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Catecholaminergic Polymorphic Ventricular Tachycardia-related Mutations R33Q and L167H Alter Calcium Sensitivity of Human Cardiac Calsequestrin

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¹ *Abbreviations:* CASQ1, skeletal muscle calsequestrin; CASQ2, cardiac calsequestrin; CPVT, catecholaminergic polymorphic ventricular tachycardia; DADs, delayed after-depolarizations; DTT, dithiothreitol; ESI, electrospray ionization; IPTG, isopropyl- β -D-thiogalactopyranoside; L167H, recombinant mutant of human cardiac calsequestrin in which Leu167 was replaced by His; pNA, *para*-nitroanilide; OD, optical density; R33Q, recombinant mutant of human cardiac calsequestrin in which Arg33 was replaced by Gln; RP, reverse-phase; RyR2, cardiac ryanodine receptor; SEC, size exclusion chromatography; SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid; TOF, time of flight; TPCK, *N*-*p*-tosyl-phenylalanine chloromethyl ketone.

Footnotes

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SYNOPSIS

Two missense mutations, R33Q and L167H, of the human cardiac calsequestrin (hCASQ2), a protein segregated to the lumen of sarcoplasmic reticulum, are linked to the autosomal recessive form of catecholaminergic polymorphic ventricular tachycardia (CPVT). The effects of these mutations on the conformational, stability and Ca^{2+} sensitivity properties of hCASQ2, were investigated. Recombinant wild-type (WT) and mutant CASQ2s were purified to homogeneity and characterized by spectroscopic (circular dichroism and fluorescence) and biochemical (size-exclusion chromatography and limited proteolysis) methods at 500 and 100 mM KCl, with or without Ca^{2+} at physiological intra-luminal concentration of 1 mM; Ca^{2+} -induced polymerization properties were studied by turbidimetry. In the absence of Ca^{2+} , 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, that became more susceptible to tryptic cleavage, in the order L167H > R33Q > WT. One mM Ca^{2+} amplified such differences: Ca^{2+} stabilized WT CASQ2 against urea denaturation and tryptic cleavage, whereas this effect is reduced in R33Q and absent in L167H. Increasing $[\text{Ca}^{2+}]$ induced polymerization and precipitation of R33Q but not that of L167H which was insensitive to Ca^{2+} . Based on CASQ2 models, we propose that the Arg33→Gln exchange made the Ca^{2+} -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.

INTRODUCTION

Mammalian calsequestrins, low-affinity, high-capacity Ca^{2+} -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 sarcoplasmic reticulum (SR) 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 Ca^{2+} concentrations [6,7]. Due to their proximity to the ryanodine receptor 2 (RyR2), CASQ matrices are thought to provide the pool of Ca^{2+} necessary to trigger myocyte contraction [3]. In this regard, polymeric CASQ represents the high capacity Ca^{2+} binding form of the protein that binds at least twice as much Ca^{2+} as monomeric CASQ [7,8]. In addition to serving as a Ca^{2+} 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 Ca^{2+} by serving as luminal Ca^{2+} sensor [10]. CASQ2, thus appears to act as an intrinsic and extrinsic regulator of Ca^{2+} release, by modulating RyR2 activity and buffering intraluminal Ca^{2+} , respectively [11].

Both intrinsic and extrinsic CASQ2 functions are affected by non-covalent polymerization of CASQ2 monomers into insoluble, high-capacity Ca^{2+} -bound forms. CASQ contains an unusually high number of acidic amino acids and its structure is characterized by the presence of three thioredoxin-like domains arranged to form a disk-like shape [6,7,12]. Polymerization of CASQ is regulated by the opposite action of Ca^{2+} and K^{+} [6,13]. In particular, Ca^{2+} promotes aggregation, whereas K^{+} inhibits Ca^{2+} -induced polymerization of CASQ by competing, at least in part, with the same binding sites on the protein surface [14-16]. Both Ca^{2+} and K^{+} induce a more compact conformation in CASQ but, at variance from Ca^{2+} , K^{+} is not able to cross-bridge CASQ molecules into linear polymers [17,18]. Light scattering data [13] indicate that at 500 mM KCl canine CASQ2 exists almost exclusively as a monomer, whereas, at 300 mM KCl, addition of 1 mM CaCl_2 leads mainly to the formation of dimers. Further reduction in [KCl] to 70 mM and addition of 1 mM CaCl_2 promote formation of higher oligomers up to hexamers. It has been proposed that gradual increasing of [Ca^{2+}] first promotes CASQ folding and then leads to the formation of front-to-front dimers, stabilised through the insertion of the extended N-terminal domain of one monomer into the hydrophobic cleft of the adjacent monomer. Further increase in [Ca^{2+}] favours polymerization through Ca^{2+} -mediated 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 "serine acidic hydrophobic" (SAH) site, on one dimer, and the positively charged "dibasic hydrophobic" (DHB) 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 catecholaminergic polymorphic ventricular tachycardia (CPVT) [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 delayed after-depolarizations (DADs) [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 for an arginine at position 33 [21]; L167H is derived from a T500A mutation, yielding the substitution of a leucine

for a histidine at position 167 [22]. Upon transient transfection, cardiomyocytes behave differently depending upon CASQ2 mutant being produced: over-expression of R33Q increases SR Ca^{2+} -storage capacity and displays gain of function effects on SR Ca^{2+} -release [21], whereas over-expression of L167H is without any effect [22]. However, the pathogenetic relationship linking mutations, structural changes of CASQ2s, spontaneous Ca^{2+} transients and arrhythmogenic DADs, remains to be elucidated.

The goal 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 wild-type human CASQ2. The present structural data indicate that the two point mutations differently affect the stability and Ca^{2+} -sensitivity of wild-type 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 QuickChange Mutagenesis Kit (Stratagene), as detailed elsewhere [22]. CASQ2 proteins were expressed from pET-5a-based plasmids in BL21 (DE3) *E. coli* cells, to yield the recombinant proteins containing a methionine at N-terminal end. For 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 µg/µl) and 0.5% (v/v) glucose and grown under gentle shaking at 37°C. Expression of recombinant proteins was induced by growing cells for three hours at 37 °C in the presence of 0.5 mM IPTG under constant shaking. Cells were harvested and re-suspended (10 ml buffer/g of cells) in 20 mM MOPS, pH 7.2, containing 5 mM DTT, 10 mM EGTA, and 0.5 M NaCl, in the presence of the protease inhibitors aprotinin (1 µg/µl), leupeptin (2 µg/µl), benzamidine (1 mM) and PMSF (100 µM). Cells lyses was carried out in a French-press (two cycles at 1300 ϕ) and centrifuged at 30000 x g for 20 min at 4°C; supernatant was collected and processed as described in [22].

The supernatant was fractionated by hydrophobic interaction chromatography (HIC) [1,22,25], on a phenyl-Sepharose column (GE Healthcare), equilibrated with the binding buffer containing 20 mM MOPS, pH 7.2, 5 mM DTT, 10 mM EGTA, and 0.5 M NaCl. Under these conditions, CASQ2 interact with 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, containing 11 mM CaCl₂. CASQ2 fractions were pooled and dialysed overnight, using a dialysis cassette (Pierce) with a cut-off of 7 kDa, against 20 mM MOPS, pH 7.2, 500 mM KCl, and then stored at -20° C. CASQ2 concentration was estimated by BioRad protein assay kit. Aliquots of CASQ2 deriving from HIC purification were equilibrated in the proper 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. Absorbance of effluent was monitored at 280 nm. The concentration of CASQ2 samples was estimated by measuring the absorbance at 280 nm and immediately used for spectroscopic studies. Experiments were carried out on three preparations of recombinant wild-type CASQ2 and two preparations each for R33Q and L167H.

The homogeneity of CASQ2 proteins was established by SDS-PAGE and RP-HPLC. Standard polyacrylamide gel electrophoresis 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 β-mercaptoethanol. RP-HPLC analyses were carried out on a C₄ analytical column (4.6 x 150 mm, 5 µm particle size, 300 Å porosity) from Grace-Vydac (The Separation Group). The column was equilibrated with 0.1% (v/v) aqueous TFA and eluted with a linear 0.1% (w/w)-TFA-acetonitrile 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 mod. 477A (Applied Biosystems) and by ESI-TOF mass spectrometry on a Mariner instrument from Perseptive Biosystems. Typically, protein samples (10 µM) were analysed by HPLC-MS, using a microbore (1 x 50 mm, 5 µm particle size) C₄ column (Grace-Vydac) equilibrated in 5% (v/v) aqueous acetonitrile-1% (v/v) formic acid and eluted in H₂O/acetonitrile (1:1), containing 1% (v/v) formic acid, at a flow-rate of 15 µl/min. The nozzle temperature was set at 140°C and the electrostatic 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]. Purity of the preparation was monitored by SDS-PAGE and analytical RP-HPLC, as detailed above.

Analytical Size Exclusion Chromatography (SEC)

The apparent molecular weight of CASQ2 proteins was estimated by SEC on a HR10/30 Superose-12 column (1 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. Absorbance of effluent was monitored at 280 nm. The column was calibrated using the low-molecular weight gel-filtration protein calibration kit (GE Healthcare). The value of void volume (V_0) and interstitial volume (V_i) were determined by loading dextran blue (2×10^3 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. Protein concentration was determined by ultraviolet (UV) absorption at 280 nm on a Lambda-2 Perkin-Elmer spectrophotometer using a molar absorptivity value of $45090 \text{ M}^{-1} \times \text{cm}^{-1}$. Second-derivative absorption spectra were recorded in the same buffer containing 500 mM KCl.

Circular Dichroism. Circular dichroism (CD) spectra were recorded on a Jasco model J-810 spectropolarimeter equipped with a thermostated 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 pathlength quartz cell, at scan speed of 10 nm/min, using a response time of 16 s. The final spectra resulted from the average of four accumulations, after base line subtraction. Near-UV spectra were recorded in a 1-cm pathlength quartz cell, at scan speed of 50 nm/min, using a response time of 2 s. The final spectra resulted from the average of 16 accumulations, after base line subtraction. CD signal was expressed as the mean residue ellipticity, calculated with the formula $[\theta] = \theta_{\text{obs}} \cdot \text{MRW} / (10 \times l \times c)$, where θ_{obs} is the observed ellipticity in degree, MRW is the mean residue molecular weight, l is the optical pathlength in cm and c the protein concentration in g/ml. Otherwise, only the base-line subtracted spectra were reported.

Fluorescence and Ca^{2+} binding. Fluorescence measurements were carried out on a Jasco spectrofluorimeter model FP-6500, equipped with a Peltier model ETC-273T temperature control system from Jasco. Spectra were recorded in a 1-cm pathlength 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 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) and recording the fluorescence intensity at the λ_{max} as a function of Ca^{2+} concentration. The protein solution was incubated at the proper 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 λ_{ex} was lower than 0.05 and therefore no inner filter effect was observed. Photobleaching due to photodegradation of Trp-residues was found 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, either 100 or 500 mM KCl, in the absence or presence of 1 mM CaCl_2 . The fluorescence intensity at the λ_{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 1-hour incubation, protein samples (2 ml, 150 nM) 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 10-fold dilution of CASQ2 stock solutions (1.5 μM) in 8 M urea with non-denaturing MOPS buffer containing 500 mM KCl. For wild-type 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 Perkin-Elmer spectrophotometer equipped with a thermostated cell-holder, connected to a Haake model F3-C water-circulating bath. Ca²⁺-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 CaCl₂ stock solutions (0.1-1 M). After calcium addition, protein samples were gently stirred for 1 min and allowed to equilibrate for 2 min and then OD at 350 nm was measured as a function of [CaCl₂]. 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-treated bovine trypsin from (Sigma) was carried out in 20 mM MOPS, pH 7.2, either 100 or 500 mM KCl, in the presence and absence of 1 mM CaCl₂. 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 stopped by 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 CaCl₂) using the chromogenic substrate S-2238 (Chromogenix), as described [28].

Computational methods

The structure of R33Q and L167H mutants was modeled on the crystallographic structure of wild-type human CASQ2, solved at 3.8 Å [12] and on that of canine ΔC27 truncated form of CASQ2, solved at 2.6 Å [7]. The side-chain conformation of Gln33 and His167 was carefully modeled on the lowest energy side-chain conformer [29], using the program WHAT-IF [30] run on an O2 workstation from Silicon Graphics.

RESULTS AND DISCUSSION

Purification and chemical characterization of wild-type and mutant CASQ2 proteins

Recombinant wild-type and two mutants of human CASQ2, R33Q and L167H, were expressed in *E. coli* cells [22] and purified by hydrophobic interaction chromatography (HIC), according to established procedures [1,25]. In the absence of Ca^{2+} , CASQs tightly bind onto a phenyl-Sepharose column, whereas addition of 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 (**Fig. 1**). Only for R33Q, a minor component was observed in the RP chromatographic analysis (**Fig. 1A**) and in the non-reducing electrophoresis (**Fig. 1B, right panel, lane 6**) as a weak band having higher molecular weight (arrowhead). Interestingly, this band disappears under reducing conditions (**Fig. 1B, left panel, lane 3**), suggesting that a disulfide-bridged dimer may be formed at the level of the single Cys53 of CASQ2. The chemical identity of recombinant CASQ2 proteins was established by N-terminal sequence analysis and mass spectrometry (not shown).

CASQ2 solutions were extensively dialysed overnight against 20 mM MOPS, pH 7.2, 500 mM KCl, in order to remove Ca^{2+} and to prevent formation of CASQ2 aggregates, otherwise observed when dialysis was carried out at lower KCl concentration (i.e., 100 mM). All CASQ2 proteins were further purified by size-exclusion chromatography (SEC) on a Superose-12 column, eluted in 20 mM MOPS, pH 7.2, 500 mM KCl. In analogy to the results obtained by RP-HPLC and PAGE (**Fig. 1B, lanes 1-2 and 4-5**), both wild-type CASQ2 and L167H elute as a single peak, whereas the chromatogram of R33Q also displays a minor component eluting at shorter retention times and containing higher-molecular weight species (see **Fig. 2**). For preparative purposes, however, only the material eluted in correspondence of the major chromatographic peak, was collected and immediately used for conformational studies.

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

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 wild-type 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).

Analytical SEC. At 500 mM KCl (**Fig. 2A**), all CASQ2 proteins elute with an apparent molecular weight higher than expected, because of the anomalous migration of CASQs in SEC [31], that likely reflects their asymmetric structure [6,12]. At 100 mM KCl (**Fig. 2B**), the apparent molecular weight of CASQ2 is increased by about 15%, in agreement with earlier 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 (**Fig. 3A**), resembles that of a mixed α/β 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 wild-type and mutant CASQ2 proteins share a similar shape and signal intensity (**Fig. 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 is identical to that of wild-type CASQ2 (**Fig. 3B**) and similar to that of other CASQs [33,34], thus indicating that the Leu167→His replacement does not appreciably affect the protein tertiary structure. The fluorescence spectra of R33Q and L167H are almost super-imposable to that of wild-type CASQ2 (**Fig. 3C**) and display a λ_{max} value at short wavelengths (i.e., 334 nm), consistent with the crystallographic structure of human CASQ2 [12],

showing that on average Trp-residues are embedded in chemical environments of medium polarity. For comparison, the emission spectrum of wild-type CASQ2 recorded under denaturing conditions (i.e. 8M urea) is also reported in **Fig. 3C** (see also **Fig. 4B**). The λ_{\max} value is red-shifted to 351 nm and the emission intensity significantly decreased, while the contribution of Tyr-residues appears as a shoulder at 303 nm. These data indicate that in 8M urea Trp-residues become exposed to the solvent and that the Tyr-to-Trp energy transfer is absent, as expected for a fully unfolded protein. Finally, the conformational properties of CASQ2 mutants were also investigated by second-derivative UV spectroscopy. The similarity of the spectra of wild-type and mutant CASQ2s (**Fig. 3D**) is an additional strong indication that in 500 mM KCl, mutations do not significantly alter the overall protein conformation.

By decreasing KCl concentration from 500 to 100 mM, the ellipticity of wild-type CASQ2 at 218 nm is reduced by 15%, with a concomitant increment of the 207-nm minima (**Fig. 4A**), suggestive of a partial unfolding of CASQ2. These results parallel those previously obtained with CASQ1 that at low ionic strength exists in a looser, more expanded conformation [14,15,34]. Even in this case, the CD spectra of R33Q and L167H are almost super-imposable to that of the wild-type CASQ2 and the fluorescence spectra display a λ_{\max} value identical to that observed at 500 mM, but the signal intensity is decreased by about 10% (**Fig. 4B**), in qualitative agreement with previous data obtained with CASQ1 [15] and canine CASQ2 [13]. Notably, the spectrum of R33Q is identical to that of wild-type CASQ2, whereas that of L167H is less intense by about 20% and the λ_{\max} value is red-shifted by ~ 4 nm, indicating that on average Trp-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, where the protein predominantly exists in the monomeric state. Similar conclusions hold for R33Q in 100 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 (**Fig. 5A**) and the fluorescence intensity (**Fig. 5B, C**) at the λ_{\max} as a function of urea concentration. Reversibility of denaturation was always > 90%.

For wild-type CASQ2, two shallow transitions are observed at either 500 (**Fig. 5A**) or 100 mM KCl (**Fig. 5A, inset**), with values of $[\text{urea}]_{1/2}$ of about 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 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 is observed at 100 mM KCl with a $[\text{urea}]_{1/2}$ of 5.0 ± 0.3 M (**Fig. 5B**), whereas at 500 mM KCl (**Fig. 5B, inset**) $[\text{urea}]_{1/2}$ is increased to 6.0 ± 0.3 M and another transition appears at about 3.3 ± 0.3 M, similarly to what observed for the CD signal (**Fig. 5A**). Hence, increasing salt concentration does not significantly affect the stability of the secondary structure, but it promotes a more compact and stable tertiary fold in wild-type CASQ2.

As compared to wild-type CASQ2, both mutations have marginal, if any, effect on the stability of CASQ2 secondary structure at 500 mM KCl (**Fig. 5A**). When denaturation was monitored by recording the fluorescence intensity as a function of urea concentration, different results were obtained at 100 and 500 mM KCl. At lower salt concentration, wild-type and mutant CASQ2 proteins display a similar denaturation profile, with a single transition occurring at $[\text{urea}]_{1/2}$ of 5.0 ± 0.3 M (**Fig. 5B**). At high KCl concentration, the stability of wild-type and R33Q to urea is increased up to 6.0 ± 0.3 M, whereas the stability of L167H remains similar to that determined at 100 mM KCl (**Fig. 5C**). Thus, contrary to wild-type 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, likely reflecting the looser structure and higher conformational heterogeneity of CASQ 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]. Here, the susceptibility of wild-type and mutant CASQ2 proteins to trypsin was evaluated in 100 and 500 mM KCl. Proteolysis digests were analyzed by SDS-PAGE and stained with either Coomassie Brilliant Blue or with the "Stains-all" dye, a cation carbocyanine dye that selectively stains blue Ca^{2+} -binding proteins whereas all other proteins are stained pink-red [27].

Electrophoretic analysis reported in **Fig. 6** shows that for wild-type CASQ2 five major fragments are reproducibly obtained in 500 mM KCl and that only polypeptides at ~ 40 and 17 kDa stain blue, i.e., bind Ca^{2+} . Comparative analysis of the time course reaction (**Fig. 6A**) reveals that, at high salt concentration, proteolysis of R33Q is faster than that of the wild-type CASQ2, even though the proteolytic patterns are similar. In the case of L167H, instead, the rate of trypsin digestion is very much increased and the proteolytic fragments generated are markedly different (arrowheads), indicating that the Leu167 \rightarrow His replacement alters 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 **Fig. 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 (**Fig. 6B**), the digestion of wild-type and mutant CASQ2 proteins is much faster than that carried out in 500 mM KCl. Moreover, contrary to what observed at high salt concentration (**Fig. 6A**), no significant difference in the proteolysis rate between wild-type and CASQ2 mutants is detected in 100 mM KCl, in agreement with the fact that wild-type and mutant CASQ2 proteins display similar urea stability (see **Fig. 5B**).

Our results 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 wild-type protein and minimal for L167H.

Effect of mutations on the Ca^{2+} sensitivity of CASQ2 at high and low KCl concentration.

As mentioned above, in 500 mM KCl, wild-type canine CASQ2 exists in the monomeric form and is rather insensitive to Ca^{2+} concentration [13]. Instead, at lower KCl concentration (0-100 mM), CASQs are more prone to form aggregates [8] and are much more responsive to Ca^{2+} -induced polymerisation [1,13]. Here, the effects of Ca^{2+} 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 wild-type and mutant CASQ2 proteins shows a biphasic profile as a function of CaCl_2 (**Fig. 7A**). In particular, at $0 < [\text{Ca}^{2+}] < 0.5$ mM the fluorescence of CASQ2s decreases, whereas at $[\text{Ca}^{2+}] > 0.5$ mM the signal intensity increases with a sigmoidal shape, reaching saturation at 7-10 mM $[\text{Ca}^{2+}]$. 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 $[\text{Ca}^{2+}]$ likely portrays only the spectroscopic effect resulting from the displacement on the protein surface of the larger K^+ by the smaller and doubly-charged Ca^{2+} , without any significant protein conformational change. Higher $[\text{Ca}^{2+}]$ induces a more compact structure in CASQ2 that is coupled to the burial of some Trp-residues in the protein interior, with a resulting fluorescence increase. Upon reduction of KCl to 100 mM, the Ca^{2+} -dependence of the fluorescence signal is complex. In particular, the Ca^{2+} concentration at which the fluorescence of CASQ2 proteins starts increasing is reduced to 0.1-0.2 mM (**Fig. 7B**) and beyond 0.8 mM CaCl_2 the fluorescence signal of wild-type CASQ2 decreases linearly. In the case of R33Q, this value is doubled to 1.6 mM CaCl_2 , but the overall shape of the fluorescence change is retained. The fluorescence of L167H, instead, displays a markedly different Ca^{2+} -dependence, with a sigmoidal curve reaching a plateau at 7 mM. Interestingly, for wild-type CASQ2 and R33Q the solution becomes opalescent upon increasing $[\text{Ca}^{2+}]$, whereas for L167H there is no visible precipitation.

The interpretation of the fluorescence data reported in **Fig. 7** may be complicated by overlapping effects, including a) structural reorganization induced by K^+ and Ca^{2+} on the monomeric species, b) formation of soluble aggregates emitting fluorescence more intensely, and c) subtraction of CASQ2 species from the solution due to precipitation. Nevertheless, it is conceivable to propose that at 500 mM the concentration of K^+ is so high that the competing Ca^{2+} ion is no longer able to effectively bind to and induce polymerization/precipitation of CASQ2 proteins. On the other hand, at 100 mM KCl, wild-type CASQ2 and R33Q bind Ca^{2+} and, beyond certain Ca^{2+} concentrations, form high-molecular weight polymers that precipitate out the solution and decrease the fluorescence signal. Instead, the Leu167→His transition impairs productive binding of Ca^{2+} in the resulting mutant, that becomes unable to form insoluble polymers.

Stability to urea. The effect of Ca^{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, 1 mM $CaCl_2$). For wild-type CASQ2 (**Fig. 8A**), addition of 1 mM Ca^{2+} alters the urea-induced unfolding by several aspects. Firstly, 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 one. Likely, the latter corresponds to the unfolding of the Ca^{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 $CaCl_2$), canine CASQ2 predominantly exists as a mixture of higher oligomers, i.e., $(CASQ2)_n$, [13], that likely are less stable than the monomeric species, as usually observed for other oligomeric proteins [36]. Hence, increasing denaturant concentration would shift the equilibrium $(CASQ2)_n \rightleftharpoons n \cdot CASQ2$ 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^{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 cooperative [38]. Similar considerations also apply to the unfolding of R33Q (**Fig. 8B**), for which the amplitude of the first transition is much larger than that observed for wild-type CASQ2. However, Ca^{2+} does not seem to increase the stability of the mutant in the monomeric form, since $[urea]_{1/2}$ remains essentially constant. Strikingly, addition of Ca^{2+} has negligible, if any, effect on the urea-induced unfolding of L167H (**Fig. 8C**), indicating that the energetics of the monomer structure is insensitive to Ca^{2+} and that addition of 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 wild-type and mutant CASQ2 proteins, in agreement with the different structural perturbations caused by either mutation.

Limited proteolysis. When proteolysis was carried out at low salt concentration (i.e., 100 mM KCl), addition of Ca^{2+} protects wild-type and mutant proteins to a different extent against tryptic cleavage, following the order: wild-type > R33Q > L167H (cfr. **Fig. 6B, C**), 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 to that obtained without Ca^{2+} . This is taken as an 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 SR [5,40,41].

Here, the propensity of wild-type 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 (**Fig. 8D**). Turbidity is caused by scattering of light and

detects all particles with a hydrodynamic radius greater than the wavelength of the incident light. Thus, the OD 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, turbidity of wild-type and CASQ2 mutants increases with a sigmoidal shape characterized by (a) a lag phase, in which no precipitation is observed, (b) a growth phase, in which the formation of precipitate sharply increases for small increase of $[Ca^{2+}]$, and (c) a steady phase, in which the concentration of insoluble forms remains 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 wild-type 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 CASQ2 and provides a convenient, albeit simplistic, parameter for quantitative characterization of Ca^{2+} -binding interaction. The value of EC_{50} for wild-type CASQ2 was estimated as 0.87 ± 0.04 mM. The affinity of the mutants R33Q and L167H was decreased by more than five-fold, with EC_{50} values of 4.7 ± 0.1 and 5.0 ± 0.1 mM, respectively. More importantly, the two mutants display markedly different Ca^{2+} -dependent precipitation. In fact, to reach the plateau, R33Q requires much higher $[Ca^{2+}]$ than wild-type. Nevertheless, the extent of precipitation at high $[Ca^{2+}]$ is similar to that of wild-type 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 polymerisation. Analysis of the crystallographic structure of human CASQ2 [12] reveals that the presence of the highly conserved Arg33 on the surface of 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 Asp29 and Glu85 (**Fig. 9A**). In particular, the latter residue is located at the C-terminal end of the helix V46-E85 and, therefore, is potentially destabilizing because of unfavourable charge-helix dipole interaction [42]. Hence, formation of the R33-E85 salt bridge may reduce this effect. In the R33Q, however, abrogation of these ionic interactions (**Fig. 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 polymerise with a back-to-back orientation and therefore higher $[Ca^{2+}]$ is required for obtaining precipitation of R33Q.

Replacement of the apolar Leu167 in the core of the second domain with the polar His-residue may alter the native conformation of CASQ2 by weakening the hydrophobic effect (**Fig. 9C**). In addition, modelling studies reveal that His167 bumps against the C-terminal helix 233-245 in the second domain (**Fig. 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-DBH interaction and therefore impair the formation of the back-to-back interface crucial for triggering polymerisation. This interpretation well explains why L167H is almost completely insensitive to Ca^{2+} -induced polymerisation/precipitation, even at high $[Ca^{2+}]$ (**Fig. 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 present data 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 weight (**Fig. 10A**), near-UV CD spectra, i.e., protein three-dimensional structure (**Fig. 10B**), Ca^{2+} -dependent fluorescence changes (**Fig. 10A**) and Ca^{2+} -dependent polymer formation (**Fig. 10A**), native CASQ2 has the same tertiary structure and shows similar Ca^{2+} -induced conformational modifications as compared to recombinant wild-type CASQ2 (cfr. **Figs. 2B, 3B, 8A and 8D**, respectively; see also legend to **Fig. 10**). Since post-translational modifications do not significantly alter such relevant CASQ2 features, the differences observed between the recombinant wild-type and mutant CASQ2 proteins are solely referable to the specific amino acid substitution.

Spectroscopic data 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 data show that, although the overall CASQ2 folding state remains unchanged upon mutation, the amino acid exchanges significantly enhance the conformational flexibility of CASQ2 mutants, that become more susceptible to tryptic cleavage in the order L167H > R33Q > wild-type. 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 wild-type 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 Arg33 → Gln exchange makes the formation of front-to-front dimers more difficult, whereas the replacement of Leu167 with His 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 form high molecular weight aggregates unresponsive to Ca^{2+} . Quite to the contrary, we found that, although the Arg → Gln conversion shifts the Ca^{2+} -dependence of CASQ2 polymerization to higher $[\text{Ca}^{2+}]$, maximal effect is preserved and R33Q does polymerize at higher $[\text{Ca}^{2+}]$. With respect to L167H, instead, we found this mutant to exist as a monomer at all Ca^{2+} concentrations and not to form high molecular weight aggregates. Discrepancies between the results of Kim et al. [12] and ours 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 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 wild-type cardiomyocytes transfected with either mutant [21,22] are rationalized, and the pathogenesis of CPVT is further discussed.

Conspicuous over-expression of R33Q increased 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 SR Ca^{2+}

storage capacity and that changes of Ca^{2+} -dependent conformation, without disrupting the tertiary structure of the monomer, render R33Q capable of Ca^{2+} -dependent association with RyR2 but unable of proper RyR2 regulation [21]. In fact, based on single channel analysis [46], we have shown that R33Q failed to inactivate RyR2 as intra-SR $[\text{Ca}^{2+}]$ decreased. Recently, two knock-in transgenic models of recessive CPVT have been generated, $\text{CASQ2}^{\text{R33Q/R33Q}}$ mice [Rizzi N, Liu N, Napolitano, C, Nori, A., Turcato F, Colombi, B., Bicciato S, Arcelli, D., Bigioggera M, Scelsi, M, Villani, L, Volpe, P., Priori SG., unpublished work] and $\text{CASQ2}^{\text{D307/D307H}}$ mice [47]. In both cases, a dramatic decrease of mutant CASQ2 up to 50% and 90%, respectively, was associated to a normal level of relative mRNAs. It is tempting to speculate that R33Q because of its higher conformational flexibility, as shown here, might undergo accelerated *in vivo* degradation via proteasome, thus reducing effective CASQ2 content within the SR lumen. *In vivo*, the molecular pathogenesis of R33Q-linked CPVT may rely on two distinct synergistic mechanisms: lack of regulation of RyR2 and decreased intraluminal Ca^{2+} buffering. Understanding the intracellular pathways of accentuated R33Q turnover requires additional experimental work.

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

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REFERENCES

- 1 Mitchell, R. D., Simmerman, H. K. and Jones, L. R. (1988) Ca²⁺ binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. *J. Biol. Chem.* **263**, 1376-1381
- 2 Scott, B. T., Simmerman, H. K., Collins, J. H., Nadal-Ginard, B. and Jones, L. R. (1988) Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J. Biol. Chem.* **263**, 8958-8964
- 3 Beard, N. A., Laver, D. R. and Dulhunty, A. F. (2004) Calsequestrin and the calcium release channel of skeletal and cardiac muscle. *Prog. Biophys. Mol. Biol.* **85**, 33-69
- 4 Meissner, G. (1975) Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta.* **389**, 51-68
- 5 Franzini-Armstrong, C., Kenney, L. J. and Varriano-Marston, E. (1987) The structure of calsequestrin in triads of vertebrate skeletal muscle: A deep-etch study. *J. Cell Biol.* **105**, 49-56
- 6 Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K. and Kang, C. H. (1998) Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. *Nat. Struct. Biol.* **5**, 476-483
- 7 Park, H., Park, I. Y., Kim, E., Youn, B., Fields, K., Dunker, A. K. and Kang, C. (2004) Comparing skeletal and cardiac calsequestrin structures and their calcium binding: A proposed mechanism for coupled calcium binding and protein polymerization. *J. Biol. Chem.* **279**, 18026-18033
- 8 Tanaka, M., Ozawa, T., Maurer, A., Cortese, J. D. and Fleischer, S. (1986) Apparent cooperativity of Ca²⁺ binding associated with crystallization of Ca²⁺-binding protein from sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **251**, 369-378
- 9 Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y. M. and Jones, L. R. (1997) Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor proteins of the cardiac junctional sarcoplasmic reticulum membrane. *J. Biol. Chem.* **272**, 23389-23397
- 10 Gyorke, I., Hester, N., Jones, L. R. and Gyorke, S. (2004) The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. *Biophys. J.* **86**, 2121-2128
- 11 MacLennan, D. H. and Reithmeier, R. A. (1998) Ion tamers. *Nat. Struct. Biol.* **5**, 409-411
- 12 Kim, E., Youn, B., Kemper, L., Campbell, C., Milting, H., Varsanyi, M. and Kang, C. (2007) Characterization of human cardiac calsequestrin and its deleterious mutants. *J. Mol. Biol.* **373**, 1047-1057
- 13 Park, H., Wu, S., Dunker, A. K. and Kang, C. (2003) Polymerization of calsequestrin. implications for Ca²⁺ regulation. *J. Biol. Chem.* **278**, 16176-16182
- 14 Ikemoto, N., Bhatnagar, G. M., Nagy, B. and Gergely, J. (1972) Interaction of divalent cations with the 55,000-dalton protein component of the sarcoplasmic reticulum. studies of fluorescence and circular dichroism. *J. Biol. Chem.* **247**, 7835-7837
- 15 Ikemoto, N., Nagy, B., Bhatnagar, G. M. and Gergely, J. (1974) Studies on a metal-binding protein of the sarcoplasmic reticulum. *J. Biol. Chem.* **249**, 2357-2365
- 16 MacLennan, D. H. (1974) Isolation of a second form of calsequestrin. *J. Biol. Chem.* **249**, 980-984
- 17 Aaron, B. M., Oikawa, K., Reithmeier, R. A. and Sykes, B. D. (1984) Characterization of skeletal muscle calsequestrin by ¹H NMR spectroscopy. *J. Biol. Chem.* **259**, 11876-11881
- 18 Slupsky, J. R., Ohnishi, M., Carpenter, M. R. and Reithmeier, R. A. (1987) Characterization of cardiac calsequestrin. *Biochemistry.* **26**, 6539-6544
- 19 Lahat, H., Pras, E., Olender, T., Avidan, N., Ben-Asher, E., Man, O., Levy-Nissenbaum, E., Khoury, A., Lorber, A., Goldman, B., Lancet, D. and Eldar, M. (2001) A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced

polymorphic ventricular tachycardia in bedouin families from israel. *Am.J.Hum.Genet.* **69**, 1378-1384

20 Postma, A. V., Denjoy, I., Hoorntje, T. M., Lupoglazoff, J. M., Da Costa, A., Sebillon, P., Mannens, M. M., Wilde, A. A. and Guicheney, P. (2002) Absence of calsequestrin 2 causes severe forms of catecholaminergic polymorphic ventricular tachycardia. *Circ. Res.* **91**, e21-6

21 Terentyev, D., Nori, A., Santoro, M., Viatchenko-Karpinski, S., Kubalova, Z., Gyorke, I., Terentyeva, R., Vedamoorthyrao, S., Blom, N. A., Valle, G., Napolitano, C., Williams, S. C., Volpe, P., Priori, S. G. and Gyorke, S. (2006) Abnormal interactions of calsequestrin with the ryanodine receptor calcium release channel complex linked to exercise-induced sudden cardiac death. *Circ. Res.* **98**, 1151-1158

22 Raffaele di Barletta, M., Viatchenko-Karpinski, S., Nori, A., Memmi, M., Terentyev, D., Turcato, F., Valle, G., Rizzi, N., Napolitano, C., Gyorke, S., Volpe, P. and Priori, S. G. (2006) Clinical phenotype and functional characterization of CASQ2 mutations associated with catecholaminergic polymorphic ventricular tachycardia. *Circulation.* **114**, 1012-1019

23 Terentyev, D., Viatchenko-Karpinski, S., Gyorke, I., Volpe, P., Williams, S. C. and Gyorke, S. (2003) Calsequestrin determines the functional size and stability of cardiac intracellular calcium stores: Mechanism for hereditary arrhythmia. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11759-11764

24 Viatchenko-Karpinski, S., Terentyev, D., Gyorke, I., Terentyeva, R., Volpe, P., Priori, S. G., Napolitano, C., Nori, A., Williams, S. C. and Gyorke, S. (2004) Abnormal calcium signaling and sudden cardiac death associated with mutation of calsequestrin. *Circ. Res.* **94**, 471-477

25 Cala, S. E. and Jones, L. R. (1983) Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca²⁺-dependent elution from phenyl-sepharose. *J. Biol. Chem.* **258**, 11932-11936

26 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685

27 Campbell, K. P., MacLennan, D. H. and Jorgensen, A. O. (1983) Staining of the Ca²⁺-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye "stains-all". *J. Biol. Chem.* **258**, 11267-11273

28 De Filippis, V., De Dea, E., Lucatello, F. and Frasson, R. (2005) Effect of Na⁺ binding on the conformation, stability and molecular recognition properties of thrombin. *Biochem. J.* **390**, 485-492

29 De Filippis, V., Sander, C. and Vriend, G. (1994) Predicting local structural changes that result from point mutations. *Protein Eng.* **7**, 1203-1208

30 Vriend, G. (1990) WHAT IF: A molecular modeling and drug design program. *J. Mol. Graph.* **8**, 52-6, 29

31 Cozens, B. and Reithmeier, R. A. (1984) Size and shape of rabbit skeletal muscle calsequestrin. *J. Biol. Chem.* **259**, 6248-6252

32 Brahms, S. and Brahms, J. (1980) Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J. Mol. Biol.* **138**, 149-178

33 Baksh, S., Spamer, C., Oikawa, K., McCubbin, W. D., Heilmann, C., Kay, C. M. and Michalak, M. (1995) Zn²⁺ binding to cardiac calsequestrin. *Biochem. Biophys. Res. Commun.* **209**, 310-315

34 Ostwald, T. J., MacLennan, D. H. and Dorrington, K. J. (1974) Effects of cation binding on the conformation of calsequestrin and the high affinity calcium-binding protein of sarcoplasmic reticulum. *J. Biol. Chem.* **249**, 5867-5871

35 Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E. and Zambonin, M. (1997) Probing the partly folded states of proteins by limited proteolysis. *Fold.Des.* **2**, R17-26

36 Price, N.C. (1993) Assembly of multi-subunit structures in *Mechanisms of protein folding* (Pain R.H., ed.), pp. 160-193, IRL Press.

37 Arnold, F. H. and Zhang, J. H. (1994) Metal-mediated protein stabilization. *Trends Biotechnol.* **12**, 189-192

- 38 Dill, K. A., Phillips, A. T. and Rosen, J. B. (1997) Protein structure and energy landscape dependence on sequence using a continuous energy function. *J. Comput. Biol.* **4**, 227-239
- 39 Maurer, A., Tanaka, M., Ozawa, T. and Fleischer, S. (1985) Purification and crystallization of the calcium binding protein of sarcoplasmic reticulum from skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4036-4040
- 40 Maguire, P. B., Briggs, F. N., Lennon, N. J. and Ohlendieck, K. (1997) Oligomerization is an intrinsic property of calsequestrin in normal and transformed skeletal muscle. *Biochem. Biophys. Res. Commun.* **240**, 721-727
- 41 Wagenknecht, T., Hsieh, C. E., Rath, B. K., Fleischer, S. and Marko, M. (2002) Electron tomography of frozen-hydrated isolated triad junctions. *Biophys. J.* **83**, 2491-2501
- 42 Hol, W. G. (1985) Effects of the alpha-helix dipole upon the functioning and structure of proteins and peptides. *Adv. Biophys.* **19**, 133-165
- 43 O'Brian, J. J., Ram, M. L., Kiarash, A. and Cala, S. E. (2002) Mass spectrometry of cardiac calsequestrin characterizes microheterogeneity unique to heart and indicative of complex intracellular transit. *J. Biol. Chem.* **277**, 37154-37160
- 44 Kiarash, A., Kelly, C. E., Phinney, B. S., Valdivia, H. H., Abrams, J. and Cala, S. E. (2004) Defective glycosylation of calsequestrin in heart failure. *Cardiovasc. Res.* **63**, 264-272
- 45 Ram, M. L., Kiarash, A., Marsh, J. D. and Cala, S. E. (2004) Phosphorylation and dephosphorylation of calsequestrin on CK2-sensitive sites in heart. *Mol. Cell. Biochem.* **266**, 209-217
- 46 Qin, J., Valle, G., Nani, A., Nori, A., Rizzi, N., Priori, S. G., Volpe, P. and Fill, M. (2008) Luminal Ca²⁺ regulation of single cardiac ryanodine receptors: Insights provided by calsequestrin and its mutants. *J. Gen. Physiol.* **131**, 325-334
- 47 Song, L., Alcalai, R., Arad, M., Wolf, C. M., Toka, O., Conner, D. A., Berul, C. I., Eldar, M., Seidman, C. E. and Seidman, J. G. (2007) Calsequestrin 2 (CASQ2) mutations increase expression of calreticulin and ryanodine receptors, causing catecholaminergic polymorphic ventricular tachycardia. *J. Clin. Invest.* **117**, 1814-1823

FIGURE LEGENDS

Figure 1 Characterization of purified human recombinant CASQ2 proteins

Wild-type human CASQ2 and relative mutants were purified as described in Experimental. RP-HPLC analysis (A) of wild-type CASQ2 (2), L167H (3), R33Q (1) at 25°C. Wild-type CASQ2 and mutants were analyzed by SDS-PAGE (B, left panel) and non-denaturing/non-reducing gel (B, right panel): lanes 1 and 4, wild-type CASQ2; lanes 2 and 5, L167H; lanes 3 and 6, R33Q; lanes 7 and 8, minor and major peaks of R33Q, respectively, obtained by size-exclusion chromatography (see also Fig. 2). Molecular mass standards for SDS-PAGE (in kDa) are indicated on the left hand side. Arrowhead, on the right hand side, points to the putative dimeric form of R33Q.

Figure 2 Size-exclusion chromatography of wild-type and mutant CASQ2 proteins

Chromatographic analyses of wild-type CASQ2 (—) and mutants L167H (····) and R33Q (---) were carried out on a Superose-12 column eluted in 20 mM MOPS pH 7.2, containing 500 mM KCl (A) and (B) 100 mM KCl. a) Blue dextran; b) dimer of BSA; c) BSA; d) ovalbumin; e) chymotrypsinogen and f) RNase are used as protein standards for retention times and are indicated at the top of panel A. In the case of R33Q, only the material eluted in correspondence of the major peak was collected and used for further analysis.

Figure 3 Conformational characterization of recombinant CASQ2 proteins at 500 mM KCl

Far- (A) and near-UV (B) CD spectra of wild-type CASQ2 (○), L167H (●), and R33Q (Δ). Measurements were carried out at a protein concentration of 2.22 μM and 10 μM in the far- and near-UV CD, respectively. Fluorescence (C) and second-derivative UV (D) spectra of wild-type CASQ2 (—), L167H (····) and R33Q (---). The emission spectrum of wild-type CASQ2 was also recorded in 8 M urea (-.-). Fluorescence and second-derivative UV spectra were taken at a protein concentration of 0.15 μM and 2.22 μM, respectively. All spectra were taken at 25°C in 20 mM MOPS, pH 7.2, 500 mM KCl.

Figure 4 Conformational characterization of recombinant CASQ2 proteins at 100 mM KCl

Far-UV CD (A) and fluorescence (B) spectra of wild-type CASQ2 (○; —), L167H (●; ····), and R33Q (Δ; ---). The emission spectrum of wild-type CASQ2 was also recorded in 8 M urea (-.-). 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, 100 mM KCl.

Figure 5 Conformational stability of recombinant CASQ2 proteins at either 500 mM or 100 mM KCl

Urea-induced unfolding of wild-type CASQ2 (○, ●) and L167H (□, ■), and R33Q (Δ, ▲) mutants was monitored by far-UV CD (A and inset) and by fluorescence spectroscopy (B and inset, C) at either 100 mM (filled symbols) or 500 mM (empty 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 data are reported as fluorescence intensity at λ_{\max} and as θ/θ° or F/F° , where θ° and F° are the ellipticity and fluorescence values of CASQ2 without urea.

Figure 6 Limited proteolysis of recombinant CASQ2 proteins with trypsin

SDS-PAGE of proteolysis of wild-type 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 CaCl_2 . 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 stopped by 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 R250 (B and C) or Stains-all (A).

Arrowheads on the right hand side of panel A, point to specific polypeptides derived from L167H only. Molecular mass standards (in kDa) are indicated on the left hand side.

Figure 7 Effect of $[Ca^{2+}]$ on the intrinsic fluorescence of recombinant CASQ2 proteins

The fluorescence intensity of wild-type CASQ2 (\circ), and L167H (\bullet), and R33Q (Δ) mutants at λ_{max} was monitored as a function of $[CaCl_2]$ in 20 mM MOPS, pH 7.2, containing either 500 mM (A) or 100 mM (B) KCl. Measurements were taken at 25°C and at protein concentration of 0.1 μ M.

Figure 8 Effect of $[Ca^{2+}]$ on the urea stability and on precipitation of recombinant CASQ2 proteins

Urea-induced unfolding of wild-type CASQ2 (A; \circ , \bullet), R33Q (B; Δ , \blacktriangle) and L167H (C; \square , \blacksquare) was monitored by recording the fluorescence intensity at the λ_{max} as a function of [urea]. All spectra were taken at 25°C in 20 mM MOPS, pH 7.2, 100 mM KCl, at a protein concentration of 0.15 μ M, in the absence (empty symbols) and presence (filled symbols) of 1 mM $CaCl_2$. The data are reported as F/F° , where F° is the fluorescence of CASQ2 without urea. The OD values at 350 nm (D) of wild-type CASQ2 (\circ , \bullet), L167H (\square , \blacksquare) and R33Q (Δ , \blacktriangle) were measured at 25°C as a function of $[CaCl_2]$ in 20 mM MOPS, pH 7.2, containing either 100 mM (empty symbols) or 500 mM KCl (filled symbols). Measurements were obtained at a protein concentration of 2.25 μ M. Note that at 500 mM KCl, no absorbance was detected for any samples, as shown by the flat curve over the entire range of $CaCl_2$.

Figure 9 Amino acid side-chain exchange in the human CASQ2 mutants R33Q and L167H

The structure of R33Q and L167H mutants was modelled on the crystallographic structure of wild-type human CASQ2 [12]. The side-chain conformation of Gln33 and His167 was carefully modelled on the lowest-energy rotamer. The ribbon drawing was generated using WebLab Viewer software. (A) In the wild-type CASQ2, Arg33 forms two salt-bridges with Asp24 and Glu85. Abrogation of these interactions in the R33Q mutant (B) makes the formation of front-to-front dimers more difficult. (C) In the wild-type CASQ2, Leu167 is tightly packed in the protein core, but in the L167H mutant (D), this residue bumps against the C-terminal helix 233-245 of the second domain, forming the negative SAH site of CASQ2. Destabilization of this helix, in turn, may impair the formation of the back-to-back interface by weakening the interaction between the negative SAH and the positive DBH site.

Figure 10 Properties of native CASQ2

Chromatographic analysis (A) on a Superose-12 column eluted in 20 mM MOPS, pH 7.2, 100 mM KCl. The apparent molecular weight of native CASQ2, estimated by analytical SEC, is similar to that estimated for the recombinant wild-type CASQ2 (see Fig. 2B), even though the peak width is larger, likely 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, 100 mM KCl is super-imposable, in both shape and signal intensity, to that of recombinant wild-type CASQ2 (see Fig. 3B), i.e., post-translational modifications do not alter the three-dimensional structure of CASQ2. The fluorescence intensity (C) and OD values at 350 nm (D) of native CASQ2 were monitored as a function of $[CaCl_2]$ in 20 mM MOPS, pH 7.2, 100 mM KCl. Native CASQ2 has similar Ca^{2+} -dependent fluorescence profile and Ca^{2+} -dependent precipitation properties as compared to recombinant wild-type CASQ2 (cfr. Fig. 7B and 8D, respectively). All measurements were carried out at 25°C and at protein concentration of 10 μ M, 0.1 μ M and 2.25 μ M in B, C and D, respectively.

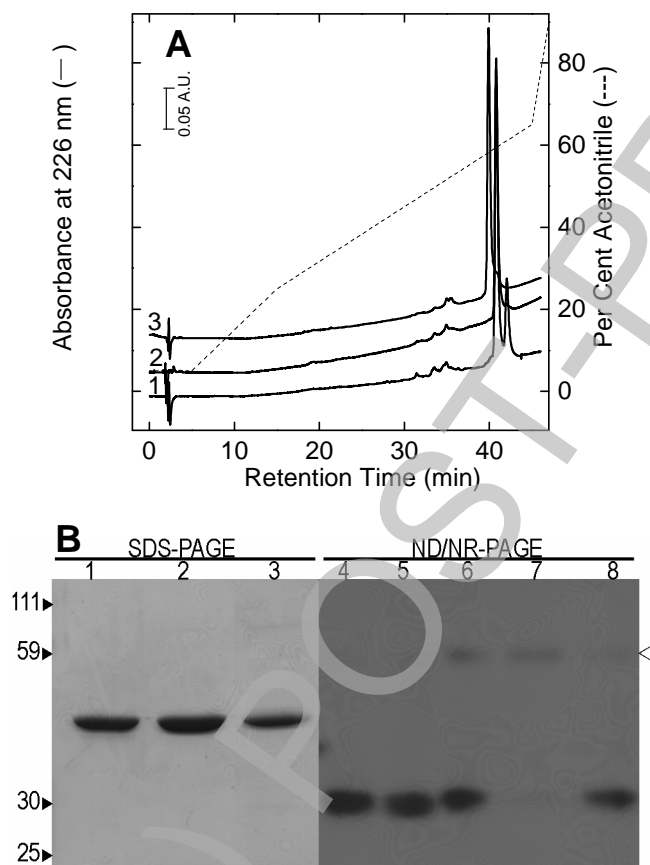
Figure 1, Valle et al., 2008

Figure 2, Valle et al., 2008

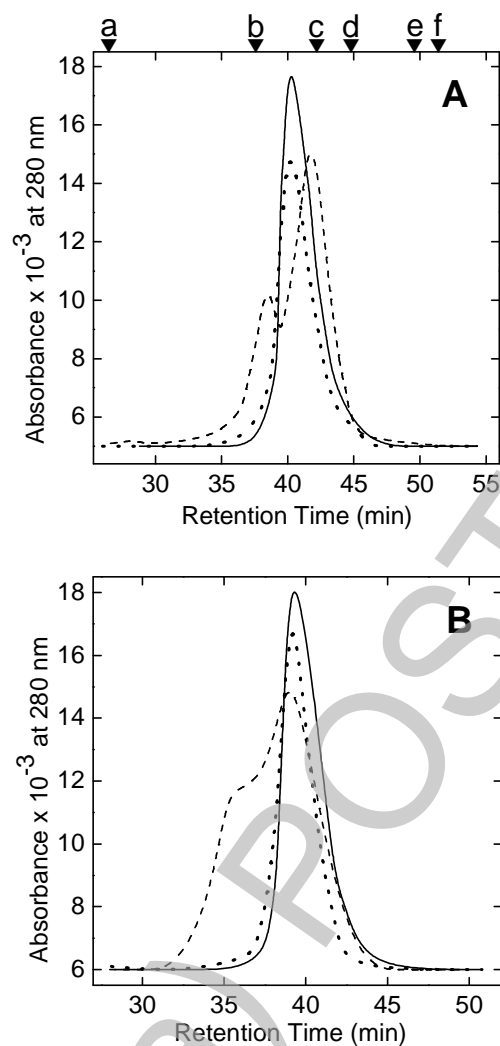


Figure 3, Valle et al., 2008

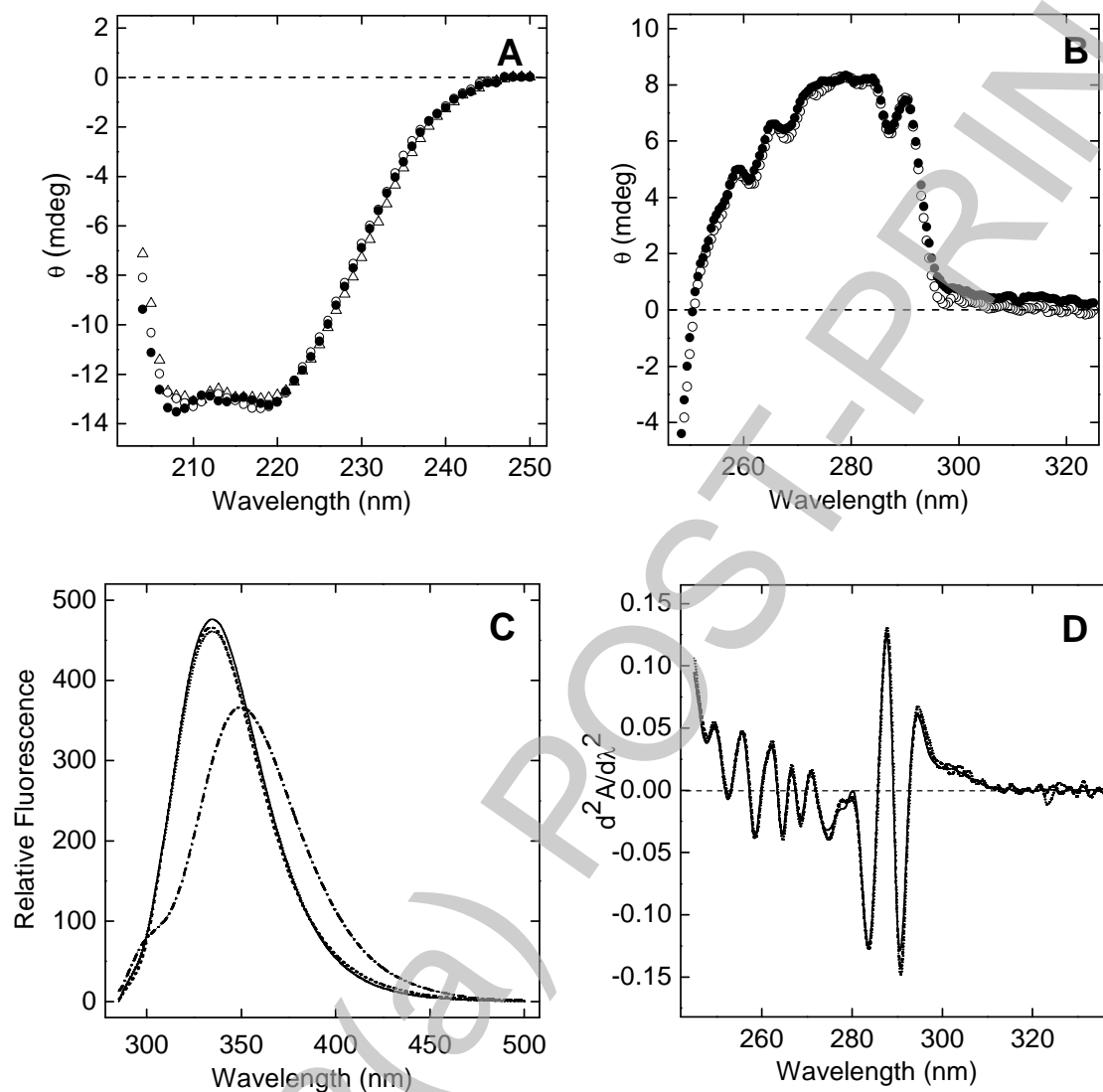


Figure 4, Valle et al., 2008

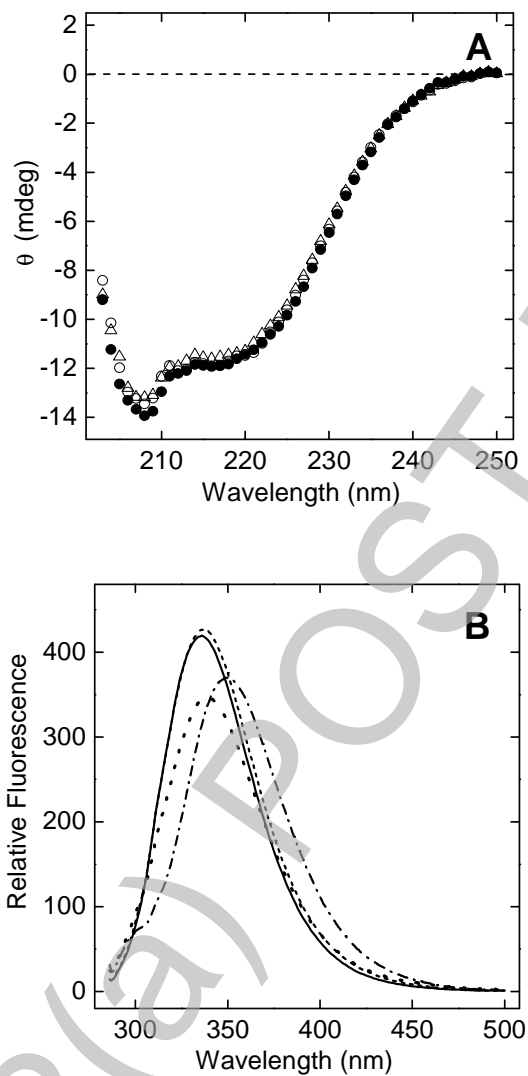


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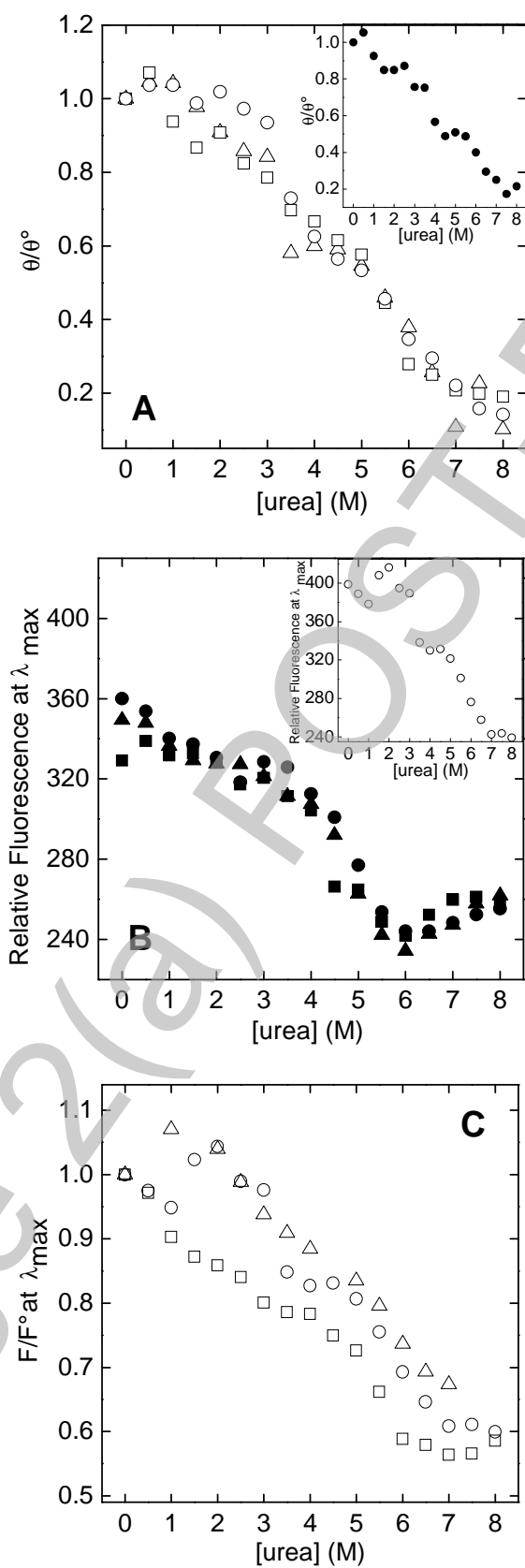
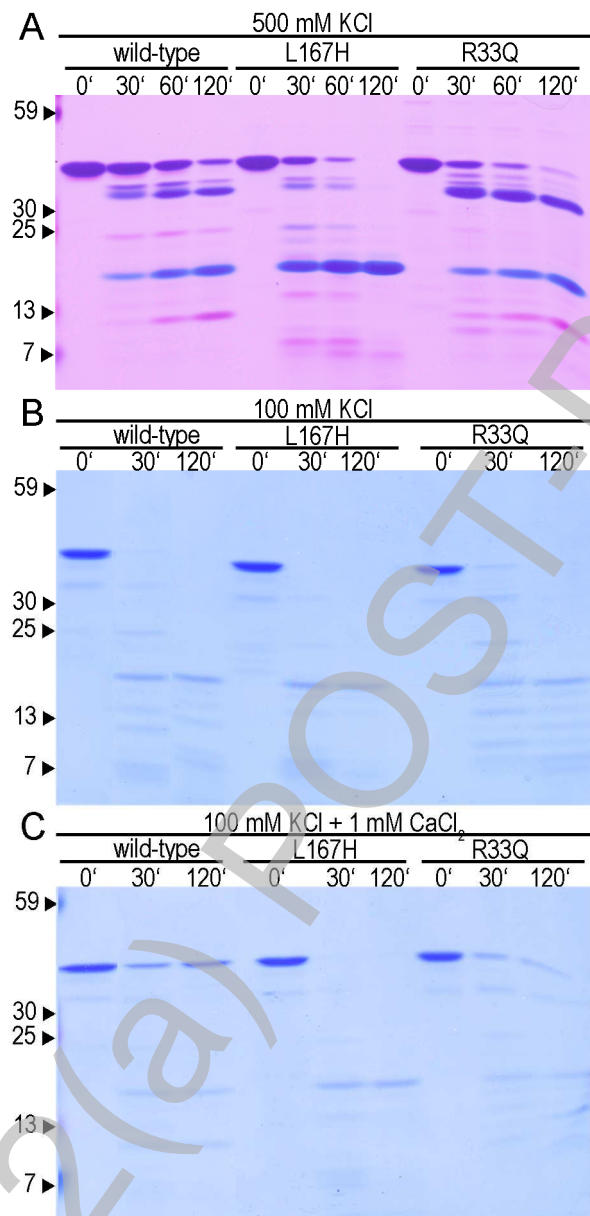


Figure 6, Valle et al., 2008



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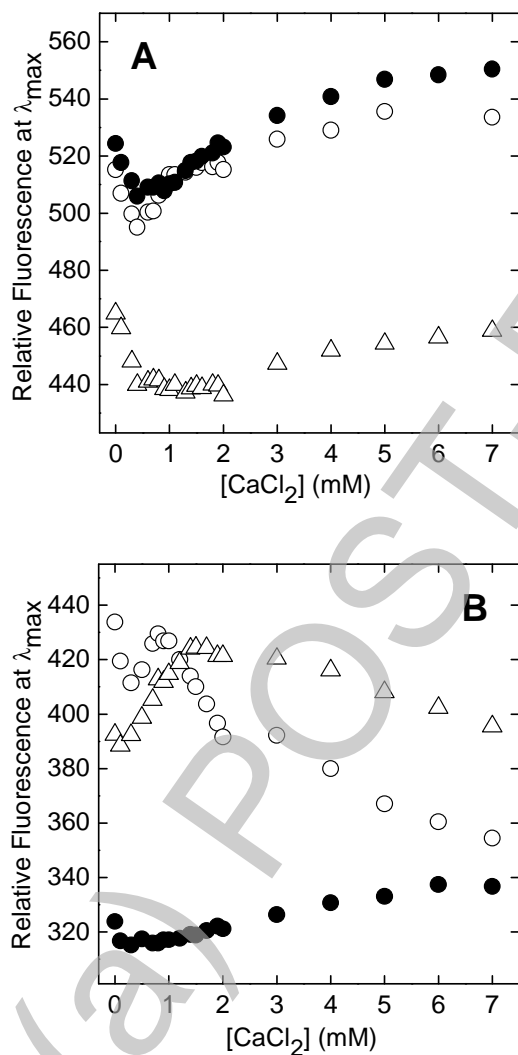


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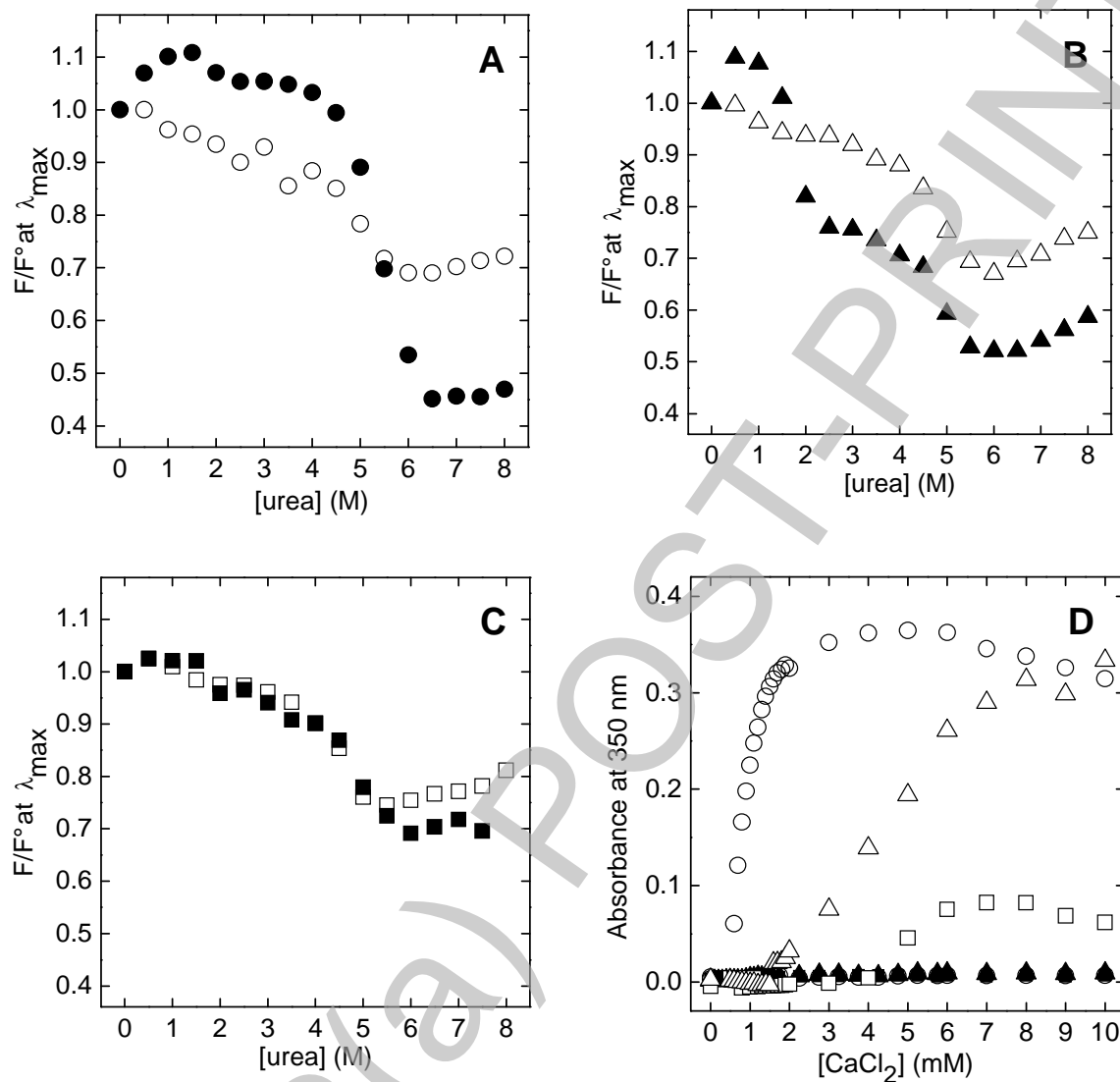


Figure 9, Valle et al., 2008

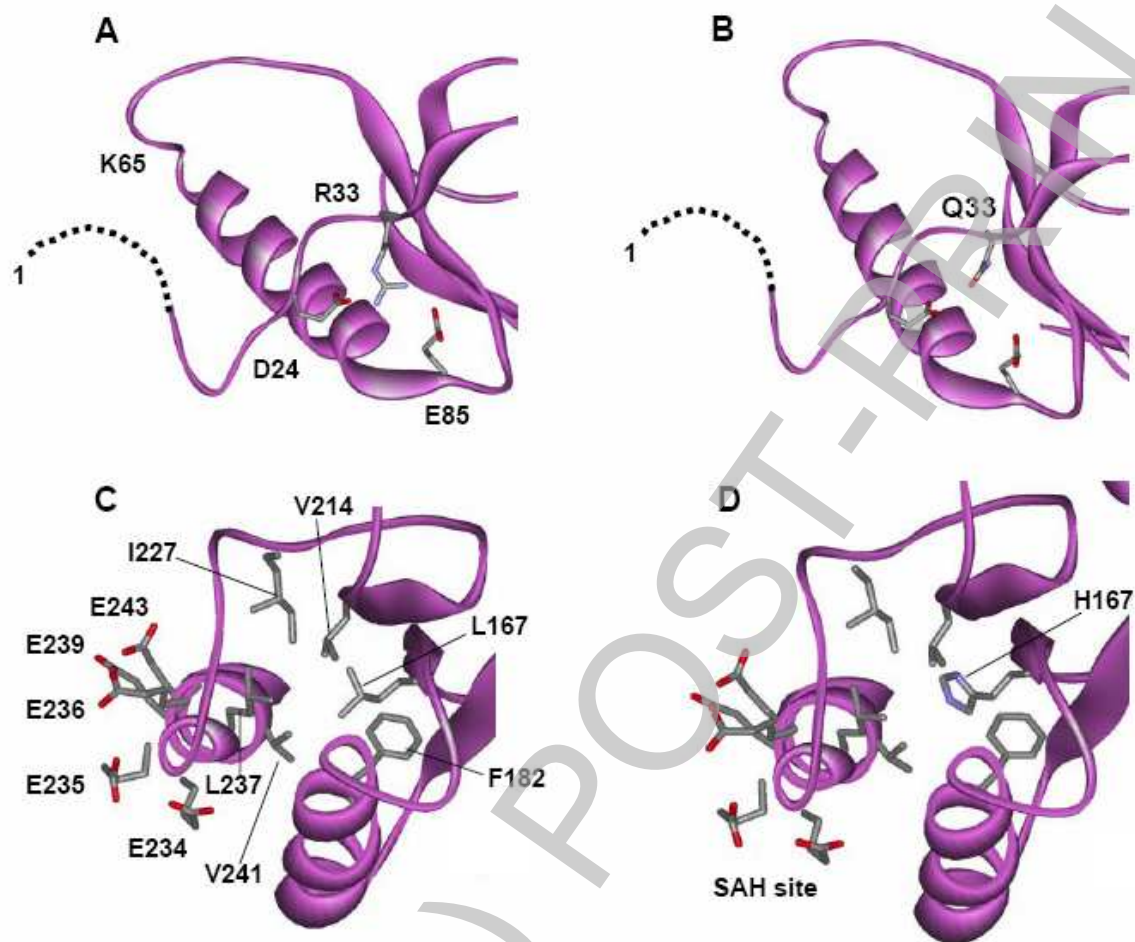
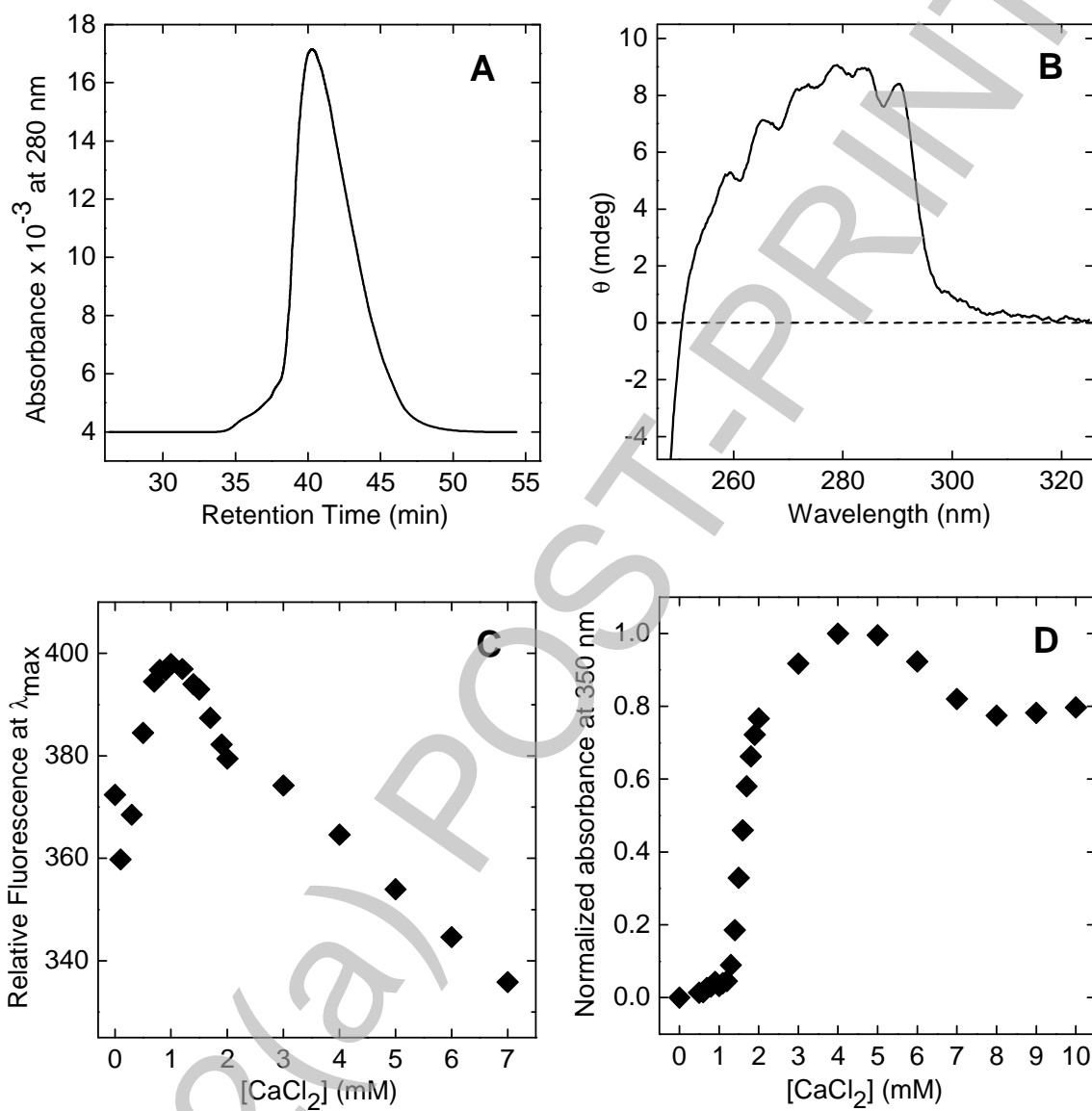


Figure 10, Valle et al., 2008



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