be observed. To investigate this possibility, we measured the pKₐ values of Glu-19 and His-130. The pKₐ value of His-130 shifted from 6.13 ± 0.02 to 6.73 ± 0.17 (using data obtained from Glu-19) or 6.54 to 6.4 (using data obtained by extrapolating from His-130 data). Glu-19 also experienced a pKₐ perturbation of 0.36 (5.06 ± 0.03/0.02 to 4.70 ± 0.01) and 0.53 units (5.23 ± 0.01 to 4.70 ± 0.01) when compared with apo/Ca²⁺ and cTnI(147–163)-bound states, respectively. The elevated pKₐ of Glu-19 when cNTnC is in complex with cTnI(147–163) may be the result of its proximity to the carboxyl group of Asp-152 from cTnI(147–163) (Ca–Cy distance: 7.3 Å, Protein Data Bank code 1j1e, and 10.6 ± 1.7 Å, Protein Data Bank code 1mxl).

If the interaction between Glu-19 and His-130 was solely responsible for the elevated pKₐ of His-130, then their ∆pKₐ values should be identical. Differences in the pKₐ shifts of His-130 and Glu-19 can be explained by several phenomena. First, the pKₐ of Glu-19 was measured at a [cNTnC]/[sTnI(115–131)] ratio of ~4:1; using Equation 4 the fraction of cNTnC bound to sTnI(115–131) ranges from 0.65 at pH 7.5 to 0.86 at pH 6.1. Therefore, at a fraction bound of one the pKₐ of His-130 would be
expected to be lower than what was actually measured. Extrapolation of Glu-19 in a similar manner as described for His-130 yielded a $pK_a$ range for bound of 4.64 to 4.51 ($pK_a$ of 0.42 and 0.55, respectively, close to that determined for His-130.

Second, the carboxylate of Asp-119 on sTnI in the x-ray structure is only 3.7 Å from N4 of His-130. If this interaction occurs when sTnI(115–131) is bound to cNTnC, then it will also contribute to an increase in the $pK_a$ of His-130. Glu-16 is also near His-130 (10.1 Å), and it may be involved in driving sTnI binding to cNTnC, because electrostatic forces can span distances >10 Å. Conversely, in the NMR and x-ray structures of cardiac troponin, these interactions do not occur, presumably because the histidine is replaced by an alanine (19, 20). Comparison of the crystal structures of the cardiac and skeletal troponin complexes in Fig. 8 illustrates that in the cardiac system Ala-162 is much further from Glu-15 and Glu-19 (11.8 and 16.4 Å, respectively). Thus, it is likely that when sTnI(115–131) binds to cNTnC, it adopts a conformation more similar to the skeletal complex.

The observed pH dependence of sTnI(115–131) binding is directly related to the ionization state of His-130, which can be explained in part by an interaction made between Glu-19 of sTnI and His-130 of sTnI(115–131). At first glance, this result seems to imply that muscle with sTnI would have enhanced Ca$^{2+}$ sensitivity at low pH. However, functional studies of cardiac myofilaments replaced with sTnI demonstrate...
that although the negative inotropic effects of acidosis are partially suppressed, the Ca\(^{2+}\) sensitivity is not enhanced when compared with values at neutral pH (12, 13). Given that we were explicitly interested in probing the role His-130 played in dictating sTnI(115–131) binding to cTnC, and as it is well established that the Ca\(^{2+}\) binding to cTnC is reduced as pH is lowered (6–8), we worked with excess Ca\(^{2+}\) to ensure Ca\(^{2+}\) saturation, even at the lower pH values. Moreover, the increased affinity of sTnI(115–131) measured at low pH in this report is not large enough to fully compensate for the reduced Ca\(^{2+}\) affinity at low pH. To illustrate this, we calculated the free energies of Ca\(^{2+}\) and sTnI(115–131) binding to cTnC. The free energy (ΔG) of sTnI(115–131) binding to cTnC can be determined as shown in Equation 6,

\[
\Delta G = RT \ln K_0
\]

where \(R\) is the ideal gas constant, and \(T\) is the temperature in Kelvin. Using this equation, the ΔG of sTnI(115–131) binding at pH 6.1 is approximately −5.6 kcal/mol and at pH 7.5 is approximately −4.7 kcal/mol, corresponding to a ΔΔG of 0.9 kcal/mol. Using the values for Ca\(^{2+}\) binding to cTnC at pH 7 and pH 6 as reported by Liou and Chang (8), Ca\(^{2+}\) binding is −2.4 kcal/mol less favorable at pH 6 than pH 7. Therefore, the enhanced binding of sTnI(115–131) at low pH would not entirely offset the reduced binding of Ca\(^{2+}\) to cTnC. We propose that the reduction in Ca\(^{2+}\) affinity of cTnC at low pH would be only partially compensated for by the concomitant enhancement in the affinity of sTnI for cTnC. This interpretation is consistent with findings that sTnI enhances the Ca\(^{2+}\) affinity of cTnC at low pH values by its enhanced affinity for cTnC (8).

In this study, we show that the histidine of sTnI makes an interaction with cTnC, and it is likely that cTnI(A162H) forms a similar interaction. It is worth mentioning that there may be some detrimental effects of increasing Ca\(^{2+}\) sensitivity during acidosis. Transgenic mice containing the hypertrophic cardiomyopathy mutation, E180G, in tropomyosin (Tm-E180G) do not experience a substantial decline of Ca\(^{2+}\) sensitivity during acidosis (60). Although it might be expected that the reduced pH sensitivity of Tm-E180G would be beneficial because heart failure would be reversed, the increased muscle contractility seems to contribute to a worsening of heart disease (60). Therefore, although it seems to be intuitive that one would want to enhance contractility in a weakened heart, this may not always be the case. However, in vivo studies done on transgenic mice expressing cTnI(A162H), which also have a reduced decrease in Ca\(^{2+}\) sensitivity during acidosis, do not have the same deleterious effects as Tm-E180G. The ability of cTnI(A162H) to be down-regulated by phosphorylation may help explain why this mutation has less severe consequences than Tm-E180G (15).

The fact that the interaction between His-130 and Glu-19 is important in stabilizing the interaction between sTnI(115–131) and cTnC has implications not only for explaining why the neonatal heart contractility is less sensitive to low pH but also for the development of pharmaceuticals. The design of molecules that become charged at low pH and then stabilize the interaction between cTnI and cTnC could be beneficial for the treatment of ischemic heart failure. Finally, although the protonation state of His-130 modifies the affinity of sTnI(115–131) for cTnC, it is also possible that its charge state may have other functional effects in an intact muscle fiber. For example, the protonation may both enhance the interaction between sTnI and cTnC and diminish sTnI binding to actin as has also been proposed (15).

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