

Significance of Troponin Dynamics for Ca²⁺-mediated Regulation of Contraction and Inherited Cardiomyopathy*

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Background: Ca²⁺ binding to the thin filament protein troponin strictly regulates muscle contraction.

Results: Ca²⁺ affects measured and mapped dynamics extensively within separated areas of cardiac troponin, in a structurally revealing manner.

Conclusion: Troponin dynamics are critical to its function.

Significance: Mechanistic discovery regarding troponin action improves understanding of normal, adaptive, and pathological cardiac contraction, including heritable cardiomyopathy.

Ca²⁺ dissociation from troponin causes cessation of muscle contraction by incompletely understood structural mechanisms. To investigate this process, regulatory site Ca²⁺ binding in the NH₂-lobe of subunit troponin C (TnC) was abolished by mutagenesis, and effects on cardiac troponin dynamics were mapped by hydrogen-deuterium exchange (HDX)-MS. The findings demonstrate the interrelationships among troponin's detailed dynamics, troponin's regulatory actions, and the pathogenesis of cardiomyopathy linked to troponin mutations. Ca²⁺ slowed HDX up to 2 orders of magnitude within the NH₂-lobe and the NH₂-lobe-associated TnI switch helix, implying that Ca²⁺ greatly stabilizes this troponin regulatory region. HDX of the TnI COOH terminus indicated that its known role in regulation involves a partially folded rather than unfolded structure in the absence of Ca²⁺ and actin. Ca²⁺-triggered stabilization extended beyond the known direct regulatory regions: to the start of the nearby TnI helix 1 and to the COOH terminus of the TnT-TnI coiled-coil. Ca²⁺ destabilized rather than stabilized specific TnI segments within the coiled-coil and destabilized a region not previously implicated in Ca²⁺-mediated regulation: the coiled-coil's NH₂-terminal base plus the preceding TnI loop with which the base interacts. Cardiomyopathy-linked mutations clustered almost entirely within influentially dynamic regions of troponin, and many sites were Ca²⁺-sensitive. Overall, the findings demonstrate highly selective effects of regulatory site Ca²⁺, including opposite changes in protein dynamics at opposite ends of the troponin core domain. Ca²⁺ release triggers an intramolecular switching mechanism that propagates extensively within the extended troponin structure, suggests specific movements of the TnI inhibitory regions, and prominently involves troponin's dynamic features.

The principal direct trigger for muscle relaxation in striated muscles, including the heart, is the dissociation of Ca²⁺ from

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the thin filament protein, troponin (1–6). More specifically, it is Ca²⁺ dissociation from specific site(s) of the troponin subunit TnC that is essential for relaxation. If troponin is removed experimentally, muscles contract regardless of Ca²⁺ (7). With troponin present, contraction is strictly dependent upon Ca²⁺ binding to TnC. To investigate this central, partially understood (8–14) regulatory mechanism, the present study widely examines and quantitatively maps the effects of regulatory site II Ca²⁺ on cardiac troponin's dynamics.

Peptide backbone NH hydrogens exchange with H₂O solvent hydrogens or exchange instead with solvent deuterium when a protein is immersed in D₂O. The hydrogen bonding of protein folding suppresses, very greatly, the rate of such hydrogen-deuterium exchange in secondary structures. Hydrogen-deuterium exchange rates vary greatly within and between proteins, quantitatively mirroring the great variation in flexibility and local dynamics that is characteristic of proteins. Measurements of the local rates of exchange across a molecule provide a quantitative dynamic map of that protein's many distinct regions (15–19).

In 2010, we reported (20) first application to troponin of the approach again used here: hydrogen-deuterium exchange-mass spectrometry (HDX²-MS). HDX-MS was used to obtain dynamic maps of troponin at 25 °C, in the presence of either saturating or subsaturating Ca²⁺ concentration. This earlier report found troponin to be highly dynamic generally, undergoing rapid HDX in many regions, and yet also to contain regions of high stability that exhibited very slow HDX. We also observed that troponin's dynamics were affected by the free Ca²⁺ concentration, in a complex manner. However, the effects of Ca²⁺ could not be assessed comprehensively, because HDX rates were so fast that 50% of exchange preceded the first time point. Furthermore, site II was not fully emptied in the lower Ca²⁺ concentration condition.

We later reported (21) a considerably more incisive dynamic map for Ca²⁺-saturated troponin, obtained by using 10 °C rather than 25 °C HDX conditions. Using a lower temperature

² The abbreviations used are: HDX, hydrogen-deuterium exchange; TnI, TnC, and TnT, troponin I, C, and T, respectively; CBMII, calcium binding mutant (TnC site II).

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to slow the intrinsic HDX rate, it was possible to characterize many troponin residues for the first time and to assess others very much better than previously.

In the present report, these lower temperature conditions are used to map the effect of regulatory site Ca^{2+} on troponin dynamics. To accomplish this, we developed an approach in which very high Ca^{2+} occupancy of sites III and IV in the TnC COOH-lobe was assured and unaffected, whereas site II in the NH_2 -lobe was completely and specifically emptied. To keep sites III and IV unaffected, we employed 1 mM CaCl_2 , the same concentration used previously for HDX of Ca^{2+} -saturated troponin. To empty site II, we used Ala mutations in selected residues of the metal-coordinating EF-hand loop (22, 23).

As shown below, the results provide a new picture of troponin dynamics in relation to regulatory function. Among the new findings is the demonstration that Ca^{2+} highly selectively alters troponin's dynamic behavior. Troponin consistently is stabilized locally near the Ca^{2+} site and consistently is destabilized in a region at the farthest end of the troponin core domain. The mechanistic implications of this and many other HDX findings are discussed. Finally, the data comprehensively and quantitatively validate the concept that cardiomyopathy-inducing mutations cluster in dynamic regions of troponin.

EXPERIMENTAL PROCEDURES

Protein Preparation—Human cardiac CBMII troponin core domain was prepared from bacterially expressed TnI, D65A/E66A TnC, and COOH-terminal TnT fragment 183–288. The three subunits were mixed in a 1:1:1 ratio and then reconstituted into a ternary complex by serial dialysis (20). The complex was isolated by ion exchange chromatography using a Resource S column and an AKTA system (GE Healthcare), concentrated by a Centricon apparatus (Millipore), and stored at -80°C before use.

HDX and Exchange Analysis by HPLC-Electrospray Ionization Fourier Transform-Ion Cyclotron Resonance MS—Exchange was performed as described previously (20, 21). Briefly, thawed protein samples were placed into exchange buffer (10 mM NaH_2PO_4 , pH 7.0, 0.1 M NaCl) by a spin column. HDX was initiated by mixing with 9 parts of the same, 10°C buffer but in D_2O . Aliquots were removed at different time points to be acid-quenched with pH 2.5 phosphate buffer and flash frozen in liquid nitrogen. Samples were stored at -80°C .

For analysis, quickly thawed troponin samples were digested with an equal mass of pepsin for 5 min on ice. They were then immediately injected into a micropeptide trap (Microchrom), which was attached to a C18 HPLC column, which was connected to the Fourier Transform-Ion Cyclotron Resonance MS. Peptide envelopes were easily recognizable by correspondence to the possible postexchange m/z of pre-HDX-identified peptides. The centroid mass of each peptide was determined using MagTran (24). The fragments are the same as those described previously for unmodified troponin examined under the same HDX conditions (21), with three exceptions. Time course measurements for TnT 264–288, TnI 79–85, and TnC 81–97 were substituted for TnT 263–288, TnI 78–88, and TnC 82–97, respectively, because data for the latter set were not consistently detected in the CBMII troponin MS spectra.

Curve Fitting of Exchange Kinetic Data—Before curve fitting, corrections for backward D/H exchange were applied as reported previously (21), averaging 23%. Transition sizes and rate constants were determined by non-linear least squares curve fitting of exponential peptide mass increases over time, using Scientist (Micromath). These parameter and error estimate determinations were performed either simply on individual peptide data or globally on grouped data from peptides wherever there was peptide overlap. The globally fit groups of peptides for CBMII troponin were: TnC (28–57, 36–56, 48–56) and (118–132, 122–130, 122–132); TnI (27–53, 29–49, 29–53), (54–66, 54–78, 62–85, 79–85), (97–109, 97–116), (125–134, 125–152), (156–169, 162–169), and (191–197, 191–210); TnT (224–242/225–243 (which are effectively indistinguishable after exchange), 230–243, 237–243) and (243–263/244–263 (which are effectively indistinguishable after exchange), 244–250, 249–263, 251–263), where peptide groups are enclosed in parentheses.

As previously (21), transition magnitudes averaged half the length of the assignable regions in which they were detected. Correspondingly, most assignable regions contained more than one exchange transition. From HDX data alone, one cannot determine the location of a transition within a somewhat larger assignable region that is defined by the size of the peptide(s). The resulting, ~ 5 -residue uncertainty in localization was addressed as previously (22) to produce the assignments in the present study. In other words, the different transitions were ordered within an assignable region to correspond as much as possible with the troponin high resolution structural data (25). For example, faster exchange was assigned where crystallographic B -factors were higher or particularly where no structure was detected by crystallography. The latter consideration produces an unambiguously justified assignment for HDX occurring prior to 5 s (*i.e.* without protection).

Calculation of Protection Factors—Measured exchange rates were converted to protection factors, which indicate the degree of HDX slowing relative to rates expected if troponin were unfolded. Protection factors reflect local folding stability, measured in the context of the globally folded molecule. More specifically, protection factors were calculated by comparison of measured HDX rates with the geometric mean of the model peptide unprotected HDX rates (26) for each residue in the assignable region. Values for these means ranged from 0.4 to 1.8 s^{-1} . Because this is a narrow range relative to the much wider variation in HDX rates, Fig. 5 would be altered little if exchange rate values were mapped instead of protection factors.

RESULTS

Troponin causes cessation of cardiac contraction when Ca^{2+} is dissociated from one specific site: site II in the TnC subunit's NH_2 -lobe. To investigate the effects of regulatory Ca^{2+} with the highest specificity, the present study compares HDX of unmodified troponin with HDX of troponin with D65A/E66A TnC. Such "CBMII" mutation prevents Ca^{2+} binding and closely reproduces the normal apo state structure, as shown by detailed NMR study of a very similar NH_2 -lobe construct (27). CBMII and its skeletal muscle counterpart potentially inhibit thin filaments in fibers and in solution. They have been examined in

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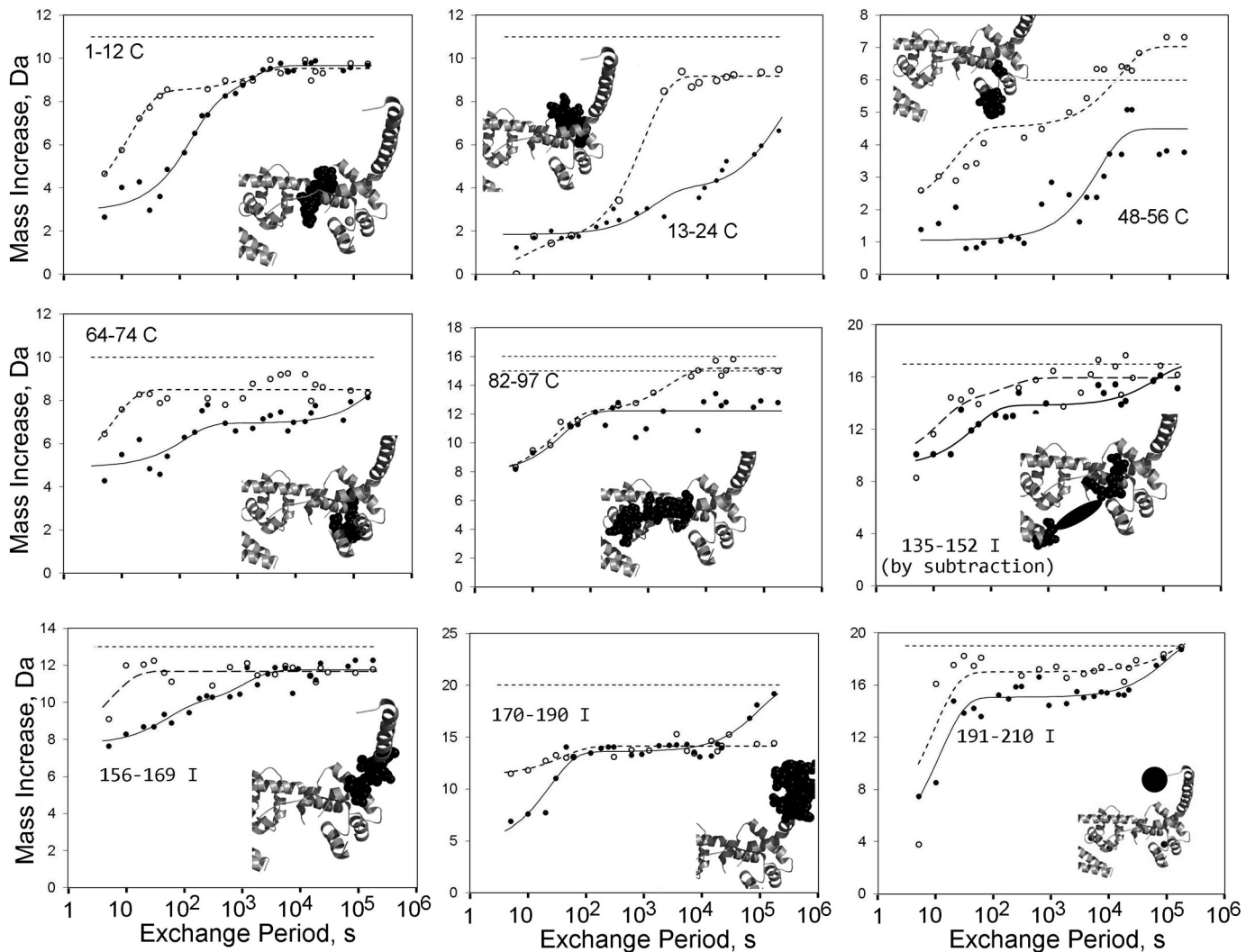


FIGURE 1. HDX kinetics within troponin's regulatory head region. Troponin was exposed to D_2O for times varying between seconds and days. Hydrogen-deuterium exchange resulted in the graphed increases in the masses of fragments generated by pepsin digestion performed post- D_2O exposure. *Open circles*, data for CBMII troponin with inactivated Ca^{2+} site II. *Filled circles*, data from Ref. 21 for unmodified troponin, with Ca^{2+} bound at site II. *Dashed and solid lines*, best fit curves. In each *panel*, the location of the graphed peptide within troponin is shown in *black* in a *cropped thumbnail image*. (Fig. 5A shows the same orientation with annotations.) The faster TnC transitions for CBMII troponin in each *panel*, compared with HDX for unmodified troponin, indicate the absence of a large stabilizing effect that occurs when site II is occupied by Ca^{2+} . The illustrated peptides from TnI have a similar effect, but the shifts are smaller in magnitude. Also, note that no TnI peptide is fully exchanged before the first time point, indicating some degree of protection from exchange in each peptide. For CBMII troponin, graphed data were acquired successfully for TnC 81–97 rather than TnC 82–97, which was assessed for unmodified troponin. TnI 135–152 results were obtained by subtraction of the *data points* and *fitted curves* shown in Fig. 4 for TnI 125–152 and TnI 125–134. *Dotted lines* at the top of each *panel* indicate maximal possible exchange for the graphed peptide(s).

many biochemical and physiological experiments (22, 23, 28–35) because they abrogate Ca^{2+} binding to the NH_2 -lobe without altering Ca^{2+} binding to the TnC COOH-lobe.

Effects of Site II Ca^{2+} on the Dynamic Properties of Troponin's Regulatory Head Region—The regulatory head region of troponin (25) contains two components: (i) the NH_2 -lobe of TnC and (ii) the COOH-region of subunit TnI. The TnI COOH-region interacts with the NH_2 -lobe in a strictly Ca^{2+} -dependent manner (25, 36, 37). This reversible interaction is the initial, immediate vicinity aspect of the regulatory Ca^{2+} switch that alters thin filament structure so as to control contraction.

Fig. 1 shows HDX as a function of time for peptides derived from the regulatory head region. In each *panel*, HDX data and best fit curves are shown for both CBMII and unmodified troponin. Measured time points spanned from 5 s to 48 h, allowing

measurement of a wide range of faster and slower dynamics. The graphed increases in mass over time report hydrogen-deuterium exchange at peptide backbone NH groups. (Side chain hydrogens either do not exchange or else revert to hydrogen before mass measurement.)

Three broad generalizations are helpful for interpreting the graphs. First, high values at 5 s generally indicate unprotected NH groups. Hydrogen exchanges to deuterium prior to the first time point at sites that have no protein folding-mediated protection from exchange. Second, the time required for an observed HDX transition reflects the relative folding stability of that region. The faster the rate, the weaker the local stability and the faster are the dynamics. Finally, failure to complete HDX by the last, 48 h measurement indicates the most locally stable, least dynamic property that can be characterized.

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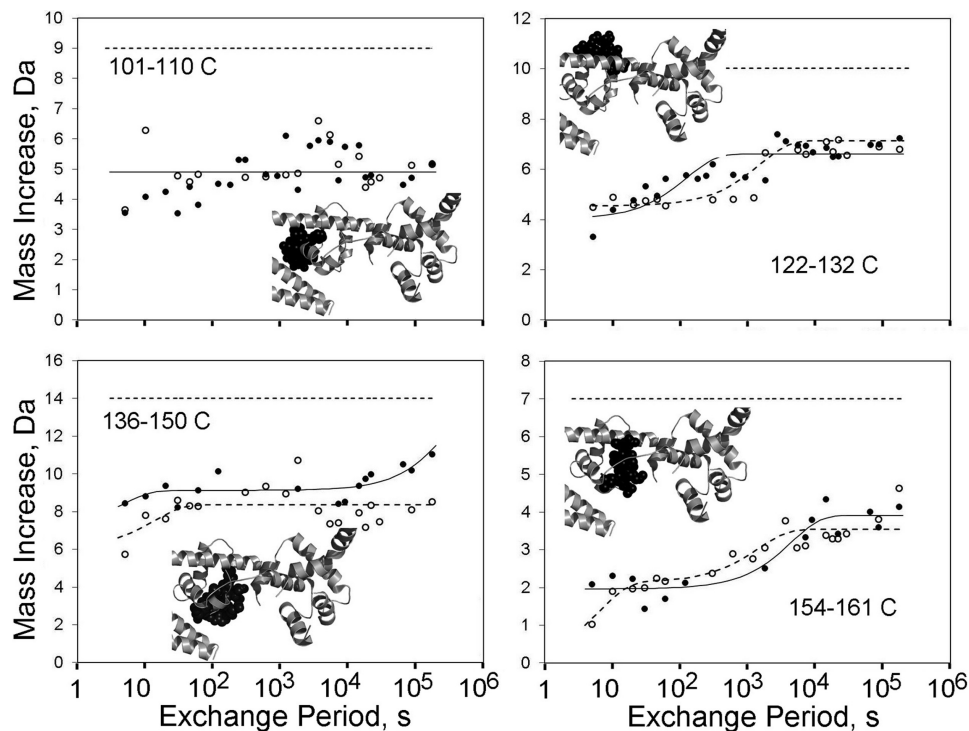


FIGURE 2. **HDX kinetics within the TnC COOH-lobe.** As in Fig. 1, the replacement of NH hydrogens by deuterium was measured after variable durations of troponin exposure to D_2O . Ca^{2+} had little effect in these experiments on the HDX occurring in peptides derived from the TnC COOH-lobe. Regardless of Ca^{2+} at site II, most HDX occurred either before 5 s or after 48 h. Symbols, lines, and thumbnail images are the same as in Fig. 1.

HDX of TnC NH_2 -lobe peptides are shown in five of the Fig. 1 panels. The feature most immediately evident is that many NH hydrogens exchanged more slowly when Ca^{2+} was bound to TnC. Each of these five panels contains at least one transition that occurred earlier when CBMII troponin was studied (open circles) than for unmodified troponin (filled circles). Ca^{2+} binding to an intact, unmodified site II slowed many HDX transition rates, and no TnC NH_2 -lobe region had a statistically significant increase in dynamics in the presence of site II Ca^{2+} . Thus, Ca^{2+} stabilized many portions of the TnC NH_2 -lobe. The time domain shifts were 1–2 orders of magnitude, indicating that this effect is a large, prominent aspect of regulation. In fact, the 2-order of magnitude shifts in the dynamics of these peptides may be reporting the fractional occupancy of site II with Ca^{2+} . This inference comes from the fact that the experimental 1 mM CaCl_2 concentration is about 100-fold greater than the site II K_d for unmodified troponin under these D_2O conditions (20).

HDX rates in the TnC NH_2 -lobe are influenced not only by Ca^{2+} directly but also by the Ca^{2+} -dependent association of the ~ 10 -residue TnI switch helix to the NH_2 -lobe. HDX for the switch helix itself is shown within Fig. 1. In short, the switch helix is stabilized by Ca^{2+} , undergoing faster HDX in the CBMII context than in unmodified troponin.

This is shown in two of the Fig. 1 panels (TnI 156–169 and TnI 135–152). Both peptides include parts of the TnI 150–160 switch helix. CBMII data for each included a pre-20 s HDX transition that was faster than in unmodified (*i.e.* Ca^{2+} -binding) troponin. It may at first seem puzzling how this transition can be assigned to the switch helix. However, HDX of TnI 162–169 is very fast, occurring before 5 s, regardless of Ca^{2+} (not shown). Also, the troponin atomic structure indicates that TnI

137–147 is dynamic and unfolded (25). Once these residues are assigned to undergo HDX in < 5 s, one can infer that the switch helix is the primary location of the Ca^{2+} -stabilized residues in both 135–152 and 156–169.

Complex, Ca^{2+} -affected dynamics for the COOH-terminal 40 residues of TnI are shown in Fig. 1 TnI panels 170–190 and 191–210. In (Ca^{2+} -free) CBMII troponin this region is not tethered to the TnC NH_2 -lobe by the switch helix. Interestingly, this untethered state does not yield a fully unfolded structure for the TnI COOH terminus. Rather, half of TnI 170–190 exhibits rather high protection from HDX. Also, much of TnI 191–210 exhibits protection, albeit weakly.

These findings indicate that at 10°C in the absence of both actin and Ca^{2+} , a large part of the TnI COOH terminus is dynamically folded rather than completely unfolded. The ability of this region to form specific structure is consistent with observations that many alternative point mutations in the TnI COOH terminus induce cardiomyopathy (38–41). A specific but weakly folded structure is consistent with NMR data (42, 43) but is distinct from the very interesting proposal that the region functions as an intrinsically disordered domain (44).

Small Effect of Site II Ca^{2+} on the Dynamics of the TnC COOH-lobe—Unlike the NH_2 -lobe, the TnC COOH-lobe was not prominently stabilized by site II Ca^{2+} . Rather, peptides describing HDX of this region were very similar for CBMII troponin and for unmodified troponin (Fig. 2). In each peptide shown, about half of the NH groups exchanged prior to the first 5 s time point; they were unprotected. Also for each peptide, many of the NH hydrogens remained unexchanged after the last, 48 h time point; they were highly protected from exchange. Small transitions were in some cases seen, with effects of Ca^{2+}

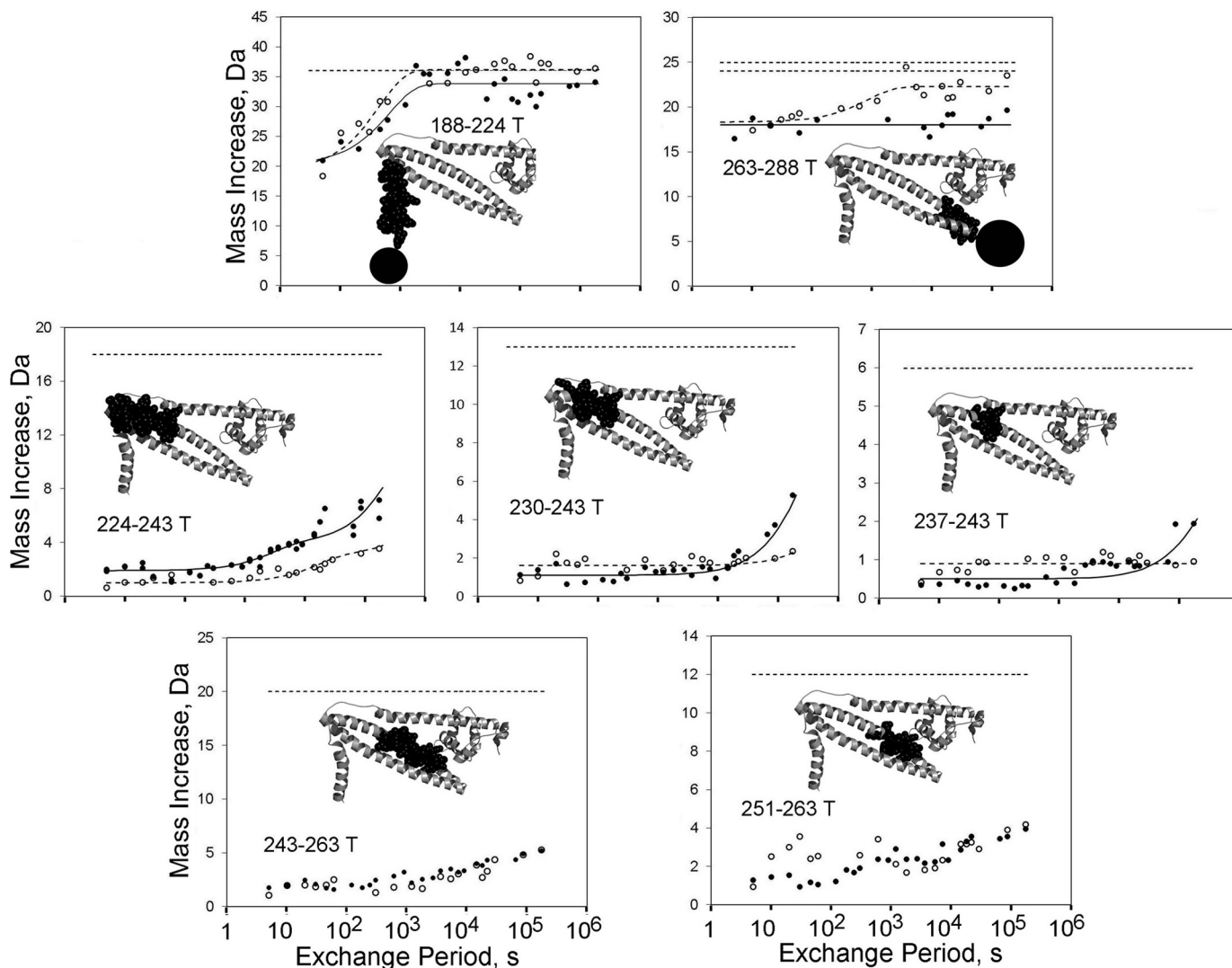


FIGURE 3. **HDX kinetics within TnT.** TnT HDX data are shown from the same experiments shown in Figs. 1, 2, and 4. TnT helix 1 was relatively dynamic and unaffected by Ca^{2+} . The TnT strand of the coiled-coil was highly stable, with very slow exchange, and with variable effects of Ca^{2+} depending upon the region of the coiled-coil. Ca^{2+} increased the dynamics of the base of the coiled-coil, had no effect for the middle, and stabilized the COOH terminus of the coiled-coil TnT strand. Symbols, lines, and thumbnail images are the same as in Fig. 1. For CBMII troponin, data were acquired successfully for TnT 264–288 rather than data for TnT 263–288, which were acquired for unmodified troponin.

not reaching statistical significance. The results imply the absence of any large scale effects of site II on the COOH-lobe.

However, these data do not exclude the possibility of limited or localized effects of site II on COOH-lobe dynamics. In fact, there is reason to believe that one portion of the TnC COOH-lobe is stabilized by Ca^{2+} . When studied at 25 °C, TnC 154–161 has an HDX transition that is slower in the presence of millimolar as opposed to micromolar Ca^{2+} (20). This transition does not appear in the current data set shown in Fig. 2, because it requires longer than 48 h to occur. The importance of this part of TnC is highlighted by the observation that the TnC G159D mutation causes human cardiomyopathy (45), contractile dysfunction (46), and altered interactions with TnI (47–49). Finally, these data do not address how troponin may be affected by site III and IV occupancy by Mg^{2+} rather than Ca^{2+} .

Selective Effects of Site II Ca^{2+} on the Dynamics of Core Domain TnT—HDX patterns within TnT were revealing in general and also in relationship to site II. TnT helix 1, compris-

ing residues 204–220, is the most NH_2 -terminal portion of TnT known clearly to have a well folded structure in the absence of other thin filament proteins. TnT peptide 188–224 contains helix 1 and had an observable HDX transition that was finished within 5 min (Fig. 3). This indicates moderately weak folding of helix 1. The log of the protection factor (Table 1) is in the range 1.76–1.92, providing an estimate of the local protein folding constant; it was slightly less than 100. Site II Ca^{2+} had no statistically significant effect on the HDX rate of TnT helix 1. However, its fast dynamics are consistent with some other role in regulation, as previously demonstrated by studies of both phosphorylation and cardiomyopathy-linked mutation (50–52).

In an unanticipated result, the base of the TnT strand of the TnT-TnI coiled-coil was sensitive to Ca^{2+} ; it was stabilized by CBMII. This action can be seen in the late time points of TnT 224–243 and its subpeptide TnT 230–243 (Fig. 3) and to a smaller extent in the HDX data for subpeptide 237–243.

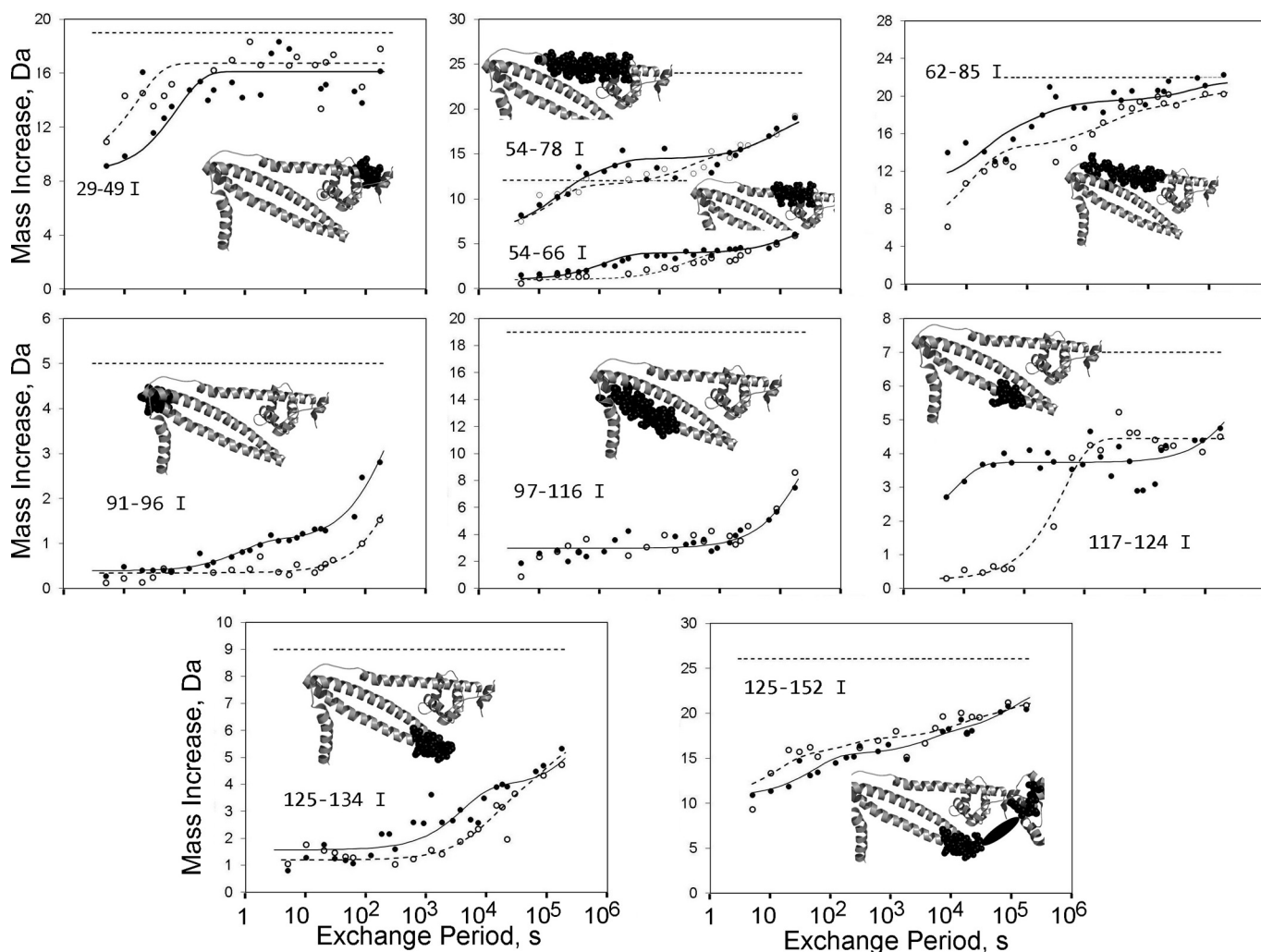


FIGURE 4. **HDX kinetics within TnI peptides outside the regulatory head region.** Shown are HDX data for additional TnI peptides, derived from the experiments also shown in Figs. 1–3. Abolishing Ca^{2+} binding (CBMII troponin) destabilized the TnI helix 1 NH_2 terminus (TnI 29–49 data) and had selective effects on specific other regions of TnI. Many regions were unaffected, but in other regions, CBMII acted to slow HDX, indicating local stabilization in the absence of Ca^{2+} . See “Results.” Symbols, lines, and thumbnail images are the same as in Fig. 1.

Despite the structurally remote location of TnT region 224–237 relative to the NH_2 -lobe of TnC, site II affected the HDX rate. A conservative extrapolation of the 224–243 and 230–243 data would indicate that half of the NH groups were stabilized by the absence of Ca^{2+} binding to site II. As shown below, this observation was validated by similar results in TnI.

The above data do not reflect a global effect on the TnT strand, however. This is demonstrated by results for other peptides. Specifically, TnT 243–263 and 251–263 showed identical, very low HDX for 48 h regardless of Ca^{2+} . TnT 237–243 was mostly similar (no effect of Ca^{2+} except for a 1-Da difference at 48 h). Thus, the mid- and post-mid-portions of the TnT coiled-coil strand do not appear to be affected by Ca^{2+} .

Interestingly, there is an opposite effect of Ca^{2+} on the dynamics of the coiled-coil’s other end, its COOH terminus. Here, as seen in Fig. 3 data for TnT 263–288, Ca^{2+} stabilized rather than destabilized the TnT strand. For unmodified troponin containing site II Ca^{2+} , HDX of TnT 264–288 never reached completion after 48 h in D_2O . This implies that the end of the coiled-coil (which extends to \sim TnT 271) was highly stable. For CBMII troponin, on the other hand, the end of the

coiled-coil was not as stable; HDX of virtually the entire 263–288 peptide finished within 1 h in D_2O .

Selective Effects of Site II Ca^{2+} on the Dynamics of TnI—TnI 91–96 (Fig. 4) showed faster exchange in unmodified troponin than in CBMII troponin. Thus, the TnI strand at the NH_2 -terminal base of the coiled-coil was stabilized by Ca^{2+} release. This finding parallels data for the corresponding TnT region, as described above. In a separate but additional similarity to TnT, the immediately succeeding part of the coiled-coil strand (TnI 97–116) was Ca^{2+} -insensitive, stable, and very slowly exchanging.

In other parts of the coiled-coil, however, the TnI and TnT strands differed. For the TnI strand but not the TnT strand, Ca^{2+} release-mediated stabilization extended to regions other than the coiled-coil base. Specifically, both TnI 117–124 and TnI 125–134 had HDX transitions that were slower in the CBMII context than in unmodified troponin. The corresponding TnT region did not exhibit this behavior.

In describing the structure of troponin for the first time, Takeda *et al.* (25) noted that the base of the coiled coil has a hydrophobic interaction with the preceding TnI loop. Also, a

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hydrogen bond network links TnI Arg-79 to the coiled-coil (53). TnI 62–85 HDX data in Fig. 4 show that CBMII stabilized this TnI loop. The relatively fast, Ca^{2+} -slowed transition in 62–85 is not present in 54–78, so it must reside in loop residues TnI 79–85. Consequently, it can be concluded that site II Ca^{2+} release has the same, stabilizing effect on all three of the interacting components: both TnT and TnI near the coiled-coil base and also the preceding TnI loop.

Finally, portions of TnI helix 1 were Ca^{2+} -sensitive. A Ca^{2+} effect on the helix's NH_2 terminus was demonstrated by the HDX data for TnI 29–49. The data for this peptide indicated two regions, with different kinetics from each other. One region exchanged before 5 s, regardless of Ca^{2+} . This fast HDX can be attributed to the first half of the peptide, which was not well ordered by crystallography. More interestingly, TnI 29–49 contained a measurable pre-60 s HDX transition that was slowed (*i.e.* stabilized) by Ca^{2+} . This Ca^{2+} -stabilized region must represent the second half of the peptide, which is the NH_2 -start of TnI helix 1. The possible structural basis for this stabilization is discussed below.

Most of the remainder of TnI helix 1 was unaffected by Ca^{2+} . HDX data for the long helix 1 peptide TnI 54–78 was similar for CBMII troponin and unmodified troponin. However, one part of helix 1 was stabilized by Ca^{2+} release. Close examination of the results for TnI 54–66, 54–78, and 62–85 indicate that the same, 3–4-Da, Ca^{2+} -shifted transition was present for all three peptides. Thus, residues 63–66, which comprise the only segment present in the data for all three peptides, became more dynamic when Ca^{2+} was bound to TnC site II. HDX of this part of TnI helix 1 was slower in the CBMII complex (*i.e.* in the absence of site II Ca^{2+}).

Comprehensive Map of the Dynamics of Ca^{2+} -free, CBMII Troponin—The processed HDX kinetic results for all regions of troponin are compiled in Table 1 and illustrated in Fig. 5. In addition to HDX rates, Table 1 indicates what those observed kinetics imply about HDX slowing. Specifically, the rate constants were used to calculate how much HDX was slowed compared with HDX rates for unfolded, unprotected model peptides (26). The log of the fold slowing yields the listed protection factors, which are illustrated by *color coding* in both *panels* of Fig. 5.

In Fig. 5A, the (Ca^{2+} -free) CBMII troponin results were mapped onto the only high resolution structure available for cardiac troponin, the Ca^{2+} -saturated core domain (25). (The TnI switch helix and helix 4 should be assumed to have a different structure than shown; they would be dissociated from the TnC NH-lobe in CBMII troponin.) Fig. 5B shows the same results (*No Ca^{2+}* represents CBMII) mapped onto the linear amino acid sequence. Additionally, Fig. 5B shows findings (Ca^{2+}) derived from our published HDX data (21) for Ca^{2+} -saturated troponin.

Fig. 5A shows that in the absence of Ca^{2+} , the most stable portions of troponin (*violet* and *blue coloring*) are the first half of the coiled-coil, parts of the TnC COOH-lobe, and COOH-lobe-associated segments of TnI helix 1. This generalization parallels HDX dynamic mapping results obtained in the presence of Ca^{2+} (21); the most stable regions remain the same, regardless of site II. Other portions are more dynamic and in fact tend to be very much more dynamic.

Many regions of troponin in Fig. 5 are *colored red*, indicating very flexible, loosely folded, or unfolded structure. This particularly includes those regions not identified by x-ray crystallography, which are enclosed by *large black rectangles* in Fig. 5B. The crystallographically undetected TnI COOH terminus is a notable exception. Much of it is weakly folded (*orange*) rather than unfolded (*red*).

Violet-shaded regions are much more stable, and they exchanged little hydrogen for deuterium after 48 h of immersion in D_2O . This slow exchange, indicating slow dynamics, was particularly evident in the coiled-coil region. Interestingly, the dynamics of the coiled-coil were not the same for TnT and TnI. Part of TnI was more dynamic. This demonstrates that the TnI strand has flexibility that is independent of any unfolding of the TnT strand.

The paired with (+) *versus* without (–) Ca^{2+} linear representation of the HDX data in Fig. 5B helps to visualize what parts of each troponin subunit have Ca^{2+} -influenced dynamics. *Color differences* (*i.e.* dynamic differences) are seen especially but not exclusively in the TnC NH_2 -lobe. In general, the changes in this domain are clear measurable effects, as established by the large curve shifts in Fig. 1 with supporting error estimates in Table 1. Fig. 5B also shows that Ca^{2+} affected several other distinct locations within troponin, outside the TnC NH_2 -lobe. Finally, it illustrates that the dynamics of many other regions were in fact Ca^{2+} -independent; site II had either a small effect or no effect on dynamics for many sections of each subunit.

Dynamic Map of Troponin Mutation Sites Linked to Inherited Cardiomyopathy—The *asterisks* in Fig. 5B show the locations (from recent reviews (54–56)) of missense mutations that have been linked to heritable cardiomyopathies. Numerous mutations lie within regions shown to have dynamics that were affected by Ca^{2+} . However, this was not a consistent pattern. Many other mutation sites were not demonstrably Ca^{2+} -sensitive by HDX.

Nevertheless, there was indeed a striking pattern that the comprehensive map in Fig. 5B illustrates. There was a conspicuous tendency for disease-linked mutations to fall within dynamic parts of this dynamic protein. Previous reports (57–60) and reviews (54, 55) have noted the connection between troponin flexibility and cardiomyopathy mutation sites; most cardiomyopathy-inducing troponin mutations occur in regions that are thought to be dynamic. Here this concept was comprehensively and quantitatively validated. Like very few other methods, HDX makes it possible to measure, compare, and map dynamics of an entire molecule, regardless of any gaps in high resolution structural information. The troponin HDX data show that a full 94% of the 50 mapped mutations lay within peptides that were dominated by relatively fast dynamics, indicated by *red, orange, or yellow*. Furthermore, two-thirds of the mapped mutations occurred in regions (in *red*) that had no detectable protection (*i.e.* no detectable folded structure) under at least one condition: in the presence of Ca^{2+} , the absence of Ca^{2+} , or both.

Map of Troponin Regions Stabilized by Regulatory Site Ca^{2+} —Fig. 6 shows a more binary representation of the effects of Ca^{2+} on the dynamics of troponin, across the full span of the core domain's extended structure. Regions stabilized by Ca^{2+} bind-

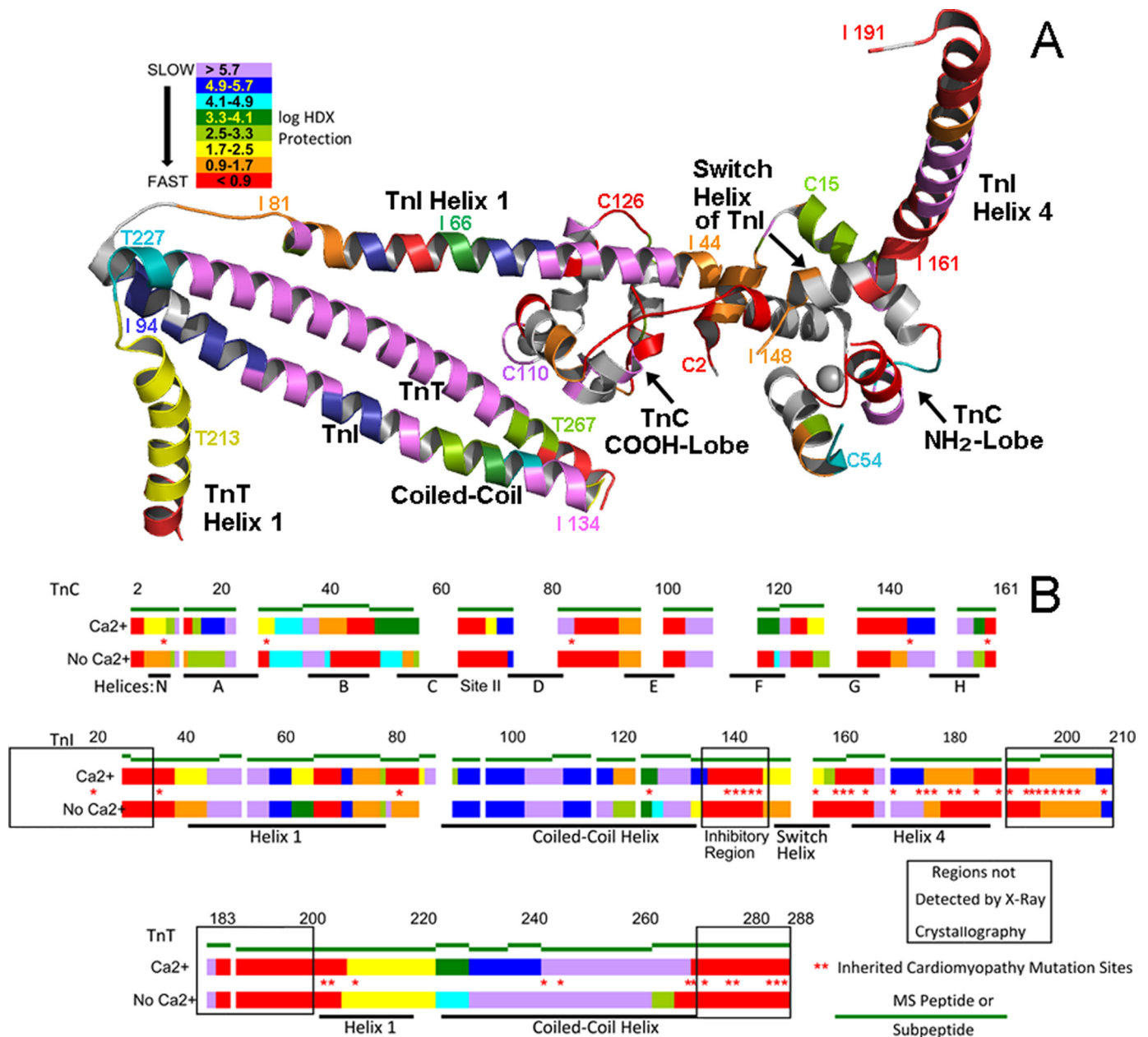


FIGURE 5. Dynamic maps of cardiac troponin in the absence and presence of site II Ca^{2+} . Shown is a summary of all of the results, using a logarithmic scale to convey the wide range of observed dynamic behavior. *A* and *B* show the same color coding. Red, highly dynamic unprotected regions, generally undergoing HDX in < 5 s. Violet, highly stable regions that have slow dynamics. Intermediate dynamics are shown according to the degree of protection from HDX, which was calculated from the measured HDX rates (Table 1). *A*, CBMII troponin (Ca^{2+} -free) findings mapped onto the cardiac troponin three-dimensional structure. Residue number annotation sites were chosen for ease of illustration. Orientation is the same as in Figs. 1–4. In *B*, the same CBMII troponin data (indicated by No Ca^{2+}) as well as data for unmodified troponin (indicated by Ca^{2+}) are shown mapped onto the linear amino acid sequence positions within each troponin subunit. Green line segments indicate the examined peptides or subpeptides, providing a lower limit (*i.e.* maximum error) to the spatial resolution of the assignments. Within each green line segment, the Ca^{2+} dynamic regions are positioned to minimize the differences from the No Ca^{2+} map. Asterisks indicate sites of cardiomyopathy-inducing missense mutations. Note that they cluster almost entirely within the more dynamic regions of troponin and that many sites are Ca^{2+} -sensitive.

ing (blue) or by Ca^{2+} release (red) or not changed by Ca^{2+} (gray) are mapped onto the atomic structure of troponin. Fig. 6*A* shows changes as mapped onto cardiac Ca^{2+} -saturated troponin. Fig. 6*B* shows mapping of the same changes onto homologous residues of the skeletal muscle troponin Mg^{2+} -EGTA atomic model (37).

When illustrated in this manner, the specificity of the effects is clear, as is the directionality of the observed differences. The blue regions to the right within Fig. 6*A* indicate Ca^{2+} -induced

stabilization within the regulatory TnC NH₂-lobe to which Ca^{2+} binds as well as stabilization in the associating switch helix. (See Fig. 5 for annotation of the switch helix location). These findings indicate relatively local actions of Ca^{2+} within the regulatory head region. They are relatively proximate effects and are direct features of the regulatory switching mechanism.

Near to the regulatory head but not included within it, two other regions are stabilized by Ca^{2+} : the NH₂-end of TnI helix 1

Effects of Regulatory Site Ca^{2+} on Troponin Dynamics

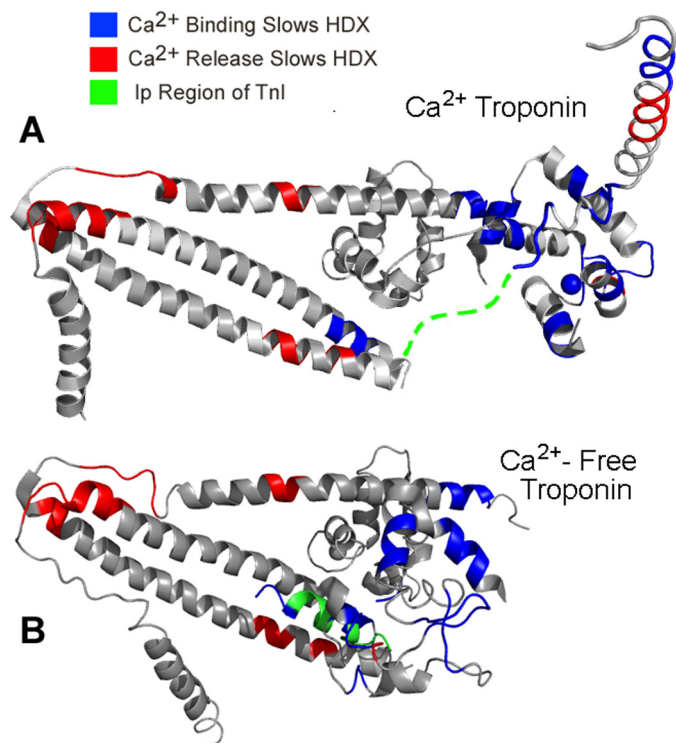


FIGURE 6. Selective, opposite effects of Ca^{2+} on the dynamics of different parts of troponin. The figure shows the estimated locations of the areas that were more stable and less dynamic in either the Ca^{2+} (blue) or Ca^{2+} -free (red) condition. *A*, results mapped onto the cardiac troponin Ca^{2+} structure. *B*, the same results mapped onto the skeletal muscle troponin Mg^{2+} -EGTA structure. TnI 137–147 is shown as a green dashed line in *A*. Note the greatly altered position of this region in *B*, where the corresponding residues are again shown in green. In *A*, unaffected areas are shown in gray, and unassessed areas are shown in white. *Ip Region*, inhibitory peptide region.

and the COOH-end of the TnT coiled-coil strand. Each of these effects is located proximate to the regulatory head region of troponin, close enough to suggest that direct contacts of some sort explain the observations.

Two direct interactions can be invoked that have the potential to explain the HDX-detected, Ca^{2+} -dependent stabilization of helix 1. First, the HDX findings may reflect weak interactions between helix 1 and the TnC NH_2 -lobe that were noted in the crystallography study (25). Second, the 30-residue, cardiac-specific NH_2 terminus of TnI is believed to attach to the Ca^{2+} -TnC NH_2 -lobe (61–64). Such tethering could stabilize the start of TnI helix 1.

The functional importance of the end of helix 1 to the muscle Ca^{2+} switch is supported not only by Fig. 6 but also by independent results. K36Q mutation, immediately preceding the helix, diminishes sensitivity to Ca^{2+} and causes dilated cardiomyopathy. Also, protein kinase C phosphorylation of two residues near the helix 1 NH_2 terminus alters contractile regulatory function (65–67).

Ca^{2+} -dependent direct contacts could, in principle, explain the stabilization of the COOH-end of the coiled-coil TnT strand. However, no specific structural foundation is known to support such a possibility. Whatever the mechanism, it is unclear whether this coiled-coil end stabilization applies to both strands of the coiled-coil or only to TnT. The HDX data of the relevant TnI peptides leave too much ambiguity in kinetic

assignments to define the behavior of the TnI strand's COOH terminus.

Map of Troponin Regions Stabilized by Ca^{2+} Release—Troponin regions that were stabilized by abolishing Ca^{2+} binding are indicated in red in Fig. 6. To understand the structural context for these observations, the cardiac HDX findings were mapped in Fig. 6*B* onto the only available structure for troponin lacking regulatory site Ca^{2+} : the atomic model of skeletal muscle troponin crystallized in Mg^{2+} -EGTA conditions. In this structural model, the TnI switch helix and helix 4 were not detected. Panels *A* and *B* of Fig. 6 look qualitatively different from each other primarily because these parts of TnI are absent in *B* and also because the TnC NH_2 -lobe is in a rather different location from the cardiac result, above the plane of the page.

Fig. 6*B* shows that the green shaded TnI inhibitory peptide region (*Ip*) corresponding to the disordered (dashed line) cardiac TnI 137–147 of Fig. 6*A*, is folded back above and almost anti-parallel to the coiled-coil. Because Ca^{2+} -free cardiac troponin may have a similar structure, this skeletal muscle troponin model is critical for considering the present results. One immediate implication would be that such a structure could underlie the stabilizing effect of Ca^{2+} removal on portions of the COOH-half of the coiled-coil (seen in red just below the green helix in Fig. 6*B*).

Furthermore, this orientation of the TnI inhibitory peptide region in Fig. 6*B* suggests an explanation for the local stabilization indicated in red at the base of the coiled-coil in the Ca^{2+} -free state. We propose that the effect results from contacts with the TnI COOH terminus that occur once Ca^{2+} is released from site II. It is striking that the most COOH-terminal portion of TnI shown in the model has folded back in the needed direction for this to occur, anti-parallel to the coiled-coil. Beyond this point, ~60 COOH-terminal residues are absent in the atomic model. We propose that they interact with this region at the base of the coiled-coil to an extent sufficient to produce the observed slowing of HDX in CBMII troponin. The alternative possibility is that the effects at the base of the coiled-coil are due to transmission of the site II Ca^{2+} signal along the coiled-coil. This seems somewhat less likely, because there are intervening sequence regions that are Ca^{2+} -insensitive.

Similar structural possibilities apply for the observed effects of site II on TnI 63–66 within helix 1. One cannot say from these data whether the observed Ca^{2+} sensitivity indicates a direct contact or is mediated by subtle changes in the nearby TnC COOH-lobe. In either case, two lines of evidence indicate that this region is indeed part of the regulatory site Ca^{2+} switch mechanism despite its location far from site II. First, the observation is robust. The same Ca^{2+} -shifted transition kinetics were observed in three peptides. Second, this precise location is functionally implicated by a previous experiment: mutation A65H shifts the pCa_{50} for tension development and alters cross-bridge-dependent activation of contraction (68).

DISCUSSION

HDX rates are functionally significant; they quantitatively reflect local folding stability and dynamics. They are not mere markers of solvent accessibility. Therefore, the troponin data in this report have considerable implications both for the details

and also for the broad mechanism of troponin's regulatory function.

The details include the finding that Ca²⁺ stabilizes the troponin regulatory head region, especially the TnC NH₂-lobe, but also the switch helix, the nearby end of TnI helix 1, and the coiled-coil terminus. Other regions of the core domain were stabilized not by Ca²⁺ binding but instead by Ca²⁺ release. Other details include the observation that the TnI COOH terminus carries out its known inhibitory actions by adopting a partially folded structure in the absence of Ca²⁺ and actin.

The identified regions of HDX rate Ca²⁺-sensitivity are part of the regulatory mechanism, in so far as the mechanism can be described within troponin itself. They have altered structural energetics and can be considered part of the Ca²⁺ switch. Furthermore, previous, particular experimental results serve to validate this interpretation. Phosphorylation and/or mutational studies show effects on regulation for each Ca²⁺-sensitive HDX region that is now newly identified outside the regulatory head.

The detailed findings point toward several broad mechanistic implications. The Ca²⁺-sensitive regions are widely rather than narrowly distributed within the core domain. Thus, Ca²⁺ release triggers an intramolecular switching mechanism that propagates extensively within troponin's extended structure. Also, the current study proves that the mechanistically important regions of troponin are weakly or even reversibly folded. Little energy would be needed for any local changes in structure, including any folding-unfolding transitions, that accompany their Ca²⁺-regulated binding to targets (actin, tropomyosin, intratroponin targets, or Ca²⁺ itself). The regulatory switching mechanism prominently involves troponin's dynamic properties.

Via comprehensive, quantitative maps, the data validate the concept that cardiomyopathy-inducing mutations cluster in dynamic regions of troponin. Inherited heart disease due to troponin mutations takes the form of any of the more general cardiomyopathic syndromes: hypertrophic, dilated, or restrictive cardiomyopathy. Detailed study of disease-linked mutations yields functionally complex, mutation-specific results (69). In this highly diverse context, any consistency is notable. Furthermore, the strong clustering of mutations within dynamic regions provides a general clue for the pathogenesis of troponin-linked cardiomyopathies.

One possibility is that the dynamic regions contain the mutation sites by default; the more stable regions cannot tolerate mutations because they are too disruptive to structure and function. Knock-out alleles are not observed, and mutation-induced disease severity usually allows carriers to survive well into adulthood. As a generalization for why mutations cluster in dynamic regions, however, we suggest the opposite inference. The observed clustering occurs because sequence changes in the relevant regions tend to cause the largest functional effects. They tend to be the most, rather than the least, sensitive regions of troponin. Sequence changes in these regions are more likely to be sufficiently disruptive to cause disease rather than to become clinically silent alleles.

As stated above, the mechanistically important regions of troponin are weakly folded, with low stability and low folding

energy. Therefore, point mutations in these regions can easily disrupt structure sufficiently to prevent their normal participation in the regulatory mechanism. This is consistent with the observations that any of multiple mutations in the same region may cause disease. In this sense, these regions are weak links in the structural chain required for normal troponin function.

These considerations imply that the clustering of cardiomyopathy-inducing mutations in dynamic regions has two inter-related origins. First, the mutations tend to cluster in areas of troponin that have functional roles in the regulatory mechanism, and such areas of troponin are dynamic. Also, the weak folding of these regions makes them particularly vulnerable to local, mutation-induced unfolding and structural change.

Finally, future studies will be needed to establish what mechanism mediates the observed stabilization of the base of the TnT-TnI coiled-coil in the Ca²⁺-free state. As suggested above, one possibility is localization of the TnI COOH terminus at the base of the coiled-coil (*i.e.* stabilization by an intratroponin binding event). The COOH-terminal region of TnI is a key mediator of troponin's ability to control tropomyosin position on actin so that tropomyosin sterically interferes with strong myosin attachment in relaxed muscle (70). More generally, the TnI COOH terminus is a key mediator of troponin's ability to shut off muscle contraction in the absence of Ca²⁺ (71–74). Therefore, our testable proposal is potentially critical for the structural basis of the regulatory mechanism.

Alternatively, if the base of the coiled-coil is Ca²⁺-sensitive by a different mechanism, such as structural effects transmitted along the coiled-coil, the implications for regulation still could be important. Troponin-mediated regulation is a remarkable example of action at a distance. Troponin is a very asymmetrical, extended molecule. In addition to the core domain, it includes a tail region that attaches to the actin filament at a point several actins distant from the bulk of the core domain. The troponin tail has many cardiomyopathy mutation sites (55) and is poorly folded unless attached to the thin filament. It is unknown how troponin's elongated structure is involved in regulation. However, a Ca²⁺-sensitive region as detected and shown in Fig. 6 may act, in the thin filament context, either directly to alter troponin's interactions with actin-tropomyosin (75, 76) or else as part of other transmission of the regulatory signal (77).

In summary, the findings indicate the many mechanistic connections among the details of troponin's fast structural dynamics, troponin's function as the primary on-off switch that regulates muscle contraction, and troponin's identification as a cause of heritable cardiomyopathy.

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