15

STRUCTURAL AND FUNCTIONAL ANALYSIS OF TROPONINS FROM SCALLOP STRIATED AND HUMAN CARDIAC MUSCLES

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15.1. INTRODUCTION

The Ca^{2+}-regulation of scallop striated muscle contraction, a Ca^{2+}-regulation mechanism that is linked to myosin, was first discovered by A. G. Szent-Györgyi and his colleagues.[1,2] In myosin-linked Ca^{2+}-regulation, the Ca^{2+}-receptive site is the essential light chain of myosin, and the ATPase of the scallop myofibrils has been found to be desensitized to Ca^{2+} by removal of the regulatory light chain (RLC) of myosin in response to treatment with a divalent cation chelator (EDTA). At the same time, three components of troponin and tropomyosin have also been isolated from scallop striated muscle, and several of their biochemical properties have been investigated.[3–5] In this troponin-linked Ca^{2+}-regulation, the concurrent presence of all three components of troponin (troponins C, I, and T; TnC, TnI, and TnT) and tropomyosin are necessary for the regulation of actomyosin ATPase activity.[6–10] The action of Ca^{2+} on TnC ultimately induces actomyosin ATPase activity. Troponin-linked Ca^{2+}-regulation is also desensitized by the removal of TnC in response to treatment with divalent cation chelators such as EDTA or CDTA. The mutual relation of these two types of Ca^{2+}-regulations in scallop myofibrils was then investigated as follows.[11] Desensitized scallop myofibrils were prepared by removing both RLC and TnC by treatment with a divalent cation chelator, CDTA, and the effects of reconstitution with RLC and/or TnC on the ATPase activity of the desensitized myofibrils were examined. It was then demonstrated that reconstitution with TnC does not inhibit ATPase in the absence of Ca^{2+} but instead induces ATPase activity only in the presence of Ca^{2+}, while reconstitution with RLC inhibits ATPase in the absence of Ca^{2+} and this inhibition is eliminated with increasing Ca^{2+} concentrations. These results indicate that scallop troponin has a predominantly activating effect on the contractile system. This finding is in sharp contrast to vertebrate troponin, which primarily exhibits inhibition-deinhibition type Ca^{2+}-regulating ability with slight activating action. In this article, we will discuss several biochemical properties of scallop troponin components with particu-

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lar reference to the Ca^{2+}-bound structure of the TnC C-lobe. In addition, we also present some findings from ongoing studies of the functional as well as structural consequences of mutations of human cardiac troponin I associated with restrictive cardiomyopathy (RCM).

15.2. SCALLOP TROPONIN

Scallop TnC binds only one Ca^{2+} ion at its C-terminal EF-hand motif (site IV) in C-the terminal lobe (C-lobe) due to the amino acid replacement that is critical to Ca^{2+}-binding, while vertebrate fast skeletal muscle TnC has four Ca^{2+}-binding sites: two regulatory low-affinity sites in the N-terminal lobe (N-lobe) and two high-affinity sites in the C-lobe[3–5,12,13] (Figure 15.1). Therefore, scallop TnC is a curious TnC that binds only Ca^{2+} at the C-lobe. In addition, Doi et al. have reported that the N-lobe of scallop TnC also plays a role in Ca^{2+} regulation, though the N-lobe itself has no Ca^{2+}-binding activity.[14] A chimeric protein containing the rabbit fast skeletal TnC N-lobe and scallop TnC C-lobe shows virtually no Ca^{2+} sensitivity with regard to Mg^{2+}-ATPase activity of a troponin and tropomyosin-reconstituted actomyosin. These results support the the hypothesis that the N-lobe of scallop TnC also plays an important role in the regulatory function of the TnC.

The followings are some unique characteristics of the scallop striated muscle TnI. The TnI is a polypeptide containing 292 amino acid residues (M.W. 34 678) and its molecular weight is greater by approximately 14 000 than that of the vertebrate skeletal muscle polypeptide.[15–18] The homologous sequence to vertebrate TnIs is found in the C-terminal portion. The sequence of the inhibitory region is essential to the inhibitory function, which is achieved by binding to actin or TnC, and this inhibitory region is highly conserved. On the other hand, scallop TnI has 100–133 extra residues at the N-terminus of the vertebrate TnI. This extra region has a unique sequence and contains many Glu and Arg residues. Moreover, there is no similarity between the second TnC binding

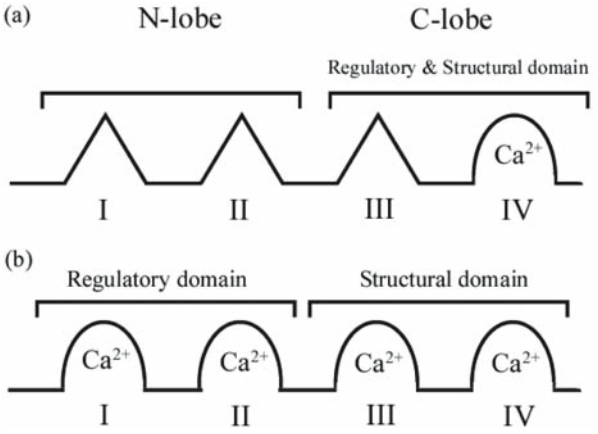

Figure 15.1. Ca^{2+}-binding sites of TnCs. (a) Ca^{2+}-binding site of Akazara scallop TnC (b) Ca^{2+}-binding sites of vertebrate fast skeletal muscle TnC.

region of the vertebrate fast skeletal muscle TnI and the corresponding region of scallop TnI. Since there are some differences between vertebrate fast skeletal muscle troponin components and those of scallop, it is possible that the molecular mechanism of Ca^{2+}-regulation by scallop troponin may be distinct from that of vertebrate striated muscle.

Many structural investigations of the structures of vertebrate cardiac and fast skeletal muscle TnCs have been carried out to increase our understanding of the molecular regulatory mechanism of Ca^{2+} and troponin.[19–23] A recent remarkable achievement was determination of the core domain crystal structures of human cardiac and chicken fast skeletal muscle troponins.[24,25] The structural information regarding invertebrate TnC, however, was limited. For instance, the coordination structures of Ca^{2+} and Mg^{2+} in scallop TnC have been investigated.[26] In Site IV of scallop TnC, the COO^- group in Glu142 has been found to bind Ca^{2+} in the bidentate coordination mode, while COO^- groups in Asp131 and Asp133 bind Mg^{2+} via the pseudo-bridging mode with low affinity (Figure 15.2). Through the use of mutagenesis, functional and structural studies of the scallop TnC E142Q mutant have demonstrated that the mutation from Glu to Gln results in a loss of Ca^{2+} binding ability and Ca^{2+}-dependent regulatory function of TnC.[13,27] These results indicate that Site IV of scallop TnC has both regulatory and structural function by through the exchange of Mg^{2+} with Ca^{2+}. Circular dichroism (CD) spectra and the gel-filtration profiles of scallop TnC have shown that the secondary structure is conserved among the three states (apo, Mg^{2+}-bound, and Ca^{2+}-bound), and the tertiary structure is in fact altered upon Ca^{2+}-binding.[26] TnC shows a more compact conformation when it binds Ca^{2+}. The concrete tertiary structural changes, however, have not been clarified.

To analyze the precise Ca^{2+}-dependent regulatory mechanism of scallop TnC, we conducted an NMR structural analysis of the scallop TnC C-lobe, which contains two EF-hand motifs (Sites III and IV) complexed with $TnI_{129–183}$.[28] Scallop $TnI_{129–183}$ is a peptide that corresponds to the N-terminal region of vertebrate fast skeletal muscle TnIs, which bind to the TnC C-lobe in the presence and absence of Ca^{2+}. Scallop TnC, TnC C-lobe, and $TnI_{129–183}$ were prepared using an *E. coli* expression system. Both TnC and the C-lobe of TnC tend to form oligomers at concentrations in the millimolar range (Figure 15.3a). On the other hand, the formation of the $TnI_{129–183}$ complex with the C-lobe of TnC suppresses the aggregation process (Figure 15.3b). We were therefore able to completely assign the backbone amide signals of the C-lobe of scallop TnC[28] (Figure 15.4). For the assignment of the NMR signals from the side-chains, HCCH-TOCSY, HCCH-COSY, 3D ^{15}N-edited NOESY, and ^{13}C-edited NOESY spectra were used. The constraints for the distances and angles were identified using the two 3D NOESY spectra and HNHA spectrum, respectively. The structure of the C-lobe of TnC in the complex with $TnI_{129–183}$, was then calculated using DYANA ver. 1.4.[29] The solution structure of Ca^{2+}-bound TnC C-lobe binding to $TnI_{129–183}$ was determined. The 3D structure showed that the C-lobe contained four

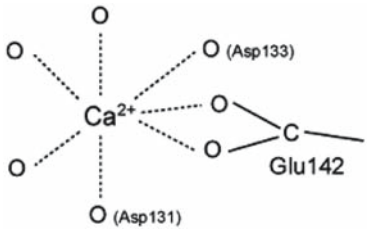

Figure 15.2. Ca^{2+}-coordination structure of scallop TnC.

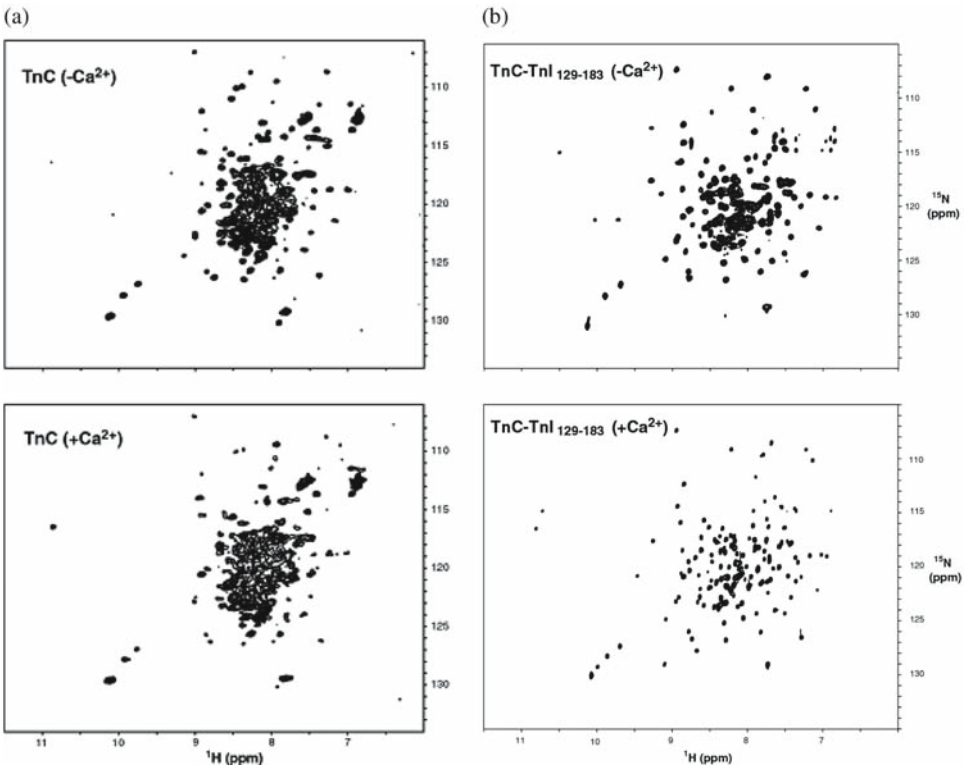

Figure 15.3. (a) ^{15}N—^{1}H HSQC spectra of scallop TnC ($-Ca^{2+}$: upper panel, $+Ca^{2+}$: lower panel) and (b) TROSY-type ^{15}N—^{1}H HSQC spectra of scallop TnC binding to $TnI_{129–183}$ ($-Ca^{2+}$: upper panel, $+Ca^{2+}$: lower panel).

α-helices and two short anti-parallel β-sheets (Figure 15.5). In general, the conformational change in the EF-hand motif due to Ca^{2+}-binding is regarded as the transition from the closed- to the open-state, which correspond to the approximately anti-parallel orientation and almost perpendicular orientation, respectively, between the two helices that are a part of the helix-loop-helix.[30] The structures of both EF-hand motifs have the open conformation. Therefore, the C-lobe of TnC is in the activated form when it binds Ca^{2+}. The conformational change to the open-state must be one of the triggers of striated muscle contraction of the scallop. However, it is not clear whether Site III, which does not bind Ca^{2+}, has a closed- or an open-conformation in the resting state in a cell (Figure 15.6). Determining the structure of the scallop TnC C-lobe in the apo state will help to clarify these details.

With regard to the biological significance of the structural transition, an interesting study was carried out by Tanaka et al.[31] They performed a comparative study of the functional roles of N- and C-terminal regions of molluscan and vertebrate TnIs (Figure 15.7). The results suggested that scallop troponin regulates muscle contraction via activating mechanisms that involve the region spanning from the TnC C-lobe binding site to the inhibitory region of TnI, and that there is no alternative binding of the TnI C-terminal region to TnC. The research also revealed that the TnI N-terminal region (from the TnC C-lobe-binding region to the inhibitory region) participates in Ca^{2+}-dependent activation. In addition, similar activation is observed for the vertebrate troponin, which contains a

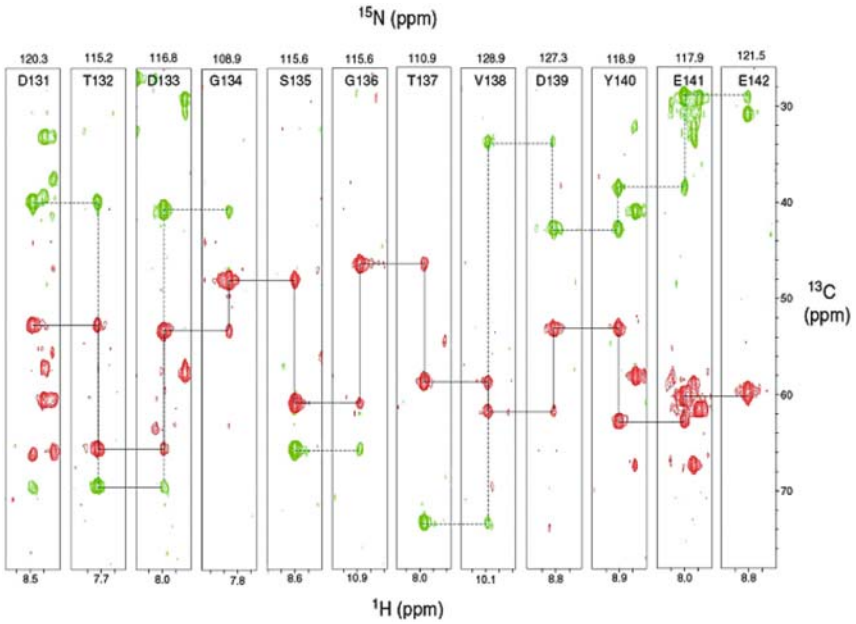

Figure 15.4. Strip-plots of the HNCACB spectrum of the Akazara scallop TnC C-lobe that binds $TnI_{129–183}$.

Figure 15.5. Amino acid sequence and the secondary structure of the Akazara scallop TnC C-lobe that binds $TnI_{129–183}$.

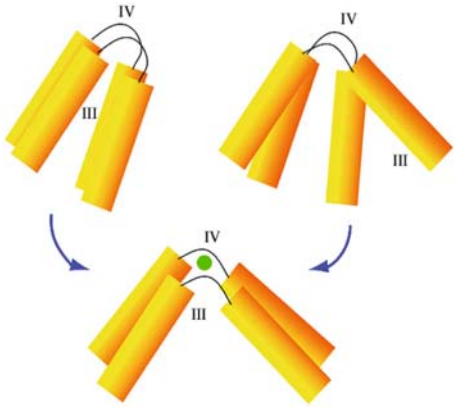

Figure 15.6. Schematic representation of the conformational changes from the closed (III)–closed (IV) state or the open (III)–closed (IV) state to the open (III)–open (IV) state.

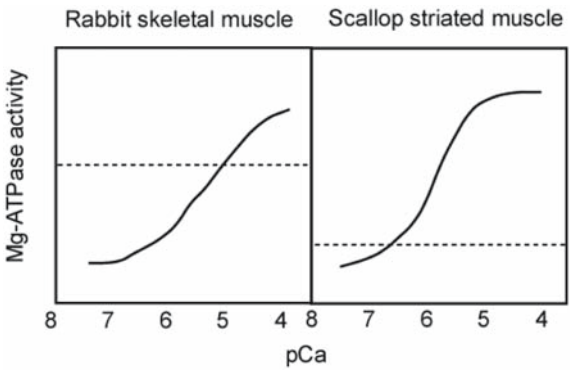

Figure 15.7. Schematic representation of the Ca^{2+}-regulation of actomyosin-tropomyosin Mg^{2+}-ATPase by rabbit and Akazara scallop reconstituted troponins at 15°C (Tanaka et al. (2005).) The activities in the absence of troponin are indicated by dashed line.

TnI-fragment. It was therefore concluded that there is a common activating mechanism between vertebrates and scallops. The activation of the scallop striated muscle might be induced by strengthening of the interaction between the TnC C-lobe and the TnC-binding region of TnI.

Based on the molecular mechanism of vertebrate striated muscle contraction, which is regulated by troponin-tropomyosin and is generally classified as the inhibition-deinhibition type, we assume that the activation effects of scallop troponin occur via the common mechanism of Ca^{2+}-regulation by mutant human cardiac troponin. The functional analyses of gene mutations for human cardiac TnT associated with hypertrophic cardiomyopathy (HCM) have revealed that Ca^{2+}-sensitization of the force generation of skinned fibers is a major functional consequence of these mutations as much as the 15 mutations causing HCM.[32–34] However, one HCM-causing mutation (F110I) does not show Ca^{2+}-sensitization but instead enhances force generation, and another mutation (E244D) shows both enhanced force generation and Ca^{2+}-sensitization.[35] This finding strongly suggests that the activating action plays some role in the genetic disorder of cardiac troponin, although the activating action of vertebrate troponin is a minor mechanism under physiological conditions. Therefore, detailed studies of scallop troponin should contribute to clarifying the pathogenic mechanism of the two TnT mutants for which the activating effect has been potentiated.

It has been demonstrated that conformational change occurs in scallop TnC when it binds Ca^{2+} at Site IV, and that it changes to an open-state that triggers muscle contraction. Therefore, further investigation of scallop troponin is essential to understanding the mechanism of the activation effect and the actin-linked Ca^{2+} regulation of invertebrate striated muscles.

15.3. CARDIAC TROPONIN

We also present herein our recent research on human cardiac troponin related to cardiomyopathy. It has been demonstrated that mutations in the genes that code for two human cardiac Tn components, cTnI and cTnT, are often responsible for cardio-

myopathy.[34,36] Cardiac TnC is a Ca^{2+}-binding component, and mutation in the gene encoding this component is also responsible for cardiomyopathy.[37] The functional consequences of cTnI and cTnT mutations linked to HCM and/or DCM have been investigated by Morimoto et al. and Ohtsuki.[31–34,38] They have shown that these mutations alter the regulatory properties of the troponin ternary complex, thereby resulting in an increase and a decrease in the Ca^{2+} sensitivity of force generation that may primarily contribute to the pathogeneses of HCM and DCM, respectively.

In addition to the two cardiomyopathies, six missense mutations (L144Q, R145W, A171T, K178E, D190G, and R192H) occurring in the human cTnI gene have been found to be associated with restrictive cardiomyopathy (RCM).[39] RCM, the least common form of the three cardiomyopathies, is characterized by restrictive diastolic dysfunction (restrictive filling and reduced diastolic volume of either or both ventricles) with normal or near-normal systolic function and wall thickness.[40] The prognosis of RCM is very poor, particularly in the young with heart failure, and patients affected by this condition often require heart transplantation. The RCM-causing mutations in cTnI occur in residues that are highly conserved among different animal species and tissues;[41–46] as such, these mutations would perturb an important physiological function of cTnI (Figure 15.1). With regard to the positions of the mutations, these mutations occur in distinct functional regions of cTnI. L144Q and R145W are positioned in the inhibitory region that binds to actin and TnC to inhibit and activate muscle contraction, respectively. HCM-causing mutations R141Q, R145G, and R145Q have also been reported to occur in the same region.[47] A171T and K178E are located near and within the second actin-tropomyosin binding region, respectively, which is necessary for the complete inhibitory activity of the inhibitory region.[48] D190G and R192H are positioned in the C-terminal region, which is also required for the complete inhibitory action of cTnI.[49]

We have shown the functional and structural consequences of RCM-causing cTnI mutations through the use of recombinant proteins.[50] In order to explore the functional consequences of cTnI mutations on force generation, each of the purified human cTnI mutants was incorporated into a skinned fiber with recombinant human cTnT and cTnC by using the troponin-exchanging method developed by Hatakenaka and Ohtsuki with a slight modification.[51–53] All six mutations shifted the force-pCa relationship curve to lower Ca^{2+} concentrations and exerted Ca^{2+}-sensitizing effects on force generation. Moreover, RCM-causing mutations were found to have considerably greater Ca^{2+}-sensitizing effects than the HCM-causing mutations. Gomes et al. have recently reported the same result by using porcine skinned cardiac muscle fibers into which human cTnIs with five RCM-causing mutations, except D190G, were incorporated.[54] With regard to ATPase activity, the six mutations also exerted Ca^{2+}-sensitizing effects, which were evident from the leftward shifts in the pCa-ATPase activity relationships. The K178E mutation exerted the most prominent Ca^{2+}-sensitizing effect on ATPase activity. These results were similar to those from the experiments on force generation. These experiments revealed that RCM-causing mutations show considerably greater Ca^{2+} sensitivity of the cardiac myofilament than HCM-causing mutations. As such, the amplitude of the Ca^{2+} sensitization might be a determinant for the phenotypic expression of RCM or HCM.

To clarify the molecular mechanisms of such drastic Ca^{2+} sensitization of cardiac myofilaments, we examined the K178E mutation from a structural perspective using CD and NMR spectroscopy. Because the full-length cTnI polypeptide tends to aggregate

easily and become insoluble at physiological ionic strength, we prepared a C-terminal cTnI fragment, $cTnI_{129–210}$. This peptide inhibits the actomyosin Mg^{2+}-ATPase activity. The CD analysis of wild-type and K178E $cTnI_{129–210}$ peptides revealed that the K178E mutation causes only a small change in the secondary structure of $cTnI_{129–210}$. To determine the component involved in the structural change following K178E substitution, wild-type and K178E $cTnI_{129–210}$ peptides were analyzed by 2D NMR. Most signals in the $^{15}N—^{1}H$ HSQC spectrum were found to be unaffected by the K178E mutation, and only a small number of peaks in the $^{15}N—^{1}H$ HSQC spectrum were found to be perturbed by the mutation. The residues changed by the mutation were K177, K(E)178, E179, and T181. These findings indicate that the structural change occurs in a restricted region surrounding the K178E mutation site. A recent NMR study of the structure of a chicken fast skeletal muscle TnI, which is complexed with the chicken skeletal muscle TnC and TnT_2, indicates that this region forms part of an anti-parallel β-sheet[55] (Figure 15.8a). This structure suggests that the K178E mutation destabilizes the β-structure critical to the physiological function of the second actin-Tm binding region in cTnI (Figure 15.8b). Therefore, the mutation caused drastic Ca^{2+} sensitization of cardiac myofilaments by inducing a subtle structural change in an unexpectedly restricted region within the cTnI molecule. We also recently found that the L144Q, A171T, D190G, and R192H mutations lead to subtle structural perturbations (Yumoto et al., unpublished results)

On the basis of these results, small molecules that desensitize the Ca^{2+}-sensitivities of force generation of cardiac muscle would likely be effective in therapy for cardiomyopathies such as HCM and RCM. Accordingly, we have screened some chemical compounds and have identified the materials that modify the Ca^{2+}-sensitivity. We have discussed the functional and structural basis of the modifier of Ca^{2+}-sensitivity in this symposium.

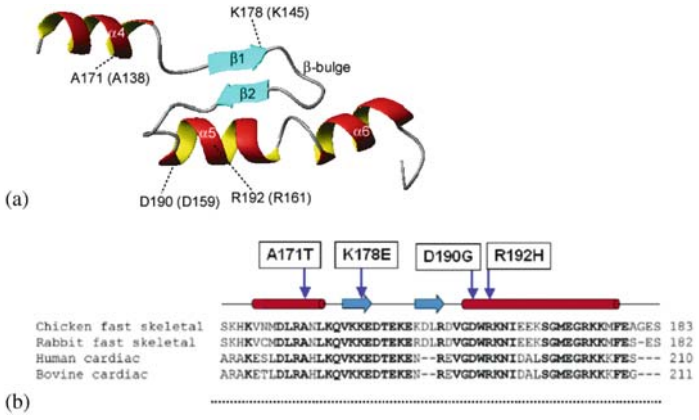

Figure 15.8. (a) The positions of RCM-causing mutations in cTnI mapped on a solution structure model of chicken fast skeletal muscle TnI C-terminal region[24] (PDB 1VDJ). The Figures are generated by MOLMOL[56]. (b) The secondary structure elements are drawn above the sequence alignments of TnIs. The common amino acids are indicated with bold characters.

15.4. ACKNOWLEDGEMENTS

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