# Effect of CASQ1 Protein Sequence Variants on Ca2+ Binding Ability

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***Abstract:*** Malignant hyperthermia (MH) is generally observed in people susceptible to volatile anesthetics, usually due to abnormalities in ryanodine receptor 1 (RYR1) leading to $Ca^{2+}$ dysregulation in the sarcoplasmic reticulum (SR). CASQ1, a $Ca^{2+}$ binding protein believed to be in the SR, also plays a regulatory role in RYR1. It has been hypothesized that mutations in CASQ1 protein may increase susceptibility to MH cases. We used the CASQ1 p.F186Y and p.I138T variants to compare $Ca^{2+}$ binding ability with CASQ1 WT. It showed that CASQ1 F and I variants have reduced $Ca^{2+}$ binding ability compared to CASQ1 WT.

***Keywords:*** Microscale Thermophoresis; Ni-NTA Chromatography

## 1. Introduction

Malignant hyperthermia (MH) is a skeletal muscle-related genetic disorder. MH is known to be associated with uncontrolled elevation of sarcoplasmic $Ca^{2+}$, usually manifested by abnormalities in ryanodine receptor 1 (RYR1), uncontrolled regulation of $Ca^{2+}$ by the sarcoplasmic reticulum (SR) in skeletal muscle, resulting in elevated sarcoplasmic $Ca^{2+}$, massive ATP depletion harmful to muscle membranes, and worse, life-threatening rhabdomyolysis and hyperkalemia [1].

20–30% of MH patients still do not have the RYR1 mutation, despite the fact that this gene is linked to a significant share of MH patients. Calsequestrin-1 (CASQ1), on the other hand, is an acidic protein with a molecular weight of roughly 45 kDa that is found in the SR, and its possession of glutamate and aspartate residues confers the ability to bind $Ca^{2+}$ [2]. In fact, it has been demonstrated in further studies that CASQ1 inhibits the activity of RYR1 in the presence of junctin and triadin proteins at a low $Ca^{2+}$ concentration of 20 μM [3]. Therefore, CASQ1 becomes one of the possible genes that play a role in MH.

Therefore, our aim in this experiment was to compare the CASQ1 p.F186Y and p.I138T variants with CASQ1 WT, and compare the binding ability of the variants with $Ca^{2+}$ by microscale thermophoresis. Thus, the mechanism of the interaction between CASQ1 protein and MH can be better understood.

## 2. Methods

## 2.1 Induced expression of CASQ1 proteins

Small-scale expression: The single colony was picked and inoculated in 2 ml of LB media containing ampicillin at 100 μg/ml. This culture was placed in an orbital incubator (Max Q 6000) and incubated overnight at 37°C with shaking at 220 rpm. The next day, 200 μl of culture was added to LB media and incubated for 2.5 hours. Added 400 μl of autoclaved glycerol 50% (v/v), snap-freeze in liquid nitrogen, and stored at -80°C.

Large-scale expression: A stock was inoculated in 4 ml of LB medium and incubated overnight at 37 °C with shaking. The next day, the overnight culture was poured into 400 ml of 2YT medium and the culture was incubated at 37 °C with shaking until the optical density reached 0.6-0.8 at 600 nm (OD600). IPTG was added to a final concentration of 0.4 mM, then the remaining culture was incubated overnight at 18 °C. The next day, the culture was transferred to a centrifuge bottle, weight equilibrated, and centrifuged at 4000g for 30 min at 5 °C. The supernatant was discarded and the cell pellet was stored at -20 °C.

## 2.2 Nickel affinity chromatography

AKTA Pure FPLC system (GE Healthcare) and HisTrap HP 1 ml column (GE Healthcare) were applied in Ni-NTA chromatography. The AKTA system and column were pre-equilibrated with the low salt His-A buffer (0.02M Na $H_2$ phosphate pH8.0; 0.5M NaCl; 10mM imidazole). The soluble fraction was loaded onto a column using a peristaltic pump, off-line at a rate of 1 ml/min. Next, the column was washed with resuspension buffer. Wash the column with low salt His-A buffer and high salt His-B buffer (0.02 M Na $H_2$ phosphate pH 8.0; 0.5 M NaCl; 0.5 M imidazole), and then again with His-A buffer. Bound proteins were eluted with increasing gradients of imidazole concentration, and the elution was recorded by absorbance at 280 nm. The fractions showing a peak of absorbance at 280 nm were analyzed by SDS-PAGE.

## 2.3 Microscale thermophoresis

The purified protein was incubated with 3X molar excess of fluorescein isothiocyanate (FITC) on ice for 1 h. The excess FITC was removed with a NAP-5 column, and the labelled protein was eluted into A buffer (50 mM Tris pH 7.5, 150 mM NaCl), then the proteins were aspirated with NT.115 standard treated capillaries and scanned with green LED at 20% power and fluorescence values were recorded. Diluted B buffer (50 mM Tris pH7.5, 150 mM NaCl, 100 mM $CaCl_2$) in A buffer and mixed 1:1 with labelled protein and incubated for 10 min at room temperature. Protein samples were mixed 1:1 with A buffer as a control. MST was performed at MST 20% power and repeated 5 times.Graphs were made using GraphPad Prism.

# 3. Result

## 3.1 Ni-NTA Chromatography Purification of CASQ1 WT and variant proteins

To study $Ca^{2+}$ binding ability, the CASQ1 protein was purified. The absorbance at 280 nm was monitored. The sample applied to the column caused an increase in the absorbance (Figure 1a、c、e). Selected fractions within the elution peaks were subjected to SDS-PAGE (Figure 1b、d、f).

In Figures 1a, c and e, we can see the presence of a very distinct blue peak in the range of 10-20 ml, representing the elution of CASQ1 protein. Subsequently, 9 elution fractions of CASQ1 WT (Figure 1b) were subjected to SDS-PAGE experiments, among which E4-E7 had bands apparently at the same position, and E5 had the widest and deepest band, associated with a molecular weight between 50 kDa and 60 kDa, which has been marked with a blue arrow. CASQ1 I (Figure 1d), E31 and E32 have the widest and deepest bands. CASQ1 F (Figure 1f), E14 and E15 have the widest and deepest bands.

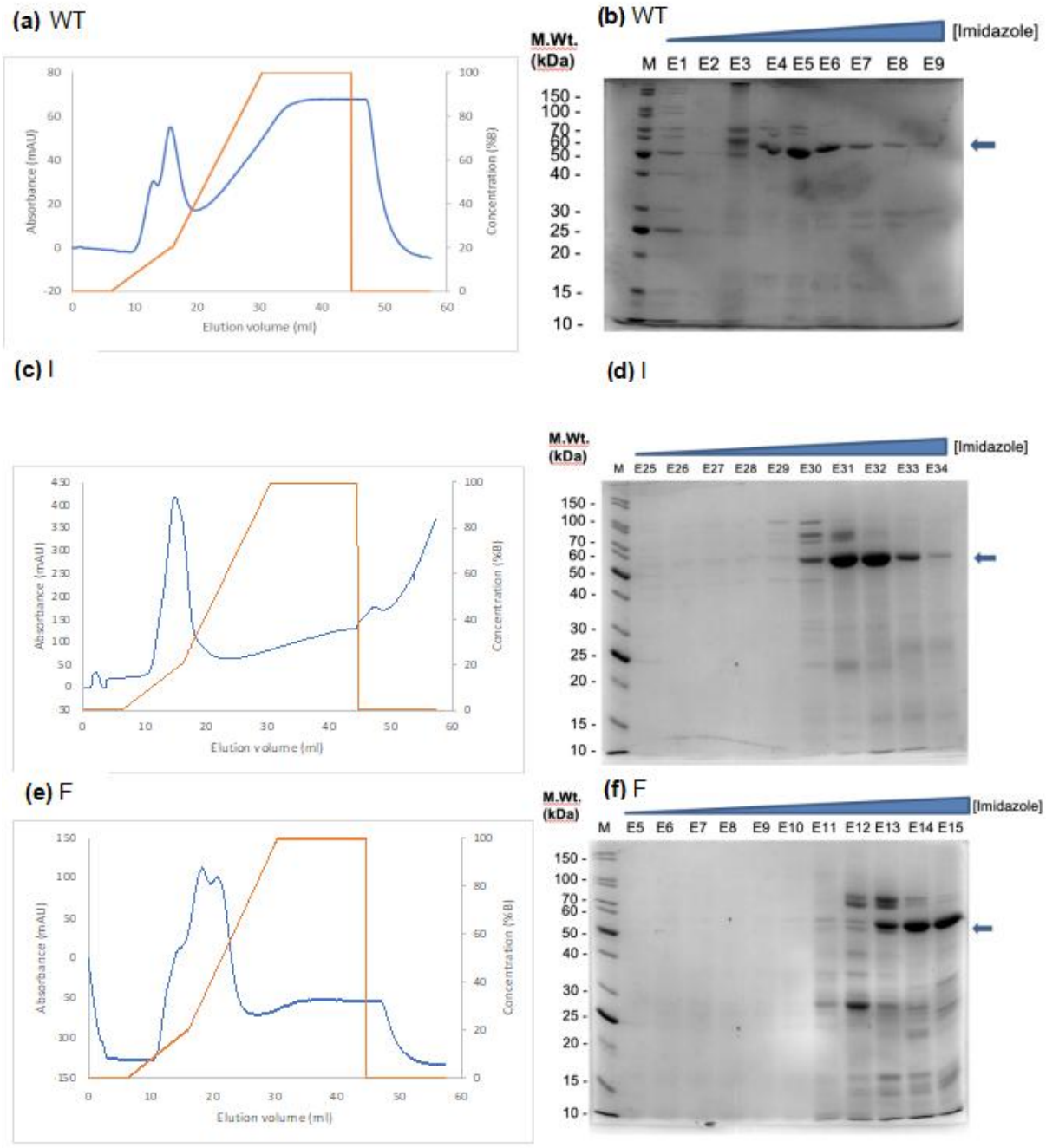
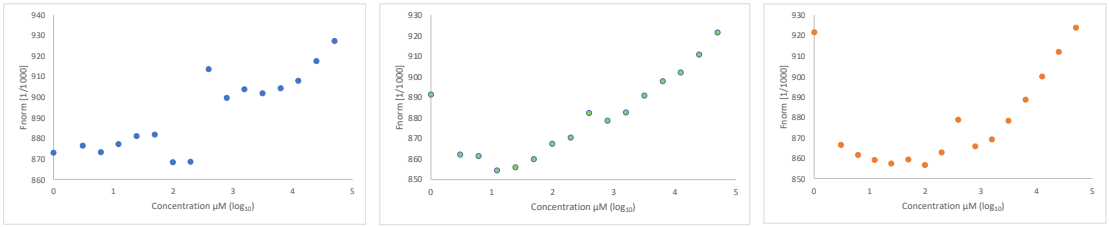



**Figure 1: Ni-NTA chromatogram and SDS-PAGE analysis of CASQ1 proteins.**

The absorbance at 280 nm (blue line) of eluted CASQ1 WT (a), CASQ1 I (c) and CASQ1 F (e) was measured in milli absorbance units (mAU). The concentration of elution buffer is shown by an orange line. The horizontal coordinate is the value of elution volume in ml. CASQ1 WT、I、F (Figure 1b、 d、f) proteins were subjected to SDS-PAGE. Lane 1 (M) indicates the unstained protein standard (NEB), and the molecular weight is noted on the leftmost side in kDa. The remaining lanes show the fraction of peak absorbance at 280 nm. The blue arrow represents the band matching the CASQ1 protein, blue triangle means the increase of imidazole concentration.

## 3.2 Microscale thermophoresis of CASQ1 WT and variant proteins

We investigated the $Ca^{2+}$ binding ability of CASQ1 protein using microscale thermophoresis. Firstly, we labeled CASQ1 WT protein with FITC at a ratio of 1:3 with a series of $Ca^{2+}$ concentrations. Because the higher the power of MST, the more the density and convective flow of the sample are affected, which may produce abnormal MST traces [4]. Therefore, we chose 20% power for MST experiments on CASQ1 proteins (shown in Figure 2a、b、c). However, when the $Ca^{2+}$ concentration was at 0.1-0.4 mM, the fluorescence signal seems to be abnormal, and this phenomenon occurs in several replications, which cannot be excluded as the cause of the machine, so we did not analyze this concentration range. The fluorescence signal of CASQ1 WT protein showed an increasing tendency between $Ca^{2+}$ concentrations of 3 mM-50 mM when the MST power was 20%. The fluorescence signal changes of CASQ1 I and F were similar to CASQ1 WT, both with an upward trend.




**(a)** **(b)** **(c)**

**Figure 2: MST of CASQ1 WT (a)、I (b) and F (c) at 20% MST power.**

Blue dots represent the fluorescence signal of CASQ1 WT at different Ca2+ concentrations converted to log10 in μM; green dots stand for CASQ1 I protein; orange dots indicate CASQ1 F protein. The Fnorm are shown in 1/1000 of the value.

In order to investigate the affinity of CASQ1 protein to Ca2+ binding, we performed 5 replicate MST and then calculated the average of dissociation constant (shown in Table 1). From the average of the dissociation constant, CASQ1 F protein has the highest value and CASQ1 WT protein has the lowest value. Therefore, we concluded that the CASQ1 WT protein has the highest affinity for binding Ca2+, followed by CASQ1 I. CASQ1 F has the lowest affinity.

**Table 1: MST data summary table.**

| | CASQ1 WT | CASQ1 I | CASQ1 F |
|---|---|---|---|
| Replicate 1 | 1890 | not fitted | not fitted |
| Replicate 2 | not fitted | not fitted | 2720 |
| Replicate 3 | not fitted | 1370 | 2370 |
| Replicate 4 | 563 | 4720 | not fitted |
| Replicate 5 | 1910 | 3400 | 7670 |
| Average | 1454.3 | 3163.3 | 4253.3 |

CASQ1 WT, I and F proteins are summarized in MST replicate experiments of the dissociation constant, with the average calculated, and nonsignificant values have been labelled as not fitted.

# 4. Discussion

The CASQ1 proteins after IPTG induction and centrifugation were purified by Ni-NTA chromatography (shown in Figure 1). In the Nickel affinity chromatogram, we could observe the presence of single or double peaks for each protein, corresponding to molecular weights between 50 kDa- 60 kDa, which may represent our expected CASQ1 protein. The expected molecular weight (MW) of the CASQ1 protein was around 45 kDa, but the results were not as expected, probably because the acidic residues in the CASQ1 protein repel the negative charges contained in SDS, which affects the normal

electrophoretic migration [5]. Subsequently, we analyzed the $Ca^{2+}$ binding ability of CASQ1 proteins using MST instrument. However, both CASQ1 I and F proteins appear to have reduced $Ca^{2+}$ binding ability compared to CASQ1 WT. In the study by Kumar et al[6], $Ca^{2+}$ binding sites in CASQ1 WT proteins have been identified with different affinities. There are also some $Ca^{2+}$-dependent high-affinity sites, and these sites show higher affinity with increasing $Ca^{2+}$ concentration. Therefore, the reduced $Ca^{2+}$ binding capacity of CASQ1 I and F variant proteins may be due to altered $Ca^{2+}$-dependent high-affinity sites. The conclusion that the binding capacity of CASQ1 I and F variant proteins to $Ca^{2+}$ were reduced in our experiments is consistent with previous reports [7], but no conclusions were drawn about the strength of the affinity between CASQ1 I and F variant in Dodds' experiments, while she performed only twice MST experiments, and the data analysis was based on average values. The number of experimental repetitions was too few and the data support for the conclusion was weak. Although we still need to further explore the effects of CASQ1 I and CASQ1 F variants in terms of $Ca^{2+}$ release at a later stage, it is reasonable to speculate that the reduced ability of the CASQ1 variant protein to polymerize has an effect on RYR1 and may lead to $Ca^{2+}$ dysregulation as a potential aetiology of MH.

# References


[1] Kim, D.-C. Malignant hyperthermia. Korean J Anesthesiol 63, 391–401 (2012).

[2] MacLennan, D. H. & Wong, P. T. S. Isolation of a Calcium-Sequestering Protein from Sarcoplasmic Reticulum. Proc Natl Acad Sci U S A 68, 1231–1235 (1971).

[3] Györke, I., Hester, N., Jones, L. R. & Györke, S. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. Biophys J 86, 2121–2128 (2004).

[4] Rainard, J. M., Pandarakalam, G. C. & McElroy, S. P. Using Microscale Thermophoresis to Characterize Hits from High-Throughput Screening: A European Lead Factory Perspective. SLAS Discov 23, 225–241 (2018).

[5] Graceffa, P., Jancsó, A. & Mabuchi, K. Modification of acidic residues normalizes sodium dodecyl sulfate-polyacrylamide gel electrophoresis of caldesmon and other proteins that migrate anomalously. Arch Biochem Biophys 297, 46–51 (1992).

[6] Kumar, A. et al. Identification of calcium binding sites on calsequestrin 1 and their implications for polymerization. Mol Biosyst 9, 1949–1957 (2013).

[7] Dodds, R. E. Defining molecular mechanisms of calcium dysregulation in malignant hyperthermia susceptibility. (University of Leeds, 2019).