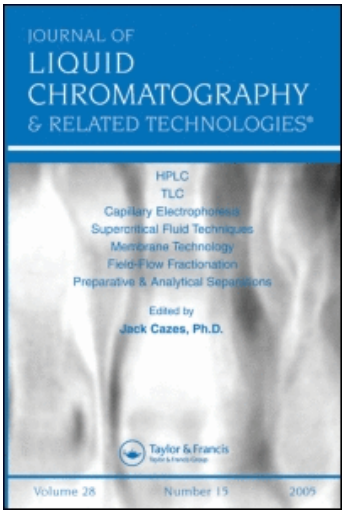
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Study of Calsequestrin Aggregation by Flow Field-Flow Fractionation with Light Scattering Detection

Susan E. Shadle[a]; Randy Rostock[a]; Lou Bonfrisco[a]; Martin E. Schimpf[a]
[a] Department of Chemistry, Boise State University, Boise, Idaho, USA

To cite this Article Shadle, Susan E. , Rostock, Randy , Bonfrisco, Lou and Schimpf, Martin E.(2007) 'Study of Calsequestrin Aggregation by Flow Field-Flow Fractionation with Light Scattering Detection', Journal of Liquid Chromatography & Related Technologies, 30: 9, 1513 — 1523
To link to this Article: DOI: 10.1080/10826070701277364
URL: http://dx.doi.org/10.1080/10826070701277364

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Liquid Chromatography & Related Technologies®, 30: 1513–1523, 2007
Copyright © Taylor & Francis Group, LLC
ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701277364

Study of Calsequestrin Aggregation by Flow Field-Flow Fractionation with Light Scattering Detection

Susan E. Shadle, Randy Rostock, Lou Bonfrisco, and Martin E. Schimpf
Department of Chemistry, Boise State University, Boise, Idaho, USA

Downloaded At: 17:43 23 January 2011

Abstract: Flow field-flow fractionation (FIFFF) with multi-angle light scattering detection indicates that calsequestrin forms even numbered aggregates, supporting the view that this Ca^{2+} binding protein aggregates through the interaction of dimers. Contrary to previous reports based on size exclusion chromatography, FlFFF further indicates that the dimer is the stable species, with very little monomer present under the conditions analyzed in this study. Increasing the concentration of K^{+} (100–700 mM) causes the dimer to be the increasingly dominant species over monomer, tetramer, and other aggregate species. Increasing the concentration of Ca^{2+} (3–10 mM) causes increased aggregation of dimers into higher order species. Finally, addition of small amounts of the anthracycline analog trifluoperazine (0.10–0.50 mM), which is known to disrupt calsequestrin function, induces severe aggregation.

Keywords: Calsequestrin aggregation, Field-flow fractionation, Light scattering detection

BACKGROUND

Calsequestrin (CSQ) is a protein that is present at high concentrations (~100 mg/mL) in the sarcoplasmic reticulum. Its function in both the sequestration and regulated release of Ca^{2+} from the sarcoplasmic reticulum is believed to be important for healthy cardiac function.[1–8] CSQ binds Ca^{2+} with moderate affinity (K_d ~1 mM)[9] and high capacity (~20–80 Ca^{2+}/CSQ

Address correspondence to Martin E. Schimpf, Department of Chemistry, Boise State University, Boise, ID 83725, USA. E-mail: mschimpf@boisestate.edu

molecule).[9–12] The high capacity Ca^{2+} binding is believed to be coupled to CSQ aggregation.[12–15] As a divalent cation, Ca^{2+} is thought to facilitate aggregation by bridging between monomers of the protein. Crystallography of CSQ shows that the protein forms linear polymers,[12,14] which are proposed to form the structural basis for CSQ aggregation, and for the organized linear structures attributed to CSQ *in vivo*.[16,17]

Studies of CSQ aggregation using size exclusion chromatography (SEC) have shown that, in response to increased Ca^{2+}, CSQ monomers first form dimers, then tetramers, then higher order aggregates.[18] The dimerization is proposed to occur *via* a "front-to-front" interface between monomers observed in the crystal structure of CSQ. The formation of tetramers is proposed to involve the dimerization of dimers through the "back-to-back" interface observed in the crystal structure.[18] In addition, the aggregation of CSQ is inhibited in the presence of increased K^+ ion concentration.[18]

Small molecules, such as trifluoperazine (TFP) and anthracyclines, are known to bind to CSQ with micromolar dissociation constants.[19,20] Although, these binding interactions may be related to cardiotoxic side effects of these drugs, their effect on CSQ aggregation and function is not understood. At relatively high concentrations (1 mM), TFP has been shown to inhibit the binding of Ca^{2+} to CSQ by $\sim$60%,[19] as well to decrease the degree of CSQ precipitation.[13] While decreased precipitation suggests that TFP inhibits CSQ aggregation in solution, no studies have specifically examined the direct effect.

In this work, we utilize flow field-flow fractionation (FlFFF) coupled with multi-angle light scattering (MALS) to study CSQ aggregation in various environments. Like SEC, FlFFF separates macromolecules according to their hydrodynamic size, but FlFFF has certain advantages over SEC for the study of protein aggregation. In both techniques, care must be taken to minimize the interaction of protein with surfaces inside the separation conduit. Such interactions, at best, decrease the separation efficiency; and in severe cases, irreversibly adsorbed material is lost, and consequently, becomes unaccounted for in the analysis. Interactions become more problematic as the size of the protein (or other macromolecule) increases, because multiple contact points that lead to prolonged or irreversible interactions occur more frequently. If ignored, such artifacts in the separation process will lead to incorrect conclusions about the makeup and behavior of the sample being analyzed. With charged macromolecules, repulsive interactions will also lead to deterioration in the separation efficiency. Therefore, it is important to use an appropriate ionic strength when analyzing such materials, in order to minimize charge repulsion without altering the native structure of the macromolecule or promoting hydrophobic interactions with the separation conduit.

Unlike SEC, which is packed with micron sized particles, the open FlFFF channel presents a minimal amount of surface area to the sample. Certainly, deleterious sample interactions can occur if experimental conditions are not optimized.[21,22] However, the force field that induces separation in FlFFF

Downloaded At: 17:43 23 January 2011

can be tuned to minimize sample interactions. The combination of low surface area and the capability for optimization allows a much wider range of solvent conditions to be utilized in FlFFF compared to SEC.[23] Furthermore, the open channel provides a gentle environment for the sample; the absence of significant shear allows for fragile macromolecules and their aggregates to be separated without degredation.[24] Consequently, FlFFF is a valuable tool for characterizing the hydrodynamic size of proteins, or for separating them for further characterization by other techniques, particularly when the effect of solvent conditions is under investigation, or when fragile or aggregated proteins are involved.[25–27] In a recent comparison of techniques,[28] both FlFFF and analytical ultracentrifugation (AUC) detected markedly higher levels of aggregation than SEC. Furthermore, SEC failed to detect many of the soluble higher order aggregates at all, and suffered from significant sample loss. Liu and coworkers[29] also found FlFFF to be superior to SEC and AUC for analyzing shear sensitive proteins.

In the work reported here, we use FlFFF with MALS detection to re-examine the aggregation of CSQ in the presence of K^+ and Ca^{2+}, and compare the results with those obtained using SEC. We also use FlFFF-MALS to study the effect of TFP on CSQ aggregation.

Downloaded At: 17:43 23 January 2011

EXPERIMENTAL

Expression and Purification of CSQ

Canine cardiac CSQ was overexpressed using *Escherichia coli* as an expression vector, then purified using previously published procedures.[30] Following the purification, the CSQ sample was dialyzed for 3 days at 4°C against 0.1 mM EGTA, with exchanges of the dialate twice daily. The purified CSQ was exchanged into 10 mM TRIS buffer (pH 7.2) containing 100 mM KCl, using an Amicon Ultrafiltration 8050 stirred cell (Millipore Corporation, Bedford, MA). The final concentration was determined by UV spectroscopy to be 1.4 mg/mL. Samples were stored in 1 mL aliquots at 4°C. Prior to analysis, the desired ionic composition of an individual CSQ aliquot was obtained through the addition of a standard buffer solution containing the ions of interest in concentrated form.

Protein Separation

Protein aggregates were fractionated by asymmetric flow field-flow fractionation using an Eclipse F separation system from Wyatt Technology (Santa Barbara, CA). The trapezoidal channel has a length of 26.5 cm and a thickness of 350 μm. The accumulation wall membrane is made of polyethersulfone, with a molecular weight cutoff of 10,000 Da. Prior to analysis, all

samples were filtered using a syringe filter containing a 0.2 μm polyethersulfone membrane (Nalge Nunc International, Rochester, NY).

All carrier liquids contained 10 mM TRIS buffer (pH 7.2) and 0.02% NaN_3 in 18 mΩ-cm water. The K^+ concentration was varied between 100–500 mM through the addition of KCl, and the Ca^{2+} concentration was varied between 0–10 mM through the addition of $CaCl_2$. The TFP concentration was varied between 0–0.50 mM. After preparation, carrier liquids were filtered through 0.02 μm Anodisc filters (Whatman International, Maidstone, England). All vessels were acid stripped using 6 N HNO_3. An inline filter, type GTTP 0.2 μm from Millipore Corp. (Billerica, MA) was placed between the pump and channel to eliminate particles originating from the pump from entering the channel. Samples were loaded using a single 100 μL injection, and focused by opposing flow to a band approximately 6 mm downstream from the injection port before elution. The focus time was 5 minutes, unless otherwise noted. The outlet flow was maintained at 1 mL/min, while the cross flow was varied according to the following program: 2 mL/min for 20 minutes, then decayed linearly to 0 mL/min over 5 minutes. Under these flow conditions, the void time of the channel is 40 seconds.

When not in use, the channel was continually flushed with 0.02% NaN_3 at a flow rate of 0.2 mL/min. Before each data collection period, the desired buffer was pumped through the system at flow rate of 0.5–1.0 mL/min for 20–30 minutes while the detector signals were allowed to stabilize.

Bovine serum albumin (BSA), obtained from Sigma-Aldrich, was analyzed daily to monitor the integrity of the channel. When the peak shape became noticeably asymmetric, or the resolution of monomer and dimer had noticeably deteriorated, the channel was rinsed with a 20% propanol-water mixture. When the rinsing procedure failed to return the expected separation efficiency, the membrane was replaced.

Downloaded At: 17:43 23 January 2011

Sample Detection and Characterization by Light Scattering

As the fractionated sample elutes from the FlFFF channel, it passes first through a DAWN EOS multi-angle light scattering (MALS) detector, followed by an Optilab DSP detector, both manufactured by Wyatt Technology (Santa Barbara, CA). Weight average molecular weight was calculated by ASTRA V analysis software from Wyatt Technology, using the Zimm model and a first order fit of the resulting Debye plots.

RESULTS AND DISCUSSION

Preliminary experiments were designed to optimize the sample load, focus time, and flow rate conditions to be used with the various carrier liquids. Figure 1 illustrates the elution profiles obtained in 3 mM Ca^{2+} and 500 mM

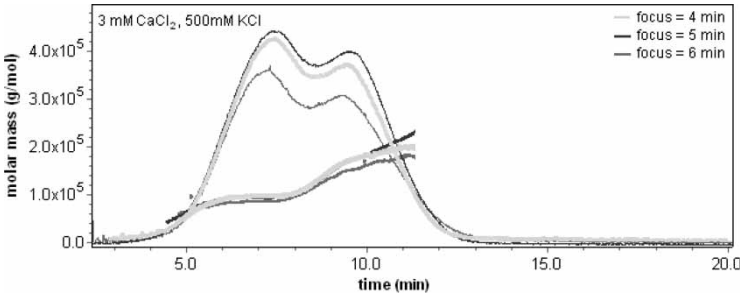


Figure 1. FlFFF-MALS analysis of CSQ in the presence of 500 mM K^+ and 3 mM Ca^{2+}, obtained using various focus times. The elution profiles were recorded by light scattered by the protein at an angle of ninety degrees relative to the incoming light beam. The sample load was 144 μg, except when focus time was 6 minutes, where the sample load was 108 μg.

Downloaded At: 17:43 23 January 2011

K^+, using focus times between 4 and 6 minutes, as recorded by the ninety degree light scattering detector. Also displayed in Figure 1 are the molar masses calculated from the MALS and dRI signals of the eluting protein fractions. The molar mass data for the different elution profiles consistently indicates two primary species with molar masses of 90,000 Da and 180,000 Da. Since the molar mass of the CSQ monomer (lacking its signal sequence)[30] is 45.3 kDa, these two species are likely the dimer and tetramer, respectively. From the trend in the molar mass plot, it appears that there may also be a small amount of monomer that elutes before 5 minutes, although deterioration of the signal makes it difficult to say with certainty.

The goal of varying the focus time is to find a range of focus times in which the elution profiles are unchanged, thereby indicating that the focusing procedure is not significantly affecting the analysis. Figure 1 illustrates that this goal was reached. The small amount of material that elutes between 2 and 5 minutes when the focus time is 4 minutes indicates incomplete focusing. We note that the smaller peak area obtained with a 6-minute focus time is due to a sample load, which was decreased from 144 μg to 108 μg by dilution of the original protein solution. Recoveries, calculated from the peak area recorded by the differential refractive index (dRI) detector, varied in no systematic way between 96 and 111%.

Based on our evaluation of elution profiles obtained with various flow rates, sample loads, and focus times, we chose the flow program outlined above, along with a focus time of 5 minutes and a sample load of 144 μg for all remaining data displayed in this report. However, it should be noted that optimum settings, particularly the cross flow rate, change with the amount of protein aggregation, and consequently, the ionic makeup of the carrier liquid. However, effects of the ionic conditions on protein aggregation

are more directly observed by comparing elution profiles obtained under a single set of conditions.

Figure 2 compares the elution profiles recorded by the dRI detector when CSQ is dissolved and analyzed in environments with increasing K^+ concentration. Displaying the dRI signal, rather than the light scattering signal, is better for visually comparing the relative amounts of the different species because the dRI signal is linear with concentration, whereas the light scattering response increases with molar mass, as well as sample concentration.

The data in Figure 2 indicates an increasing tendency toward the formation of dimers as the concentration of K^+ is increased in the absence of Ca^{2+}. At both 300 and 500 mM K^+, there is a clear indication that significant amounts of monomer exist, although the resolution of monomer and dimer is better at the lower K^+ concentration. Furthermore, while a significant amount of tetramer exists at all concentrations, the amount decreases with increasing K^+ concentration.

Kang et al.[18] also reported a decrease in CSQ aggregation with increasing concentrations of K^+. However, their analysis by SEC-MALS indicates that monomer is the dominant species in the absence of Ca^{2+}. Our initial reaction to this disagreement was that one or both of two factors are responsible–either the aggregates are not robust enough to withstand the sheer forces that exist in an SEC column, or field induced concentration of the sample at the channel wall is causing sample aggregation. However, further analysis by FlFFF using a range of flow rates and focus times indicates that in this case, at least, any aggregation induced by the FlFFF experiment is minimal. For further confirmation of the FlFFF result, the same CSQ sample in 500 mM KCl was analyzed by analytical ultracentrifugation (AUC).[31] The AUC analysis (data not shown) confirmed the presence of three species having distinct sedimentation coefficients, with the intermediate sized species being present in the greatest amount. Based on these results, and the recent report by Gabrielson, et al.[28] that SEC fails to identify aggregates

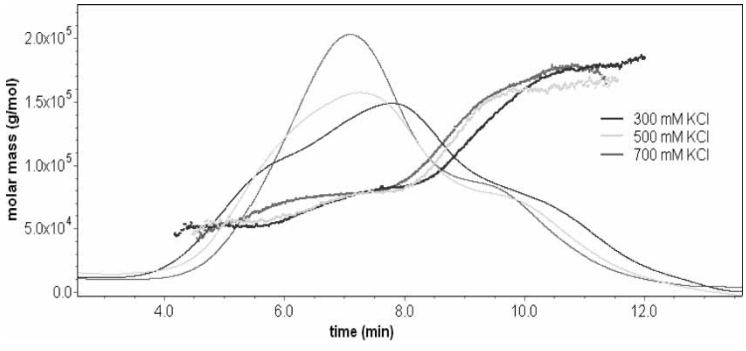


Figure 2. FlFFF-MALS analysis of CSQ in the presence of varying amounts of K^+. The elution profiles were recorded by the dRI detector.

Downloaded At: 17:43 23 January 2011

seen by both FlFFF and AUC, we are confident that dimers and tetramers of CSQ exist in significant amounts, even in the absence of Ca^{2+}. Furthermore, our results lend further support to the proposed mechanism[18] that CSQ aggregates by first forming dimers through front-to-front interactions, followed by the back-to-back interaction of dimers to produce tetramers and larger aggregates. Such a mechanism precludes the formation of trimers and other odd numbered aggregates.

In the next set of experiments, we held various concentrations of K^+ constant, while increasing the concentration of Ca^{2+}. The trends were similar, whether the K^+ concentration was 300, 500, or 700 mM. Figure 3 displays the elution profiles with a fixed K^+ concentration of 500 mM and Ca^{2+} concentrations of 0, 3, and 10 mM. As the Ca^{2+} concentration is increased from 0 to 3 mM, the protein undergoes a subtle shift, with less monomer and more dimer and tetramer. Small amounts of hexamer are also apparent. The elution of these higher order aggregates leads to a divergence in the molar mass profiles above 9 minutes because of insufficient resolution by the FlFFF channel. As the concentration of Ca^{2+} is further increased, the trend continues, and very large aggregates begin to form. Sample loss also becomes a factor, as indicated by a 40% reduction in the area of the peaks that elute within the experimental run time. Similar trends were observed at K^+ concentrations of 300 mM and 700 mM.

In the final set of experiments, we held the K^+ and Ca^{2+} concentrations at 300 mM and 3 mM, respectively, while varying the concentration of TFP. The results are illustrated in Figure 4. With the addition of 0.10 mM TFP, the distribution shifts away from monomer and dimer toward more tetramer and hexamer. Otherwise, the profiles are fairly similar, with little, if any higher order aggregates. However, a new component begins to appear as a shoulder in the elution profile before 5 minutes. As the TFP concentration is increased to 0.50 mM, large aggregates begin to appear, and the shoulder below 5 minutes becomes a significant feature in the elution profile. This

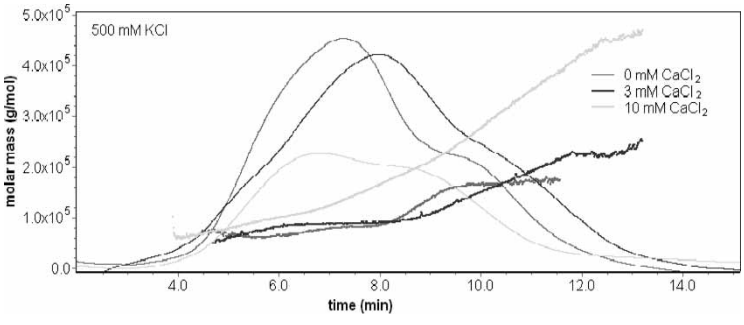


Figure 3. FlFFF-MALS analysis of CSQ in the presence of 500 mM K^+ and varying concentrations of Ca^{2+}. The elution profiles were recorded by the dRI detector.

Downloaded At: 17:43 23 January 2011

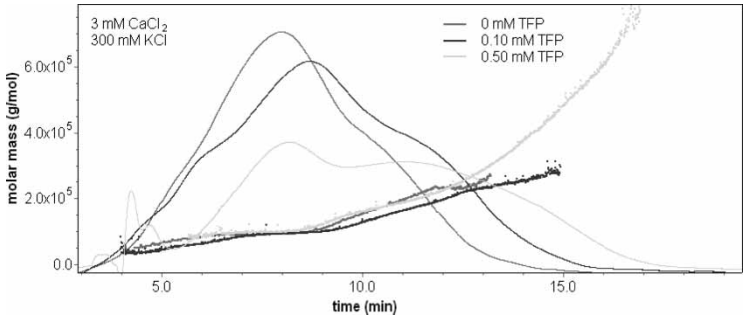


Figure 4. FlFFF-MALS analysis of CSQ in the presence of 300 mM K^+, 3 mM Ca^{2+}, and varying amounts of TFP. The elution profiles were recorded by the dRI detector.

Downloaded At: 17:43 23 January 2011

early eluting component is clearly retained by the system, since the channel void time is below 1 minute. However, the component is clearly not protein, as it fails to register on the light scattering detector. Quite possibly it is TFP that either interacts with the accumulation wall, and is therefore retained, or is released by the protein during elution through the channel. Previous models have proposed that ligand binding to CSQ would hinder dimer formation and subsequent aggregation.[19] However, the FlFFF data indicate that TFP induces CSQ aggregation. Given that TFP is known to reduce Ca^{2+} binding,[19] it is likely that the TFP dependent aggregation is in some way distinct from Ca^{2+} dependent aggregation, and results in aggregates less capable of binding Ca^{2+}.

CONCLUSIONS

Flow field-flow fractionation combined with multi-angle light scattering detection is a useful tool for studying protein aggregation. Compared to SEC, the resolution of relatively low molecular weight proteins ($<$100,000 Da) is inferior, but the gentle nature of the separation makes FlFFF-MALS particularly useful for the study of proteins that form loosely associated aggregates. Consistent with reports on other proteins, the aggregation behavior indicated by FlFFF-MALS agrees well with analytical ultracentrifugation, but has the advantage that molar masses can be calculated with more accuracy. With AUC, conversion of the measured sedimentation coefficient to molar mass requires a well defined shape factor, which can be difficult and variable for aggregates. In this work, for example, calculation of the monomer mass of calsequestrin by the AUC software was highly accurate, but because of the linear nature of the aggregates, the mass calculated for dimer and tetramer was significantly less than the true mass. Still, FlFFF-MALS has its own issues, including potential interactions with the

accumulation wall and diminishing resolution for lower mass molecules. Consequently, a range of techniques is necessary for obtaining the most complete picture of complex interacting systems.

The data presented here supports the basic tenets of the model of CSQ aggregation.[12,18,19] In particular, results indicate CSQ aggregates by first forming dimers, then tetramers and higher order aggregates. However, the dependence on K^+ concentration may be slightly different than originally proposed. In addition, the finding that TFP induces CSQ aggregation suggests that additional studies are needed to fully understand the functional effects of small molecule binding to CSQ.

ACKNOWLEDGMENTS

We thank Dawn Muhlenstein at the Biomolecular Research Center at Boise State University for supplying the AUC data, and the Murdock Charitable Trust for their contribution toward the purchase of the FlFFF channel, MALS detector, and AUC. This work was supported by the National Institutes of Health through grant R15-HL68579–01 and the INBRE/BRIN Program from the National Center for Research Resources (P20-RR16454).

Downloaded At: 17:43 23 January 2011

REFERENCES

1. Kang, C. Calsequestrin. In *Handbook of Metalloproteins*; Messerschmidt, A., Cygler, M. and Bode, W., (eds); Wiley: 2001, Vol. 3, 692–703.
2. Sato, Y.; Ferguson, D.G.; Sako, H.; Dorn II, G.; Kadambi, V.J.; Yatani, A.; Hoit, B.D.; Walsh, R.A.; Kranias, E.G. Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. J. Biol. Chem. **1998**, *273*, 28470–28477.
3. Knollman, B.C.; Knollmann-Ritschel, B.E.; Weissman, N.J.; Jones, L.R.; Morad, M. Remodeling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. J. Physiol. **2000**, *525* (2), 483–498.
4. Ihara, Y.; Suzuki, Y.J.; Kitta, K.; Jones, L.R.; Ikeda, T. Modulation of gene expression in transgenic mouse hearts overexpressing calsequestrin. Cell Calcium **2002**, *32*, 21–29.
5. Wang, W.; Cleeman, L.; Jones, L.R.; Morad, M. Modulation of focal and global Ca^{2+} release in calsequestrin-overexpressing mouse cardiomyocytes. J. Physiol. **2000**, *524*, 399–414.
6. Frank, K.F.; Mesnard-Rouiller, L.; Chu, G.; Young, K.B.; Zhao, W.; Haghighi, K.; Sato, Y.; Kranias, E.G. Structure and expression of the mouse cardiac calsequestrin gene. Basic Res. Cardiol. **2001**, *96*, 636–644.
7. Sato, Y.; Schmidt, A.G.; Kiriazis, H.; Hoit, B.D.; Kranias, E.G. Letter to the editor: re-evaluation of heart failure in transgenic mice with impaired SR Ca^{2+} release. J. Mol. Cell Cardiol. **2001**, *33*, 1757–1759.
8. Schmidt, A.G.; Kadambi, V.J.; Ball, N.; Sato, Y.; Walsh, R.A.; Kranias, E.G.; Hoit, B.D. Cardiac-specific overexpression of calsequestrin results in left

ventricular hypertrophy, depressed force-frequency relation and pulsus alternans *in vivo*. J. Mol. Cell Cardiol. **2000**, *32*, 1735–1744.
9. Slupsky, J.R.; Ohnishi, M.; Carpenter, M.R.; Reithmeier, R.A.F. Characterization of cardiac calsequestrin. Biochem. **1987**, *26*, 6539–6544.
10. Mitchell, R.D.; Simmerman, H.K.B.; Jones, L.R. Ca^{2+} binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. J. Biol. Chem. **1988**, *263*, 1376–1381.
11. Ikemoto, N.; Nagy, B.; Bhatnagar, G.M.; Gergely, J. Studies on a metal-binding protein of the sarcoplasmic reticulum. J. Biol. Chem. **1974**, *249*, 2357–2365.
12. Park, H.; Park, I.Y.; Kim, E.; Youn, B.; Fields, K.; Dunker, A.K.; Kang, C. Comparing skeletal and cardiac calsequestrin structures and their calcium binding: A proposed mechanism for coupled calcium binding and protein polymerization. J. Biol. Chem. **2004**, *279, 17*, 18026–18033.
13. He, Z.; Dunker, A.K.; Wesson, C.R.; Trumble, W.R. Ca^{2+}-induced folding and aggregation of skeletal muscle sarcoplasmic reticulum calsequestrin: the involvement of the trifluoperazine-binding site. J. Biol. Chem. **1993**, *268*, 24635–24641.
14. Wang, S.; Trumble, W.R.; Liao, H.; Wesson, C.R.; Dunker, A.K.; Kang, C. Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. Nature Struct. Biol. **1998**, *5* (6), 476–483.
15. Gatti, G.; Trifari, S.; Mesaeli, N.; Parker, J.M.R.; Michalak, M.; Meldolesi, J. Head-to-tail oligomerization of calsequestrin: A novel mechanism for heterogeneous distribution of endoplasmic reticulum luminal proteins. J. Cell Biol. **2001**, *154*, 525–534.
16. Franzini-Armstrong, C.; Protasi, F. Ryanodine receptors of striated muscles: A complex channel capable of multiple interactions. Physiol. Rev. **1997**, *77*, 699–729.
17. Saito, A.; Seiler, S.; Chu, A.; Fleischer, S. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J. Cell Biol. **1984**, *99* (3), 875–85.
18. Park, H.; Wu, S.; Dunker, A.K.; Kang, C. Polymerization of calsequestrin. implications for Ca^{2+} regulation. J. Biol. Chem. **2003**, *278* (18), 16176–82.
19. Park, I.Y.; Kim, E.; Park, H.; Fields, K.; Dunker, K.A.; Kang, C. Interaction between cardiac calsequestrin and drugs with known cardiotoxicity. Mol. Pharmacol. **2005**, *67* (1), 97–104.
20. Charlier, J.H.A.; Olson, R.D.; Thornock, C.M.; Mercer, W.K.; Olson, D.R.; Broyles, T.S.; Muhlestein, D.J.; Larson, C.L.; Cusack, B.J.; Shadle, S.E. Investigations of calsequestrin as a target for anthracyclines: structure-function comparisons of daunorubicin, daunorubicinol, and trifluoperazine. Mol. Pharmacol. **2005**, *67*, 1505–12.
21. Williams, S.K.R.; Giddings, J.C. Sample recovery. In *The Field-Flow Fractionation Handbook*; Schimpf, M.E., Caldwell, K.D. and Giddings, J.C., (eds); John Wiley and Sons: New York, 2000, 325–343.
22. Du, Q.; Schimpf, M.E. Correction for particle-wall interaction in the separation of colloids by flow FFF. Anal. Chem. **2002**, *74*, 2478–2485.
23. Schimpf, M.E.; Petteys, M. Characterization of humic materials by flow field-flow fractionation. Coll. Surf. **1997**, *120*, 87–100.
24. Wittgren, B.; Wahlund, K.-G.; Andersson, M.; Arfvidsson, C. Polysaccharide characterization by flow field-flow fractionation-multiangle light scattering: initial studies of modified starches. Inter. J. Polym. Anal. Charact. **2002**, *7* (1–2), 19–40.

Downloaded At: 17:43 23 January 2011

25. Silveira, J.R.; Raymond, G.J.; Hughson, A.; Race, R.E.; Sim, V.L.; Hayes, S.F.; Caughey, B. The most infectious prion protein particles. Nature **2005**, *437*, 257–261.
26. Song, J.H.; Kim, W.-S.; Park, Y.H.; Yu, E.K.; Lee, D.W. Retention characteristics of various proteins in flow field-flow fractionation: effects of ph, ionic strength, and denaturation. Bull. Korean Chem. Soc. **1999**, *20*, 1159–1165.
27. Luo, J.; Leeman, M.; Ballagi, A.; Su, Z.; Janson, J.C.; Wahlund, K.-G. Size characterization of green fluorescent protein inclusion bodies in *E. coli* using asymmetrical flow field-flow fractionation-multi-angle light scattering. J. Chromatogr. **2006**, *1120*, 158–164.
28. Gabrielson, J.P.; Brader, M.L.; Pelar, A.H.; Mathis, K.B.; Winter, G.; Carpenter, J.F.; Randolph, T.W. Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field-flow fractionation, and sedimentation velocity. J. Pharm. Sci. **2007**, *96*, online at DOI 10.1002/jps.20760.
29. Liu, J.; Andya, J.D.; Shire, S.J. A critical review of analytical ultracentrifugation and field-flow fractionation for measuring protein aggregation. AAPS **2006**, *8* (3), E580–589.
30. Kobayashi, Y.M.; Alseikhan, B.A.; Jones, L.R. Localization and characterization of the calsequestrin-binding domain of triadin 1; evidence for a charged β-strand in mediating the protein-protein interaction. J. Biol. Chem. **2000**, *275*, 17639–17646.
31. Analysis by Beckman XL-I analytical ultracentrifuge, Dawn Muhlenstein, Biomolecular Research Center, Boise State University.

Received February 1, 2007
Accepted February 20, 2007
Manuscript 6057

Downloaded At: 17:43 23 January 2011