Catecholaminergic polymorphic ventricular tachycardia-related mutations R33Q and L167H alter calcium sensitivity of human cardiac calsequestrin

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INTRODUCTION

Mammalian CASQs (calsequestrins), low-affinity, high-capacity Ca2+-binding proteins, are the product of two different genes, the skeletal muscle isoform (CASQ1) and the cardiac muscle isoform (CASQ2) [1–3]. CASQ is segregated to the SR (sarcoplasmic reticulum) lumen and is detected as electron-dense filamentous matrices apposed to the junctional SR membrane [4,5]. These matrices are composed of linear polymers formed by CASQ monomers and dimers at millimolar Ca2+ concentrations [6,7]. Owing to their proximity to the RyR2 (ryanodine receptor 2), CASQ matrices are thought to provide the pool of Ca2+ necessary to trigger myocyte contraction [3]. In this regard, polymERIC CASQ represents the high-capacity Ca2+-binding form of the protein that binds at least twice as much Ca2+ as monomeric CASQ [7,8]. In addition to serving as a Ca2+ sink, CASQ2 has been shown to regulate RyR2 activity directly via protein–protein interactions involving triadin and junctin [9]. In particular, CASQ2 appears to mediate the responsiveness of the RyR2 channel to luminal Ca2+ by serving as a luminal Ca2+ sensor [10]. CASQ2 thus appears to act as an intrinsic and extrinsic regulator of Ca2+ release, by modulating RyR2 activity and buffering intraluminal Ca2+ respectively [11].

Two missense mutations, R33Q and L167H, of hCASQ2 (human cardiac calsequestrin), a protein segregated to the lumen of the sarcoplasmic reticulum, are linked to the autosomal recessive form of CPVT (catecholaminergic polymorphic ventricular tachycardia). The effects of these mutations on the conformational stability and Ca2+ sensitivity properties of hCASQ2, were investigated. Recombinant WT (wild-type) and mutant CASQ2s were purified to homogeneity and characterized by spectroscopic (CD and fluorescence) and biochemical (size-exclusion chromatography and limited proteolysis) methods at 500 and 100 mM KCl, with or without Ca2+ at a physiological intraluminal concentration of 1 mM; Ca2+-induced polymerization properties were studied by turbidimetry. In the absence of Ca2+, mutations did not alter the conformation of monomeric CASQ2. For L167H only, at 100 mM KCl, emission fluorescence changes suggested tertiary structure alterations. Limited proteolysis showed that amino acid substitutions enhanced the conformational flexibility of CASQ2 mutants, which became more susceptible to tryptic cleavage, in the order L167H > R33Q > WT. Ca2+ at a concentration of 1 mM amplified such differences: Ca2+ stabilized WT CASQ2 against urea denaturation and tryptic cleavage, whereas this effect was reduced in R33Q and absent in L167H. Increasing [Ca2+]i induced polymerization and precipitation of R33Q, but not that of L167H, which was insensitive to Ca2+. Based on CASQ2 models, we propose that the Arg33 → Gln exchange made the Ca2+-dependent formation of front-to-front dimers more difficult, whereas the Leu167 → His replacement almost completely inhibited back-to-back dimer interactions. Initial molecular events of CPVT pathogenesis begin to unveil and appear to be different depending upon the specific CASQ2 mutation.

Key words: calcium binding, calsequestrin, circular dichroism, fluorescence, protein stability, proteolysis.

Abbreviations used: CASQ, calsequestrin; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed after-depolarization; DHB, dibasic hydrophilic; DTT, dithiothreitol; HIC, hydrophobic interaction chromatography; RP-HPLC, reverse-phase HPLC; RyR2, ryanodine receptor 2; SAH, serine acidic hydrophobic; SEC, size-exclusion chromatography; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid; WT, wild-type.

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cross-bridging of the dimers with a back-to-back orientation. Ca\(^{2+}\) binding would stabilize this orientation by abrogating electrostatic repulsions between the negatively charged C-terminal tail of the preformed dimers. In the nascent CASQ polymer, the back-to-back pairing of the dimers would be stabilized by favourable electrostatic interactions between the negatively charged SHI (serine acidic hydrophobic) site, on one dimer, and the positively charged DHB (dibasic hydrophobic) site on the other dimer [6,13]. This model is supported by the crystallographic structures of CASQs [6,7,12] and validated by biochemical data [13] showing that deletion of the N-terminal domain results in random aggregation of CASQ, whereas truncation of the C-terminal tail impairs oligomerization of CASQ beyond the dimeric form [13].

Seven distinct mutations of the human CASQ2 gene have been linked to the recessive autosomal variant of CPVT (catecholaminergic polymorphic ventricular tachycardia) [19–22], a familial arrhythmogenic disorder characterized by tachyarrhythmias leading to syncope and sudden cardiac death, typically occurring in young children during physical activity or emotional stress. Initial characterization of these mutations indicated that they act by disrupting normal SR Ca\(^{2+}\) handling, thereby leading to premature recovery of SR Ca\(^{2+}\) release from a luminal Ca\(^{2+}\)-dependent refractory state and causing spontaneous after-Ca\(^{2+}\) transients and arrhythmogenic DADs (delayed after-depolarizations) [22–24]. R33Q and L167H, two CPVT-related CASQ2 missense mutants, have been recently characterized in our laboratories: R33Q is derived from a CASQ2 allele carrying a mutation at nucleotide 98 (G98A) yielding the substitution of a glutamine residue for an arginine residue at position 33 [21]; L167H is derived from a T500A mutation, yielding the substitution of a glutamine residue for an arginine residue at position 167 [22]. Upon transient transfection, cardiomyocytes [21]; L167H is derived from a T500A mutation, yielding the substitution of a glutamine residue for an arginine residue at position 167 [22]. However, the pathogenetic relationship linking mutations, structural changes of CASQ2s, spontaneous Ca\(^{2+}\) transients and arrhythmogenic DADs, remains to be elucidated.

The aim of the present study was to investigate the effects of the mutations R33Q and L167H on the conformational and stability properties, as well as on the Ca\(^{2+}\) sensitivity, of recombinant WT (wild-type) human CASQ2. The present structural data indicate that the two point mutations differently affect the stability and Ca\(^{2+}\)-sensitivity of WT CASQ2 and contribute to unveil the initial events of the molecular pathogenesis of CASQ2-linked CPVT.

EXPERIMENTAL

Expression and purification of recombinant CASQ2 proteins

Mutations in the human CASQ2 gene were introduced by using the QuikChange\textsuperscript{\textregistered} mutagenesis kit (Stratagene), as described previously [22]. CASQ2 proteins were expressed from pET-5a-based plasmids in BL21 (DE3) Escherichia coli cells, to yield the recombinant proteins containing a methionine residue at the N-terminal end. For the production of large quantities of recombinant proteins, fresh colonies carrying the different constructs were inoculated into 500 ml of Luria broth in the presence of ampicillin (100 \(\mu\)g/ml) and 0.5% (v/v) glucose and grown under gentle shaking at 37°C. Expression of recombinant proteins was induced by growing cells for 3 h at 37°C in the presence of 0.5 mM IPTG (isopropyl \(\beta\)-d-thiogalactoside) under constant shaking. Cells were harvested and re-suspended (10 ml of buffer/g of cells) in 20 mM Mops (pH 7.2), containing 5 mM DTT (dithiothreitol), 10 mM EGTA and 0.5 M NaCl, in the presence of the protease inhibitors aprotinin (1 \(\mu\)g/ml), leupeptin (2 \(\mu\)g/ml), benzamidine (1 mM) and PMSF (100 \(\mu\)M). Cell lysis was carried out in a French-press [two cycles at 1300 psi (1 psi = 6.9 kPa)] and the cell suspension was centrifuged at 30000 \(\times\) g for 20 min at 4°C; the supernatant was collected and processed as described in [22].

The supernatant was fractionated by HIC (hydrophobic interaction chromatography) [1,2,25], on a phenyl–Sepharose column (GE Healthcare), equilibrated with binding buffer [20 mM Mops (pH 7.2), 5 mM DTT, 10 mM EGTA and 0.5 M NaCl]. Under these conditions, CASQ2 interacts with the phenyl–Sepharose, whereas other contaminants are eluted in the flow-through. The column was extensively washed with binding buffer, and then recombinant CASQ2 proteins were eluted with 20 mM Mops (pH 7.2), 5 mM DTT, 0.5 M NaCl, 1 mM EGTA and 11 mM CaCl\(_2\). CASQ2 fractions were pooled and dialysed overnight, using a dialysis cassette (Fishe) with a cut-off of 7 kDa, against 20 mM Mops (pH 7.2) and 500 mM KCl, and then fractions were stored at −20°C. The CASQ2 concentration was estimated using the Bio-Rad protein assay kit. Aliquots of CASQ2 deriving from HIC purification were equilibrated in the elution buffer at the desired ionic strength by gel-filtration chromatography on a HR 10/30 Superose-12 column (GE Healthcare) eluted at a flow rate of 0.3 ml/min with 20 mM Mops (pH 7.2), containing 100 or 500 mM KCl. The absorbance of the effluent was monitored at 280 nm. The concentration of CASQ2 samples was estimated by measuring the absorbance at 280 nm and the samples were immediately used for spectroscopic studies. Experiments were carried out on three preparations of recombinant WT CASQ2 and two preparations each for R33Q and L167H.

The homogeneity of CASQ2 proteins was established by SDS/PAGE and RP-HPLC (reverse-phase HPLC). Standard PAGE in the presence of SDS was performed using 10–17.5% (w/v) acrylamide gradient gels [26]. Non-denaturing and non-reducing gel electrophoresis was carried out using a modified Laemmli’s gel system without SDS and 2-mercaptoethanol. RP-HPLC analyses were carried out on a C\(_2\) analytical column [4.6 mm × 150 mm, 5 \(\mu\)m particle size, 300 A (1 A = 0.1 nm porosity) from Grace-Vydac]. The column was equilibrated with 0.1% (v/v) aqueous TFA (trifluoroacetic acid) and eluted with a linear 0.1% (v/v) TFA/acetoniitrile gradient at a flow rate of 0.8 ml/min. The absorbance of the effluent was recorded at 226 nm. The chemical identity of the purified protein was established by N-terminal sequence analysis on a protein sequencer model 477A (Applied Biosystems) and by ESI-TOF (electrospray ionization–time-of-flight) MS on a Mariner instrument from Perseptive Biosystems. Typically, protein samples (10 \(\mu\)M) were analysed by HPLC-MS, using a microbore (1 mm × 50 mm, 5 \(\mu\)m particle size) C\(_2\) column (Grace-Vydac) equilibrated in 5% (v/v) aqueous acetonitrile/1% (v/v) formic acid and eluted in water/acetoniitrile (1:1, v/v), containing 1% (v/v) formic acid, at a flow rate of 15 \(\mu\)l/min. The nozzle temperature was set at 140°C and the electrospray potential at 4.4 kV. The instrument was calibrated using the standard protein kit from Sigma.

Purification of native CASQ2

Native CASQ2 was purified from rat heart according to established procedures [18]. All animal experimentation complied with the recommendations stated in Principles of Animal Care (National Institutes of Health) and the guidelines of the animal care advisory committee of the Italian Ministry of Health. Purity of the preparation was monitored by SDS/PAGE and analytical RP-HPLC, as detailed above.

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Analytical SEC (size-exclusion chromatography)

The apparent molecular mass of CASQ2 proteins was estimated by SEC on a HR10/30 Superose-12 column (1 cm x 30 cm) equilibrated in 20 mM Mops (pH 7.2), containing 100 or 500 mM KCl and eluted with the same buffer at a flow rate of 0.3 ml/min. The absorbance of effluent was monitored at 280 nm. The column was calibrated using the low-molecular-mass gel-filtration protein calibration kit (GE Healthcare). The values of void volume ($V_0$) and interstitial volume ($V_i$) were determined by loading Dextran Blue (2 x 10^4 kDa) and the dipeptide H-Tyr-Gly-OH.

Spectroscopic techniques

UV absorption

All measurements were recorded at 25 °C in 20 mM Mops buffer (pH 7.2), containing 100 or 500 mM KCl. The protein concentration was determined by UV absorption at 280 nm on a Lambda-2 PerkinElmer spectrophotometer using a molar absorption coefficient of 45090 M$^{-1}$ cm$^{-1}$. Second-derivative absorption spectra were recorded in the same buffer containing 500 mM KCl.

CD

CD spectra were recorded on a Jasco model J-810 spectro-
polarimeter equipped with a thermostatically controlled cell holder, connected to a model RTE-111 (NesLab) water-circulating bath. CD spectra in the far-UV region spectra were recorded in a 1-mm path-length quartz cell, at a scan speed of 10 nm/min, using a response time of 16 s. The final spectra resulted from the average of four accumulations, after baseline subtraction. Near-UV spectra were recorded in a 1-cm path-length quartz cell, at a scan speed of 50 nm/min, using a response time of 2 s. The final spectra resulted from the average of 16 accumulations, after baseline subtraction. The CD signal was expressed as the mean residue ellipticity, calculated with the formula:

$$\theta = \theta_{obs} \cdot MRW/(10 \times l \times c)$$

where $\theta_{obs}$ is the observed ellipticity in degrees, MRW is the mean residue molecular mass, $l$ is the optical path-length in cm and $c$ is the protein concentration in g/ml. Otherwise, only the baseline-subtracted spectra were reported.

Fluorescence and Ca$^{2+}$ binding

Fluorescence measurements were carried out on a Jasco spectrofluorometer model FP-6500, equipped with a Peltier model ETC-273T temperature-control system from Jasco. Spectra were recorded in a 1-cm path-length quartz cell, at a scan speed of 200 nm/min by exciting the protein samples at 280 nm or 295 nm with an excitation/emission slit of 5 nm. The effect of Ca$^{2+}$ on the fluorescence of CASQ2 proteins was monitored by adding to a solution of CASQ2 (2 ml, 0.1–1 M) in 20 mM Mops (pH 7.2), containing 100 or 500 mM KCl, aliquots (2–5 μl) of CaCl$_2$ stock solutions (0.1–1 M) and recording the fluorescence intensity at the $\lambda_{max}$ as a function of the Ca$^{2+}$ concentration. The protein solution was incubated at each Ca$^{2+}$ concentration for 1 min under gentle magnetic stirring. Thereafter, protein samples were allowed to equilibrate for 2 min and excited at 295 nm using an excitation/emission slit of 5 and 10 nm respectively. Fluorescence data were corrected for sample dilution that was always lower than 2% at the end of the titration. The optical density of the solution at $\lambda_{ex}$ was lower than 0.05 and therefore no inner filter effect was observed. Photobleaching due to photodegradation of tryptophan residues was found to be lower than 2%, even after prolonged light exposure. Fluorescence data were acquired with the Slow Kinetics software from Jasco.

Urea stability

Urea-mediated denaturation of CASQ2 and its mutants was carried out at 25 °C in 20 mM Mops (pH 7.2) and either 100 or 500 mM KCl, in the absence or presence of 1 mM CaCl$_2$. The fluorescence intensity at the $\lambda_{max}$ was recorded as a function of [urea]. Samples (400 μl) of CASQ2 proteins (0.75 μM) were added to 1600 μl of urea solution at the appropriate concentration. After a 1 h incubation, protein samples (2 ml, 150 mM) were excited at 280 nm, using an excitation/emission slit of 5 nm. Reversibility of the denaturation process was evaluated by measuring the recovery of the fluorescence intensity upon a 10-fold dilution of CASQ2 stock solutions (1.5 μM) in 8 M urea with non-denaturing Mops buffer containing 500 mM KCl. For WT CASQ2, urea denaturation was also monitored by measuring the CD signal of CASQ2 solution (2 ml, 0.22 μM) at 222 nm, in the presence of 100 or 500 mM KCl, for increasing urea concentrations.

Turbidimetric measurements

Measurements were carried out at 25 °C on a double-beam model Lambda-2 PerkinElmer spectrophotometer equipped with a thermostatically controlled cell holder, connected to a Haake model F3-C water-circulating bath. Ca$^{2+}$-induced aggregation of CASQ2 proteins was monitored by adding to a solution of CASQ2 (2 ml, 2.25 μM) in 20 mM Mops (pH 7.2), containing 100 or 500 mM KCl, aliquots (2–5 μl) of CaCl$_2$ stock solutions (0.1–1 M). After Ca$^{2+}$ addition, protein samples were gently stirred for 1 min and allowed to equilibrate for 2 min, and then A at 350 nm was measured as a function of [CaCl$_2$]. Absorbance values were corrected for sample dilution (<5% of the final volume) and subtracted for the corresponding value of the buffer alone.

Limited proteolysis

Proteolysis of CASQ2 proteins with TPCK (tosylphenylalanylchloromethane)-treated bovine trypsin (Sigma) was carried out in 20 mM Mops (pH 7.2) and either 100 or 500 mM KCl, in the presence and absence of 1 mM CaCl$_2$. CASQ2 was incubated at 25 °C at a protease/CASQ2 ratio of 1:50 (w/w). At time intervals, aliquots (8 μg) of the reaction mixture were taken and the reaction was stopped by the addition of soybean trypsin inhibitor (Serva), using a 1:1 (w/w) inhibitor/trypsin ratio. Proteolysis digests were analysed by SDS/PAGE. Polyacrylamide gels were stained with Coomassie Brilliant Blue R250 (Sigma) or with the cationic carbocyanine dye ‘Stains-All’ (Sigma) [27]. The activity of trypsin was tested under the specific salt conditions used (i.e. 500 or 100 mM KCl, with or without 1 mM CaCl$_2$) using the chromogenic substrate S-2238 (Chromogenix), as described previously [28].

Computational methods

The structure of R33Q and L167H mutants was modelled on the crystallographic structure of WT human CASQ2, solved at 3.8 Å [12] and on that of the canine AC27 truncated form of CASQ2, solved at 2.6 Å [7]. The side-chain conformation of Gin$^\text{35}$ and His$^\text{167}$ was carefully modelled on the lowest-energy side-chain conformer [29], using the program WHAT-IF [30] run on an O2 workstation from Silicon Graphics.
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Figure 1 Characterization of purified human recombinant CASQ2 proteins

WT human CASQ2 and relative mutants were purified as described in the Experimental section. (A) RP-HPLC analysis of WT CASQ2 (2), L167H (3) and R33Q (1) at 25°C, A.U., arbitrary units. (B) WT CASQ2 and mutants were analysed by SDS/PAGE (left-hand panel) and non-denaturing/non-reducing gel (right-hand panel): lanes 1 and 4, WT CASQ2; lanes 2 and 5, L167H; lanes 3 and 6, R33Q; lanes 7 and 8, minor and major peaks of R33Q respectively, obtained by SEC (see also Figure 2). Molecular-mass standards for SDS/PAGE (in kDa) are indicated on the left-hand side. The arrowhead on the right-hand side points to the putative dimeric form of R33Q. ND, non-denaturing; NR, non-reducing.

RESULTS AND DISCUSSION

Purification and chemical characterization of WT and mutant CASQ2 proteins

Recombinant WT and two mutants of human CASQ2, R33Q and L167H, were expressed in E. coli cells [22] and purified by HIC, according to previously published procedures [1,25]. In the absence of Ca$^{2+}$, CASQs tightly bind to a phenyl–Sepharose column, whereas addition of a saturating CaCl$_2$ concentration (i.e. 10 mM) determines their elution from the column in a highly homogeneous form, as inferred by RP-HPLC analysis, as well as by denaturing and non-denaturing/non-reducing PAGE (Figure 1). For R33Q only, a minor component was observed in the RP chromatographic analysis (Figure 1A) and in the non-reducing electrophoresis (Figure 1B, right-hand panel, lane 6) as a weak band having a higher molecular mass (indicated by the arrowhead). Interestingly, this band disappeared under reducing conditions (Figure 1B, left-hand panel, lane 3), suggesting that a disulfide-bridged dimer may be formed at the level of the single Cys$^{37}$ of CASQ2. The chemical identity of recombinant CASQ2 proteins was established by N-terminal sequence analysis and MS (results not shown).

CASQ2 solutions were extensively dialysed overnight against 20 mM Mops (pH 7.2) and 500 mM KCl, in order to remove Ca$^{2+}$ and to prevent formation of CASQ2 aggregates, which were observed when dialysis was carried out at lower KCl concentration (i.e. 100 mM). All CASQ2 proteins were further purified by SEC on a Superose-12 column, eluted in 20 mM Mops (pH 7.2) and 500 mM KCl. In analogy to the results obtained by RP-HPLC and PAGE (Figure 1B, lanes 1, 2, 4 and 5), both WT CASQ2 and L167H eluted as a single peak, whereas the chromatogram of R33Q also displayed a minor component eluting at shorter retention times and containing higher-molecular-mass species (see Figure 2). However, for preparative purposes, only the material eluted in correspondence of the major peak was collected and used for further analysis.

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Molecular properties of cardiac calsequestrin mutants

Effect of mutations on the conformation and stability of CASQ2 at high and low KCl concentrations

Previous light-scattering studies have shown that canine CASQ2 predominantly exists in the monomeric form at 500 mM KCl, whereas at 100 mM KCl it is more prone to aggregate [8], especially upon prolonged storage and in the absence of Ca$^{2+}$ [13]. Hence, in order to establish whether CPVT-related mutations mainly affect the structure of CASQ2 monomers or they influence the formation of polymers, we investigated the conformational, stability and Ca$^{2+}$-binding properties of WT and mutant human CASQ2 proteins under salt conditions either stabilizing the monomer (i.e. 500 mM KCl) or promoting the formation of oligomers (i.e. 100 mM KCl).

Analytical SEC

At 500 mM KCl (Figure 2A), all CASQ2 proteins eluted with an apparent molecular mass higher than expected, because of the anomalous migration of CASQs in SEC [31], that probably reflects their asymmetric structure [6,12]. At 100 mM KCl (Figure 2B), the apparent molecular mass of CASQ2 is increased by approx. 15%, in agreement with previous studies showing that low KCl concentrations induce a looser conformation in CASQ structure [1,13,15,17,18].

Conformational characterization

The far-UV CD spectrum of wild-type CASQ2, recorded in 500 mM KCl (Figure 3A), resembles that of a mixed $\alpha/\beta$ protein, with two equally intense minima at 218 and 207 nm [32], and is qualitatively similar to those of canine [13,18,33] and human [12] CASQ2. The spectra of WT and mutant CASQ2 proteins shared a similar shape and signal intensity (Figure 3A), suggesting that mutations do not alter the secondary structure of CASQ2, even though the spectrum of L167H has slightly more prominent minima at 207 nm. Even the CD spectrum of L167H in the near-UV region was identical with that of WT CASQ2 (Figure 3B) and similar to that of other CASQs [33,34], thus indicating that the Leu$^{167} \rightarrow$ His replacement does not appreciably affect the protein tertiary structure. The fluorescence spectra of R33Q and L167H were almost superimposable to that of WT CASQ2 (Figure 3C) and displayed a $\lambda_{\text{max}}$ value at short wavelengths (i.e. 334 nm), consistent with the crystallographic structure of human
denaturing conditions (i.e. 8 M urea) is also shown in Figure 3(C), showing that on average tryptophan residues are located in a more polar and flexible environment.

Thus our findings demonstrate that R33Q and L167H mutations do not perturb the conformation of CASQ2 in 500 mM KCl, whereas, at lower ionic strength, L167H seems to acquire a looser conformation.

**Stability to urea**

The stability of CASQ2 proteins at 500 and 100 mM KCl was studied by recording the decrease of the CD signal in the far-UV region (Figure 5A) and the fluorescence intensity (Figures 5B and 5C) at the \( \lambda_{\text{max}} \) as a function of the urea concentration. The reversibility of denaturation was always > 90%.

For WT CASQ2, two shallow transitions were observed at either 500 (Figure 5A) or 100 mM (Figure 5A, inset) KCl, with values of \([\text{urea}]_{1/2}\) of approx. 3.3 ± 0.3 M and 6.0 ± 0.3 M, where \([\text{urea}]_{1/2}\) is the concentration of denaturant corresponding to the midpoint of the transition being considered. The biphasic nature of the unfolding process is consistent with the modular structure of the CASQ2 monomer, formed by three domains [12] that may have different urea stabilities. When the unfolding was monitored by measuring the decrease of CASQ2 fluorescence, a single transition was observed at 100 mM KCl with a \([\text{urea}]_{1/2}\) of 5.0 ± 0.3 M (Figure 5B), whereas at 500 mM KCl (Figure 5B, inset) \([\text{urea}]_{1/2}\) was increased to 6.0 ± 0.3 M and another transition appeared at approx. 3.3 ± 0.3 M, similar to what was observed for the CD signal (Figure 5A). Hence, increasing the salt concentration does not significantly affect the stability of the secondary structure, but it promotes a more compact and stable tertiary fold in WT CASQ2.

As compared with WT CASQ2, both mutations have a marginal, if any, effect on the stability of the CASQ2 secondary structure at 500 mM KCl (Figure 5A). When denaturation was monitored by recording the fluorescence intensity as a function of the urea concentration, different results were obtained at 100 and 500 mM KCl. At lower salt concentration, WT and mutant CASQ2 proteins displayed a similar denaturation profile, with a single transition occurring at \([\text{urea}]_{1/2}\) of 5.0 ± 0.3 M (Figure 5B), whereas at 500 mM KCl (Figure 5B, inset) \([\text{urea}]_{1/2}\) was increased to 6.0 ± 0.3 M and another transition appeared at approx. 3.3 ± 0.3 M, similar to what was observed for the CD signal (Figure 5A). Thus contrary to WT and R33Q, L167H is no longer able to sense the stabilizing effect of KCl on the protein tertiary structure. Notably, significant scattering of experimental data is observed at lower urea concentrations, probably reflecting the looser structure and higher conformational heterogeneity of CASQ2 proteins in the Ca\(^{2+}\)-free state [1,7,13,14,34].

**Limited proteolysis**

Proteolytic enzymes can be effectively used as probes of protein conformation and dynamics [35]. In the present study, the susceptibility of WT and mutant CASQ2 proteins to trypsin was evaluated in 100 and 500 mM KCl. Proteolytic digests
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Figure 5  Conformational stability of recombinant CASQ2 proteins at either 500 mM or 100 mM KCl

Urea-induced unfolding of WT CASQ2 (○, ●), L167H (■, □) and R33Q (△, ▲) was monitored by far-UV CD (A and inset) and by fluorescence spectroscopy (B and inset, and C) at either 100 mM (closed symbols) or 500 mM (open symbols) KCl. CD and fluorescence spectra were taken at a protein concentration of 2.22 μM and 0.15 μM respectively. All spectra were taken at 25°C in 20 mM Mops (pH 7.2), containing either 100 or 500 mM KCl. The results are reported as fluorescence intensity at λmax and as θ/θo or F/Fo, where θo and Fo are the ellipticity and fluorescence values of CASQ2 without urea.

were analysed by SDS/PAGE and stained either with Coomassie Brilliant Blue or with the Stains-All dye, a cation carboxycyanine dye that selectively stains Ca2+-binding proteins blue, whereas all other proteins are stained pink/red [27].

Electrophoretic analysis shown in Figure 6 shows that for WT CASQ2 five major fragments are reproducibly obtained in 500 mM KCl and that only polypeptides at ~40 and 17 kDa stained blue, i.e. bind Ca2+. Comparative analysis of the time course reaction (Figure 6A) revealed that, at a high salt concentration, proteolysis of R33Q was faster than that of the WT CASQ2, even though the proteolytic patterns are similar. In contrast, in the case of L167H, the rate of trypsin digestion was very much increased and the proteolytic fragments generated were markedly different (shown by arrowheads), indicating that the Leu167→His replacement altered the structural/dynamical properties of the protein, exposing new cleavable sites to trypsin. The enhanced rate of proteolysis of L167H is consistent with the lower stability of the tertiary structure of this mutant to urea-induced denaturation (see Figure 5C) in 500 mM KCl and reflects the general inverse relationship existing between conformational stability and rate of proteolysis. When proteolysis was carried out in 100 mM KCl (Figure 6B), the digestion of WT and mutant CASQ2 proteins was much faster than that carried out in 500 mM KCl. Moreover, contrary to what was observed at a high salt concentration (Figure 6A), no significant difference in the proteolysis rate between WT and CASQ2 mutants was detected in 100 mM KCl, in agreement with the fact that WT and mutant CASQ2 proteins displayed similar urea stability (see Figure 5B).

The results of the present study demonstrate that increasing KCl concentrations protect CASQ2 against tryptic cleavage and that CASQ2 proteins sense differently the protective effect of KCl, being maximal for the WT protein and minimal for the L167H mutant.

Effect of mutations on the Ca2+ sensitivity of CASQ2 at high and low KCl concentrations

As mentioned above, in 500 mM KCl, WT canine CASQ2 exists in the monomeric form and is rather insensitive to the Ca2+ concentration [13]. In contrast, at lower KCl concentrations...
Figure 6 Limited proteolysis of recombinant CASQ2 proteins with trypsin
SDS/PAGE of proteolysis of WT and CASQ2 mutants with trypsin at either 500 mM KCl (A) or 100 mM KCl in the absence (B) and presence (C) of 1 mM CaCl₂. CASQ2s were incubated at 25°C at a protease/CASQ2 ratio of 1:50 (w/w). At the indicated time intervals, aliquots (8 μg) of the reaction mixture were taken and the reaction was stopped by the addition of soybean trypsin inhibitor, using a 1:1 inhibitor/trypsin molar ratio. Proteolysis digests were analysed by polyacrylamide 10–17.5 % gradient gels that were stained with either Coomassie Brilliant Blue R250 (B and C) or Stains-All (A). Arrowheads on the right-hand side of (A) indicate specific polypeptides derived from L167H only. Molecular-mass standards (in kDa) are indicated on the left-hand side.

(0–100 mM), CASQs are more prone to form aggregates [8] and are much more responsive to Ca²⁺-induced polymerization [1,13]. In the present study, the effects of Ca²⁺ on the conformation, stability and aggregation of CASQ2 proteins were investigated by biochemical and spectroscopic tools.

Fluorescence
In the presence of 500 mM, the fluorescence of WT and mutant CASQ2 proteins showed a biphasic profile as a function of CaCl₂ (Figure 7A). In particular, at [Ca²⁺] < 0.5 mM the fluorescence of CASQ2s decreased, whereas at [Ca²⁺] > 0.5 mM the signal intensity increased with a sigmoidal shape, reaching saturation at 7–10 mM [Ca²⁺]. Interestingly, in all cases, no precipitation was observed. Considering that at 500 mM KCl CASQ2 exists as a monomer, the reduction of fluorescence observed at very low [Ca²⁺] probably portrays only the spectroscopic effect resulting from the displacement on the protein surface of the larger K⁺ by the smaller and doubly charged Ca²⁺, without any significant protein conformational change. Higher [Ca²⁺] induces a more compact structure in CASQ2 that is coupled to the burial of some tryptophan residues in the protein interior, with a resulting fluorescence increase. Upon reduction of KCl to 100 mM, the Ca²⁺-dependence of the fluorescence signal was complex. In particular, the Ca²⁺ concentration at which the fluorescence of CASQ2 proteins starts increasing was reduced to 0.1–0.2 mM (Figure 7B) and, beyond 0.8 mM CaCl₂, the fluorescence signal of WT CASQ2 decreased linearly. In the case of R33Q, this value was doubled to 1.6 mM CaCl₂, but the overall shape of the fluorescence change was retained. In contrast, the fluorescence of L167H displayed a markedly different Ca²⁺-dependence, with a sigmoidal curve reaching a plateau at 7 mM. Interestingly, for WT CASQ2 and R33Q, the solution became opalescent upon increasing [Ca²⁺], whereas for L167H there was no visible precipitation.

The interpretation of the fluorescence data reported in Figure 7 may be complicated by overlapping effects, including (i) structural reorganization induced by K⁺ and Ca²⁺ on the monomeric species,
Figure 8  Effect of [Ca\textsuperscript{2+}] on the urea stability and on precipitation of recombinant CASQ2 proteins

Urea-induced unfolding of WT CASQ2 (A, ◦), R33Q (B, △, ▲) and L167H (C, □, ■) was monitored by recording the fluorescence intensity at the λ\textsubscript{max} as a function of [urea]. All spectra were taken at 25°C in 20 mM Mops (pH 7.2) and 100 mM KCl, at a protein concentration of 0.15 μM, in the absence (open symbols) and presence (closed symbols) of 1 mM CaCl\textsubscript{2}. The results are reported as F/F\textsuperscript{◦}, where F\textsuperscript{◦} is the fluorescence of CASQ2 without urea. The A values at 350 nm (D) of WT CASQ2 (C, ◦), L167H (□, ■) and R33Q (△, ▲) were measured at 25°C as a function of [CaCl\textsubscript{2}] in 20 mM Mops (pH 7.2), containing either 100 mM (open symbols) or 500 mM KCl (closed symbols). Measurements were obtained at a protein concentration of 2.25 μM. Note that at 500 mM KCl, no absorbance was detected for any samples, as shown by the flat curve over the entire range of CaCl\textsubscript{2}.

(ii) formation of soluble aggregates emitting fluorescence more intensely, and (iii) subtraction of CASQ2 species from the solution owing to precipitation. Nevertheless, it is conceivable to propose that at 500 mM the concentration of K\textsuperscript{+} is so high that the competing Ca\textsuperscript{2+} is no longer able to effectively bind to and induce polymerization/precipitation of CASQ2 proteins. On the other hand, at 100 mM KCl, WT CASQ2 and R33Q bind Ca\textsuperscript{2+} and, beyond certain Ca\textsuperscript{2+} concentrations, form high-molecular-mass polymers that precipitate out the solution and decrease the fluorescence signal. Instead, the Leu\textsuperscript{167} → His transition impairs productive binding of Ca\textsuperscript{2+} in the resulting mutant, that become unable to form insoluble polymers.

Stability to urea

The effect of Ca\textsuperscript{2+} on the stability to urea of CASQ2 proteins was evaluated under conditions that favour oligomerization without causing visible precipitation of CASQ2 (i.e. 100 mM KCl and 1 mM CaCl\textsubscript{2}). For WT CASQ2 (Figure 8A), addition of 1 mM Ca\textsuperscript{2+} altered the urea-induced unfolding by several aspects. First, two transitions (instead of one) can be clearly identified, with values of [urea]_{1/2} of 2.0 ± 0.3 M for the first transition, and of 5.5 ± 0.3 M for the second transition. Probably, the latter corresponds to the unfolding of the Ca\textsuperscript{2+}-bound monomeric form of CASQ2, whereas the transition occurring at lower [urea] would correspond to dissociation of CASQ2 oligomers into monomers. This view is consistent with light-scattering data showing that, under similar salt conditions (i.e. 70 mM KCl and 1 mM CaCl\textsubscript{2}), canine CASQ2 predominantly exists as a mixture of higher oligomers, i.e. (CASQ2)\textsubscript{n}, [13], that probably are less stable than the monomeric species, as usually observed for other oligomeric proteins [36]. Hence, increasing denaturant concentration would shift the equilibrium (CASQ2)\textsubscript{n} ↔ n · CASQ2 towards the monomers. Thereafter, further increase of [urea] leads to monomer unfolding. Secondly, the value of [urea]_{1/2} of the second transition increases by ~0.6 M in the presence of Ca\textsuperscript{2+}, in keeping with the notion that preferential binding of metal ions to the folded/native state enhances protein stability [37]. Thirdly, the change in fluorescence intensity for the transition occurring at higher [urea] is steeper, suggesting...
that the unfolding process is more co-operative [38]. Similar considerations also apply to the unfolding of R33Q (Figure 8B), for which the amplitude of the first transition is much larger than that observed for WT CASQ2. However, Ca\(^{2+}\) does not seem to increase the stability of the mutant in the monomeric form, since [urea]\(_{50}\) remains essentially constant. Strikingly, addition of Ca\(^{2+}\) has a negligible, if any, effect on the urea-induced unfolding of L167H (Figure 8C), indicating that the energetics of the monomer structure are insensitive to Ca\(^{2+}\) and that addition of a physiological concentration of Ca\(^{2+}\) is not able to promote cross-bridging of L167H monomers.

These findings indicate that the potential stabilizing effect of Ca\(^{2+}\) is sensed differently by WT and mutant CASQ2 proteins, in agreement with the different structural perturbations caused by either mutation.

**Limited proteolysis**

When proteolysis was carried out at a low salt concentration (i.e. 100 mM KCl), addition of Ca\(^{2+}\)-protected WT and mutant proteins to a different extent against tryptic cleavage, following the order WT > R33Q > L167H (compare Figures 6B and 6C), thus demonstrating subtle changes in the conformational/flexibility properties of the mutants. The electrophoretic pattern of the proteolytic digest of L167H with 1 mM CaCl\(_2\) is identical with that obtained without Ca\(^{2+}\). This is taken as additional evidence that Ca\(^{2+}\) binding cannot evoke any significant structural change in L167H.

**Turbidimetric measurements**

Ca\(^{2+}\) binding to CASQ is coupled to the shift in equilibrium from soluble to precipitated species [6,8,13], that form fibrils or needle-like crystals *in vitro* [39]. Ribbon-like polymers of Ca\(^{2+}\)-bound CASQ have also been found *in vivo* in the terminal cisternae of the SR [5,40,41].

In the present study, the propensity of WT and mutant CASQ2 proteins to form insoluble aggregates was investigated by recording the increase of turbidity (i.e. the absorbance at 350 nm) as a function of [Ca\(^{2+}\)], at either 500 or 100 mM KCl (Figure 8D). Turbidity is caused by the scattering of light and detects all particles with a hydrodynamic radius greater than the wavelength of the incident light. Thus the A at 350 nm is proportional to the amount of insoluble forms of CASQ2. At 500 mM KCl, light scattering is negligible for all CASQ2s, in keeping with the notion that K\(^{+}\) inhibits Ca\(^{2+}\)-induced polymerization/precipitation of CASQ [13,15,17]. Conversely, at 100 mM KCl, the turbidity of WT and CASQ2 mutants increased with a sigmoidal shape characterized by (i) a lag phase, in which no precipitation was observed, (ii) a growth phase, in which the formation of precipitate sharply increased for small increases of [Ca\(^{2+}\)], and (iii) a steady phase, in which the concentration of insoluble forms remained approximately constant.

From turbidity data, we estimated the value of EC\(_{50}\) (i.e. the [Ca\(^{2+}\)] at which half of the maximal effect was observed) for WT and mutant CASQ2 proteins. Notably, Ca\(^{2+}\) binding to soluble CASQ2 is coupled to polymerization into insoluble forms, in the sense that the extent of precipitation depends on the concentration of soluble Ca\(^{2+}\)-CASQ forms [8]. Hence, although the chemical process leading to CASQ precipitation is complex and involves multiple steps, the value of EC\(_{50}\) can be related to the affinity of Ca\(^{2+}\) for CASQ and provides a convenient, albeit simplistic, parameter for quantitative characterization of the Ca\(^{2+}\)-binding interaction. The value of EC\(_{50}\) for WT CASQ2 was estimated as 0.87 ± 0.04 mM. The affinity of the mutants R33Q and L167H was decreased by more than 5-fold, with EC\(_{50}\) values of 4.7 ± 0.1 and 5.0 ± 0.1 mM respectively. More importantly, the two mutants displayed markedly different Ca\(^{2+}\)-dependent precipitation. In fact, to reach the plateau, R33Q required much higher [Ca\(^{2+}\)] than WT. Nevertheless, the extent of precipitation at high [Ca\(^{2+}\)] is similar to that of WT CASQ2. Strikingly, L167H reaches the plateau at [Ca\(^{2+}\)] > 6 mM, but it is not able to form any significant amount of insoluble aggregates, even at high [Ca\(^{2+}\)].

The effects of the mutations R33Q and L167H are interpreted with respect to (i) the impact of the amino acid exchanges on the structure of CASQ2 monomer and (ii) the influence of such modifications on Ca\(^{2+}\)-dependent polymerization. Analysis of the crystallographic structure of human CASQ2 [12] revealed that the presence of the highly conserved Arg\(^{33}\) on the surface of the CASQ2 first domain might be critical in determining the direction and stability of the N-terminal arm, through the formation of two salt-bridges with Asp\(^{29}\) and Glu\(^{85}\) (Figure 9A). In particular, the latter residue is located at the C-terminal end of the helix Val\(^{40}\)-Glu\(^{85}\) and, therefore, is potentially destabilizing because of an unfavourable charge–helix dipole interaction [42]. Hence, formation of the Arg\(^{33}\)-Glu\(^{85}\) salt bridge may reduce this effect. In the R33Q mutant, however, abrogation of these ionic interactions (Figure 9B) makes the formation of dimers with the right topology (i.e. front-to-front) more difficult, but nevertheless not impossible. Furthermore, modelling studies suggest that the mutation R33Q does not dramatically affect the ability of the newly formed dimers to polymerize with a back-to-back orientation and therefore a higher [Ca\(^{2+}\)] is required for obtaining precipitation of R33Q.

Replacement of the apolar Leu\(^{167}\) in the core of the second domain with the polar histidine residue may alter the native conformation of CASQ2 by weakening the hydrophobic effect (Figure 9C). In addition, modelling studies reveal that His\(^{167}\) bumps against the C-terminal helix (residues 233–245) in the second domain (Figure 9D). This helix is negatively charged and is the putative SAH site interacting with the positively charged DHB site of another CASQ2 molecule in a back-to-back orientation [6]. Hence, the mutation L167H may destabilize the SAH–DHB interaction and therefore impair the formation of the back-to-back interface crucial for triggering polymerization. This interpretation explains why L167H is almost completely insensitive to Ca\(^{2+}\)-induced polymerization/precipitation, even at high [Ca\(^{2+}\)] (Figure 8D).

**Concluding remarks and relevance to CPVT**

This is the first and comprehensive study of the structural, conformational and oligomeric properties of two CASQ2 mutants, R33Q and L167H, related to the onset of CPVT.

The results of the present study have been obtained on recombinant CASQ2s purified from bacteria, i.e. on proteins lacking post-translational modifications typical of eukaryotic cells. CASQ2s purified from heart tissues are reported to be heterogeneously phosphorylated and glycosylated [43–45] with possible overall effects on both surface Ca\(^{2+}\)-binding sites and protein hydrophilicity due to phosphorylation and glycosylation respectively. Thus, in order to extrapolate the present results to the current pathogenetic hypotheses for CPVT, selected experiments were carried out on native CASQ2. With respect to retention time in gel filtration, i.e. the apparent molecular mass (Figure 10A), near-UV CD spectra, i.e. protein three-dimensional structure (Figure 10B), Ca\(^{2+}\)-dependent fluorescence changes (Figure 10A) and Ca\(^{2+}\)-dependent polymer formation (Figure 10A), native CASQ2 has the same tertiary structure and shows similar Ca\(^{2+}\)-induced conformational modifications as compared with recombinant WT CASQ2 (compare Figures 2B, 3B, 8A and 8D respectively; see also the legend to Figure 10).
Since post-translational modifications do not significantly alter such relevant CASQ2 features, the differences observed between the recombinant WT and mutant CASQ2 proteins are solely referable to the specific amino acid substitution.

Spectroscopic results indicate that, in the absence of Ca\(^{2+}\) mutations do not appreciably alter the conformation of CASQ2 monomers. For L167H only, at 100 mM KCl, the observed changes in the emission fluorescence may suggest alterations in the tertiary structure. Nevertheless, limited proteolysis results show that, although the overall CASQ2 folding state remains unchanged upon mutation, the amino acid exchanges significantly enhance the conformational flexibility of CASQ2 mutants, which become more susceptible to tryptic cleavage in the order L167H > R33Q > WT. Such differences are amplified when the effect of physiological Ca\(^{2+}\) concentrations on susceptibility to proteolysis and urea-induced denaturation was investigated. In particular, 1 mM Ca\(^{2+}\) stabilizes WT CASQ2 against urea denaturation and tryptic cleavage. This effect is reduced for R33Q and virtually absent in the case of L167H. Thus the conformation of L167H is more compromised than that of R33Q. In keeping with this conclusion, increasing Ca\(^{2+}\) concentrations are still able to induce polymerization/precipitation of R33Q, but not that of L167H which is essentially insensitive to Ca\(^{2+}\). The Arg\(^{33}\) \(\rightarrow\) Gln exchange makes the formation of front-to-front dimers more difficult, whereas the replacement of Leu\(^{167}\) with a histidine residue completely blocks back-to-back polymerization.

Recently, Kim et al. [12] carried out light-scattering and Ca\(^{2+}\)-binding measurements (at 300 mM KCl) and suggested that the R33Q mutation renders the protein absolutely unable to form linear polymers and that the L167H mutant forms high-molecular-mass aggregates unresponsive to Ca\(^{2+}\). Quite to the contrary, we found that, although the Arg \(\rightarrow\) Gln conversion shifts the Ca\(^{2+}\)-dependence of CASQ2 polymerization to a higher [Ca\(^{2+}\)], the maximal effect is preserved and R33Q does polymerize at higher [Ca\(^{2+}\)]. In contrast, with respect to L167H, we found this mutant to exist as a monomer at all [Ca\(^{2+}\)] and not to form high-molecular-mass aggregates. Discrepancies between the results of Kim et al. [12] and the present study might be due to several reasons; in particular, their actual data are impinged by the heterogeneous nature of protein samples being tested; for example, light-scattering data reveal the presence of large amounts of dimers and higher polymeric species, that sometimes exceed those of the monomeric species, as in the case of L167H. These polymeric species pre-exist in the protein samples prior to Ca\(^{2+}\) addition and clearly affect scattering data, with or without proteolysis and urea-induced denaturation; for example, light-scattering data reveal the presence of large amounts of dimers and higher polymeric species, that sometimes exceed those of the monomeric species, as in the case of L167H. These polymeric species pre-exist in the protein samples prior to Ca\(^{2+}\) addition and clearly affect scattering data, with or without Ca\(^{2+}\), and all subsequent measurements [12].

At the cellular level, CPVT arises as a consequence of spontaneous Ca\(^{2+}\) release causing DADs and triggered arrhythmia. Spontaneous Ca\(^{2+}\) release has been attributed to shortened Ca\(^{2+}\) release site refractoriness caused by either altered luminal Ca\(^{2+}\)-dependent modulation by CASQ2 of the RyR2 and/or altered luminal Ca\(^{2+}\) dynamics secondary to altered Ca\(^{2+}\) buffering by CASQ2 [23]. Based on the present results, conclusions of our own recent papers on Ca\(^{2+}\) handling in WT cardiomyocytes transfected with either mutant [21,22] are rationalized, and the pathogenesis of CPVT is further discussed.

Conspicuous overexpression of R33Q increased the total SR Ca\(^{2+}\) content, despite enhancing RyR2-mediated SR Ca\(^{2+}\) leak, and this was tentatively attributed to an increased SR Ca\(^{2+}\)-storage capacity provided by the mutant protein [21]. The present results show that the preserved ability of R33Q to form polymers, required for high-capacity Ca\(^{2+}\) binding, determined increased polymers.
Figure 10 Properties of native CASQ2

Chromatographic analysis (A) on a Superose-12 column eluted in 20 mM Mops (pH 7.2) and 100 mM KCl. The apparent molecular mass of native CASQ2, estimated by analytical SEC, is similar to that estimated for the recombinant WT CASQ2 (see Figure 2B), even though the peak width is larger, probably because of the chemical heterogeneity introduced by the carbohydrate chains. Near-UV CD spectrum (B) of native CASQ2 in 20 mM Mops (pH 7.2) and 100 mM KCl is superimposable, in both shape and signal intensity, to that of recombinant WT CASQ2 (see Figure 3B), i.e. post-translational modifications do not alter the three-dimensional structure of CASQ2. The fluorescence intensity (C) and $A$ values at 350 nm (D) of native CASQ2 were monitored as a function of $[\text{CaCl}_2]$ in 20 mM Mops (pH 7.2) and 100 mM KCl. Native CASQ2 has a similar $\text{Ca}^{2+}$-dependent fluorescence profile and $\text{Ca}^{2+}$-dependent precipitation properties as compared with recombinant WT CASQ2 (compare Figures 7B and 8D respectively). All measurements were carried out at 25°C and at protein concentrations of 10 $\mu$M, 0.1 $\mu$M and 2.25 $\mu$M, respectively.

SR $\text{Ca}^{2+}$-storage capacity, and that changes of $\text{Ca}^{2+}$-dependent conformation, without disrupting the tertiary structure of the monomer, render R33Q capable of $\text{Ca}^{2+}$-dependent association with RyR2 but incapable of proper RyR2 regulation [21]. In fact, based on single-channel analysis [46], we have shown that R33Q failed to inactivate RyR2 as the intra-SR $[\text{Ca}^{2+}]$ decreased. Recently, two knock-in transgenic models of recessive CPVT have been generated, CASQ2(R33Q/R33Q) mice [N. Rizzi, N. Liu, C. Napolitano, A. Nori, F. Turcato, B. Colombi, S. Bicciato, D. Arcelli, M. Bigioggera, M. Scelsi, L. Villani, P. Volpe and S. G. Priori, unpublished work] and CASQ2(D307H/D307H) mice [47]. In both cases, a dramatic decrease of mutant CASQ2 up to 50% and 90% respectively, was associated with a normal level of relative mRNAs. It is tempting to speculate that R33Q, because of its higher conformational flexibility (as shown in the present study), might undergo accelerated $\text{in vivo}$ degradation via the proteasome, thus reducing the effective CASQ2 content within the SR lumen. $\text{In vivo}$, the molecular pathogenesis of R33Q-linked CPVT may rely on two distinct synergistic mechanisms: lack of regulation of RyR2 and decreased intraluminal $\text{Ca}^{2+}$ buffering. Understanding the intracellular pathways of accentuated R33Q turnover requires additional experimental work.

As to L167H, transient overexpression of the mutant in cardiomyocytes had no significant effects on RyR2 function nor on SR $\text{Ca}^{2+}$ content [22]; moreover, based on single-channel analysis [46], we have shown that L167H was absolutely inactive on RyR2. Taken together, these findings are fully consistent with the extensive changes in the structural and $\text{Ca}^{2+}$-dependent properties of L167H shown in the present study, i.e. L167H is not capable of polymerization-dependent high-capacity $\text{Ca}^{2+}$ binding. Although as a monomer it binds $\text{Ca}^{2+}$ [22], L167H is an essentially functionally inert protein and very probably leads to a functional knockout phenotype equivalent to a null phenotype.

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Molecular properties of cardiac calsequestrin mutants

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