by methyl moities leads to changes in the structural and properties of the reconstituted protein. This clearly indicates that LmrP does not depend on the bulk properties of the phospholipids tested but solely on the availability of one or more protons on the headgroup. We then show that a single point mutation in LmrP, D68C, is sufficient to recapitulates precisely every biochemical and biophysical effect observed when PE is replaced by PC, including energy transfer between the protein tryptophans and the lipid headgroups. We conclude that the negatively charged D68 is most likely involved in the interaction between LmrP and PE, and that such interaction is required for proton gradient sensing, substrate binding and transport. As D68 belongs to a highly conserved motif in the Major Facilitator Superfamily (which includes LacY, EmrD), this interaction might be a general feature of these transporters, and is involved in proton gradient sensing and lipid dependence.

951-Plat Solid State Nmr And Biophysical Studies On Multidrug Efflux Pumps From The Smr Family

Ines Lehner[1,2], Daniel Basting[1,2], Karsten Mörs[1,2], Clemens Glaubitz[1,2]

[1] *Goethe University Frankfurt, Frankfurt, Germany*
[2] *Centre for Biomolecular Magnetic Resonance, Frankfurt, Germany.*

Transport proteins exhibiting broad substrate specificities are major determinants for the phenomenon of multidrug resistance. One of the key problems is to understand drug recognition in the context of their structural diversity. We have used 1H MAS NOESY NMR to screen the membrane interaction of many different molecules and found a similar interaction pattern (Siarheyeva et al. 2006). This supports the hypothesis that the membrane might act as a potential selectivity filter. Efflux pumps of the small multidrug resistance family bind antibiotics and transport them across the membrane in exchange for protons. The transport cycle must involve various conformational states of the protein needed for substrate binding, translocation and release. We show the existence of an occluded substrate-transporter complex for the EmrE homologue M.tuberculosis TBsmr and its substrate ethidium bromide by fluorescence spectroscopy (Basting et al. 2007). The pH gradient needed for antiport has been generated by co-reconstituting TBsmr with bacteriorhodopsin. Our findings support a model with a single occluded intermediate state in which the substrate is highly immobile. SMR transporters are functional dimers. We have used double quantum filtered 13C MAS NMR to probe the dimerisation interface of the E.coli multidrug transported EmrE. Essential residues were selectively labelled using cell free expression. Chemical shift and line shape analysis did reveal the formation of an asymmetric homo dimer.

References

Siarheyeva, A., Lopez, J.J., Lehner, I., Hellmich, U.A., van Veen, H., Glaubitz, C. (2007) Probing the Molecular Dynamics of the ABC Multidrug Transporter LmrA by Deuterium Solid-State NMR, Biochemistry, *46*, 3075–83.

Basting, D., Lorch, M., Lehner, I., Glaubitz, C. (2007) Transport Cycle Intermediate in Small Multidrug Resistance Protein is revealed by Substrate Fluorescence. FASEB J, e-pub ahead of print.

952-Plat Dynamic Imaging The Cytosolic Ph Of Individual Phagocytosing Human Neutrophils By Shifted Excitation And Emission Ratioing Of Fluorescence (seer)

Deri Morgan, Eduardo Rios, Thomas E. DeCoursey

Rush Medical Center, Chicago, IL, USA.

Phagocytosis is the internalization of microbes by phagocytes in order to kill and digest them. Phagocytosis is accompanied by activation of the NADPH oxidase complex that transfers electrons into the phagosome to produce superoxide anion, leaving protons in the cytosol. Here we employ a highly sensitive confocal microscopy technique to examine the cytosolic pH (pH_i) of individual human neutrophils during phagocytosis.

Human neutrophils were allowed to adhere to glass coverslips, incubated with 10 μM 5-(and 6)-Carboxy SNARF-1 in HBSS for 30 min at 37°C, and washed. SEER imaging (Launikonis et al., 2005, *J. Physiol. 567*: 523) was performed by simultaneously acquiring two confocal images: F_1, Excited at 514 nm and Emitted at 500–604 nm and F_2 (Ex at 594 nm and Em at 620–715 nm). F_1/F_2 monitors $[H^+]$ with a dynamic range of ∼150. Experiments were performed at room temperature. Resting neutrophils had a pH_i of 7.03 ± 0.03 (mean ± SEM, $n = 35$). Upon the initial engulfment of an OPZ particle, pH_i decreased rapidly for ∼5 min, with a maximum rate of −0.13 ± 0.01 pH units/min ($n = 18$), reaching a minimum pH_i of 6.49 ± 0.17 ($n = 18$). Non-phagocytosing cells did not acidify. Acidification of phagocytosing cells was prevented by 20 μM DPI, implicating NADPH oxidase as the source of the protons. When incubated with 100 μM Zn^{2+} resting pH_i was 6.95 ± 0.05 ($n = 9$), the maximum rate of acidification of phagocytosing cells was −0.37 ± 0.04 units/min, and the minimum was 5.9 ± 0.04 ($n = 9$). Thus, activation of NADPH oxidase in phagocytosing neutrophils produces rapid cytosolic acidification that is limited by proton current.

Supported by NIAMS, NIH-HLBI, and Philip Morris.

Platform Y: Muscle Regulatory Proteins

953-Plat A Troponin Chimera To Study Troponin Dynamics And Interactions

Tharin M. A. Blumenschein[1], Ryan M. B. Hoffman[2], Carlos H. I. Ramos[3], Brian D. Sykes[2]

[1] *University of East Anglia, Norwich, United Kingdom*
[2] *University of Alberta, Edmonton, AB, Canada*
[3] *Universidade Estadual de Campinas (Unicamp), Campinas, Brazil.*

The troponin complex is responsible for the regulation of muscle contraction, and is composed of three subunits: troponin C, troponin I and troponin T. Troponin T is responsible for transmitting the conformational changes to the rest of the muscle thin filament, troponin I inhibits the interaction between actin and myosin, and troponin C is a Ca^{2+}-dependent switch which interacts with troponin I, removing the inhibition in the presence of calcium. While the atomic structure of most of the core region of the troponin complex has been determined by X-ray crystallography (Takeda et al., *Nature* ***424***, 35; Vinogradova et al., *Proc. Natl. Acad. Sci. USA*

102, 5038), the flexible regulatory C-terminal tail of troponin I could not be seen in the crystal structure. A structure of this region was published (Murakami et al., *J. Mol. Biol.* ***352***, 178), but later shown to account for only about 20% of the conformational population in the absence of actin (Blumenschein et al., *Biophys. J.* ***90***, 2436). A chimera containing the regulatory regions of troponin C and troponin I was previously described (Tiroli et al., *FEBS J.* ***272***, 779), and is here used to study the dynamics of these regions by nuclear magnetic resonance (NMR) spectroscopy. The single polypeptidic chain is composed of the first 91 residues of chicken skeletal troponin C and the last 85 residues of chicken skeletal troponin I, connected by a GGAGG linker. Relaxation times T_1 and T_2 confirm that this protein is monomeric in solution, as previously shown by SAXS. The much smaller size of this chimera construct, relative to the core region of the troponin complex, makes it more approachable by NMR spectroscopy, and allows us to overcome some of the difficulties encountered when working with the core complex.

954-Plat Troponin/Tropomyosin Determines Myosin Binding Target Zones Along the Thin Filament

Dipesh Patel[1], Jian-Ping Jin[2], Douglas D. Root[1]

[1] *University of North Texas, Denton, TX, USA*
[2] *Northwestern University, Evanston, IL, USA.*

Microscopic observations have indicated that target zones for myosin binding to the thin filament exist in a variety of invertebrate and vertebrate muscle types. However, debate has arisen whether these target zones are due to the troponin preventing myosin binding to some of the seven actin-binding sites per tropomyosin or some intrinsic structural property of the actin filament. To test the hypothesis that troponin/tropomyosin determines the target zones, a deletion mutant of tropomyosin was constructed that increased the frequency of troponin/tropomyosin subunits along the length of a synthetic thin filament and thus increase the inhibition of myosin binding. The mutant tropomyosin demonstrated a higher saturation point than wildtype tropomyosin for binding to actin as measured by cosedimentation assays. This data indicates that the shortened tropomyosin mutant still binds end to end along the thin filament, and its binding position is not predetermined by actin filament structure. To evaluate the impact of the mutation on myosin binding to actin, the ATPase activity was measured at fixed actin concentration and increasing concentrations of myosin S1 up to the saturation limit of the actin. The difference in S1 ATPase between the presence and absence of actin was greater in the presence of wildtype tropomyosin/troponin than in the presence of mutant tropomyosin/troponin. Thus, the increased density of troponin/tropomyosin in the mutant reduced the target zone size for S1 binding and the actin-activated S1 ATPase activity in the presence of calcium. Indeed, the amount of reduction in maximal actin-activated S1 ATPase activity was directly proportional to the size reduction in the tropomyosin deletion mutant that resulted in a reduction in the number of available S1 binding sites. This data is consistent with the determination of the target zone size and location by the troponin/tropomyosin complex.

955-Plat Conserved Destabilization of Tropomyosin at Asp 137: Functional Significance of Flexibility

John P. Sumida[1], Eleanor Wu[2], Sherwin S. Lehrer[1]

[1] *Boston Biomedical Research Institute, Watertown, MA, USA*
[2] *Boston Biomedical Research Institute/Northeastern University undergraduate Coop student, Watertown, MA, USA.*

Tropomyosin (Tm), a coiled-coil rod-like molecule, controls muscle contraction by sterically regulating the myosin-actin interaction. Tm moves between 3 states on actin either as a uniform semi-flexible molecule or as a non-uniform flexible rod. To learn if a highly conserved Asp (#137) present in the hydrophobic ridge locally destabilizes Tm and imparts flexibility we substituted hydrophobic Leu for Asp 137 and studied changes in susceptibility to proteolysis by trypsin and changes in function. We found that the control Tms that contained Asp 137 were readily cleaved at Arg 133 with $t_{1/2} = 5$ min. The Leu 137 mutant, however, was not cleaved at all under the same conditions. Actin stabilized Tm as seen by a 10-fold reduction in the rate of cleavage at Arg 133. The actin-myosin subfragment S1 ATPase was much greater for the Leu mutant compared to control both in the absence of troponin and in the presence of troponin and Ca^{2+}. It therefore appears that there is a region of local structural instability near the middle of Tm that imparts some flexibility and regulates the extent of cooperative activation of the thin filament by myosin. We thus have shown a link between a dynamic structural property of Tm and its function. This link provides a basis to understand how Tm point mutations associated with skeletal and cardiac myopathies can modulate local Tm dynamic properties to affect regulatory function.

Supported by NIH HL 22461 and AR41637

956-Plat The Structural Basis for Troponin-I Inhibition of Muscle Contraction

Agnieszka Galińska-Rakoczy[1], Patti Engel[2], Chen Xu[3], HyunSuk Jung[4], Roger Craig[4], Larry S. Tobacman[2], William Lehman[1]

[1] *Boston Univ. School Medicine, Boston, MA, USA*
[2] *Univ. Ill., Chicago, Chicago, IL, USA*
[3] *Brandeis Univ., Waltham, MA, USA*
[4] *Univ. Mass. Med. Sch., Worcester, MA, USA.*

A C-terminal "mobile" domain of TnI anchors troponin on actin in relaxed muscles and apparently interacts with and constrains tropomyosin in an inhibitory position that blocks myosin crossbridge interaction. Release of this "latching" domain from actin after Ca^{2+}-binding to TnC presumably allows tropomyosin movement away from the inhibitory position on actin, thus initiating contraction. The TnI domain is disordered in solution and not seen in the low-Ca^{2+} crystal structure of the troponin (Vinogradova *et al.*, 2005), and may only be "structured" by its binding to actin. We have used electron microscopy and image reconstruction to investigate the structural mechanism by which TnI controls tropomyosin position on actin, thereby regulating muscle contraction. To accomplish this, we

expressed the C-terminal 80 amino acids of TnI (human TnI residues 131–210) to mimic the mobile domain of TnI. The construct binds to actin and strongly inhibits actin-activated S1 ATPase at equimolar TnI:actin. Reconstructions generated from EM images of tropomyosin-free F-actin saturated with the construct reveal densities attributable to the mobile domain that lie primarily on actin subdomain 1. This position is consistent with predictions derived from models (Pirani *et al*., 2006) and EM tomograms of thin filaments (Xu *et al*., 2007). Moreover, EM reconstructions of <unl>F-actin-tropomyosin</unl> decorated with the TnI construct show additional TnI density that drapes over tropomyosin on actin. Hence the presence of tropomyosin appears to further structure the TnI domain. By lying over the tropomyosin strand on actin, the TnI construct is likely to fix tropomyosin in the blocked B-state position, which in muscle leads to relaxation. Our results support the hypothesis that muscle regulation involves a coupling of folding of the mobile TnI domain to its binding on actin-tropomyosin (Hoffman *et al*., 2006).

957-Plat Mechanism of Regulation of Native Cardiac Thin Filaments

Howard D. White[1], Betty Belknap[1], David Heeley[2]

[1] *Eastern Virginia Medical School, Norfolk, VA, USA*
[2] *Memorial University of Newfoundland, St, Johns, NL, Canada.*

We have used double mixing stopped flow fluorescence to measure the acceleration of product dissociation (mdADP) from the active site of cardiac myosin-S1 by native porcine cardiac thin filaments. At pCa <4 and one rigor myosin bound per seven actin subunits, the kinetics of the fluorescence decrease associated with the dissociation of deoxymantADP (mdADP) from cardiac actomyosin-S1-mdADP-Pi is biphasic over a wide range of thin filament concentrations. The rapid phase increases to a maximum of 30 s^{-1} and the slow phase to 3 s^{-1} at saturating concentrations of thin filaments. The rapid phase is a measure of the rate of phosphate dissociation from actomyosin-mdADP-Pi. At pCa >7 in the absence of rigor myosin bound to the thin filament, the rate of product dissociation is fit by a single exponential with a maximum rate of 0.6 s^{-1} at saturating thin filament concentration, which corresponds to 50 fold regulation of the rate of product dissociation. In the presence of either calcium (pCa <4) or bound rigor myosin-S1 the rate of product dissociation is activated 50–75 percent of the maximal rate obtained with both ligands. The apparent affinity of M-ADP-Pi for cardiac thin filaments during steady state ATP hydrolysis shows less than a 1.5 fold sensitivity to a decrease in calcium from pCa 4 to 7, which indicates that the primary regulatory mechanism is associated with phosphate dissociation. This data supports a mechanism similar to that previously determined for regulation of skeletal thin filaments (Heeley et. al PNAS, *99*, 16731, 2002 and JBC *281*,668, 2006) in which both calcium and myosin binding shift the equilibrium between active and inactive conformations of the thin filament but there are some quantitative differences.

This work has been supported by NIH HL84604, AHA, and the CIH.

958-Plat Substitution of Cardiac Troponin C into Rat Skeletal Muscle Fibers Increases Thin Filament Responsiveness to Strong-Binding Crossbridges

Karen L. Reece, Daniel P. Fitzsimons, Jitandrakumar R. Patel, Marion L. Greaser, Richard L. Moss

University of Wisconsin Medical School, Madison, WI, USA.

The contributions of troponin C (TnC) isoforms to cooperative activation of the thin filament were examined in rat skinned single psoas fibers that had been treated to extract skeletal TnC, reconstituted with cardiac TnC, and subsequently activated in the presence or absence of a strong-binding, non-force generating derivative of myosin subfragment 1 (NEM-S1). Substitution of cardiac TnC reduced the steepness of the force-pCa relationship relative to control fibers containing endogenous skeletal TnC, indicating a decrease in the apparent cooperativity of activation, but this replacement had no effect on either the calcium sensitivity of force or the rate of force redevelopment during maximal activation. In the presence of NEM-S1, skinned fibers containing cardiac TnC exhibited greater increase in calcium independent force and calcium sensitivity of force compared to fibers containing skeletal TnC. NEM-S1 treatment also accelerated the rate of force redevelopment at intermediate levels of activation to a greater degree in fibers containing cardiac TnC, but had no effect on the kinetics of force development in maximally activated preparations. These findings indicate that muscle-specific isoforms of TnC contribute to cooperative activation at submaximal calcium concentrations by modulating thin filament responsiveness to strong-binding crossbridges. Since cardiac TnC is in a more open conformation in the absence of calcium than skeletal TnC, substitution of cardiac TnC into a skeletal muscle fiber makes the skeletal thin filament more responsive to strong-binding crossbridges at submaximal calcium concentrations.

This work was supported by R01-HL61635.

959-Plat Decreased Ca2+-binding in a Skeletal Troponin C Mutant Disrupts Cooperative Interactions between Thin Filament Regulatory Units in Rabbit Psoas Muscle

Anita E. Beck, Alice W. Ward, Bertrand C.W. Tanner, Kareen L. Kreutziger, Michael Regnier

University of Washington, Seattle, WA, USA.

Experimental and computational research from our group suggests that cooperative activation of force in skeletal muscle is dominated by spatial coupling between troponin and tropomyosin. To study the effect of compromised Ca^{2+} kinetics on these protein interactions we created a I60Q mutant rabbit skeletal troponin C (sTnC) that increases Ca^{2+} dissociation rate from the whole sTn complex ~14-fold (79.69±2.33s^{-1}), compared to the same measurements using stopped-flow spectroscopy with the native sTn complex. Extracting

endogenous sTnC and reconstituting with I60Q sTnC in demembranated rabbit psoas fibers (n=8) caused a decrease in maximal force (F_{max}) to 65±3.4% of WT and a dramatic loss of Ca^{2+} sensitivity (pCa_{50}) of 1.15 pCa units from 5.74±0.05 to 4.59±0.05 at 15°C with 2.5μm sarcomere length. The slope of the force-pCa curve decreased from 2.1±0.1 to 1.7±0.1. At maximal activation (pCa 4.0), phosphate (P_i) sensitivity of force and the rate of force redevelopment (k_{tr}) significantly increased with I60Q sTnC. The reduced F_{max}, pCa_{50} and slope and increased Pi sensitivity is similar to our previous measurements where thin filaments were reconstituted with a mixture of 20% purified sTnC and 80% D28A, D63A sTnC (no Ca^{2+} binding at the N-terminus). This 20% to 80% mixture reduced nearest neighbor regulatory unit (comprising 1 troponin, 1 tropomyosin, and 7 actins) coupling by spatial means. We conclude I60Q sTnC compromises spread of activation along the thin filament though kinetic means, even though the spatial relationship between neighboring regulatory units is maintained. Our results support the hypothesis that coupling between thin filament regulatory units dominates cooperative thin filament activation, and suggests it may limit force development kinetics when creatine phosphate levels decrease and phosphate levels increase (as in fatigue).

Supported by HL61683, HL65497.

960-Plat Structural and Functional Implications of Familial Hypertrophic Cardiomyopathy Mutations in cTnT

Rachel K. Moore, John Wilson, Edward Manning, Pia Guinto, Gary Gerfen, Jil Tardiff

Albert Einstein College of Medicine, Bronx, NY, USA

Familial Hypertrophic Cardiomyopathy (FHC), a primary disease of the cardiac sarcomere, is a common cause of sudden cardiac death in young people. Many disease-causing mutations in the thin filament protein cTnT are found in TNT1, a tropomyosin binding region. Residues 160–163, which fall within a highly charged region (RREEEENRR), represent a mutational hotspot where the α-helix may unwind to provide flexibility necessary for function. The flexibility of this putative hinge region may be affected differently by mutation of these residues, and these differences may provide insight into the structure of TNT1 and the molecular mechanism of FHC. Our SDSL-EPR data suggest that the C-terminal portion of this region has loop-like characteristics in the troponin complex. In order to study the effects of these mutations, cTnT residues 120–220 were modeled *in silico* and the molecular dynamics of the mutations were compared to wild type. Interestingly, preliminary MD simulations indicate that deletion of residues 160E and 163E may have the greatest effect on the flexibility of this region, but with opposite effects. Δ160E decreases simulated flexibility, while Δ163E increases flexibility. The positions of these mutations in the hinge region may affect troponin complex assembly, consequently altering protein flexibility and impairing function. Residue 160E may be a part of a helical region of the protein, while 163E is located in a less structured portion, accounting for the different effects predicted for these mutations. To evaluate our MD studies *in vivo*, a transgenic mouse model of Δ163E has been generated, and the physiologic effects will be compared to the existing murine Δ160E model. Additionally, further EPR studies will be performed on WT, Δ160E, and Δ163E to elucidate the structure about this hinge region and the effects of these mutations on troponin complex structure.

Platform Z: Fluorescence Spectroscopy II

961-Plat Study Of Nuclear Receptor-Coregulator Interactions In Live Cells By Two Photon Two Colour Fluorescence Cross Correlation Spectroscopy

Julien Savatier[1,2], Stéphan Jalaguier[2], Emmanuel Margeat[1], Vincent Cavaillès[2], Catherine A. Royer[1]

[1] *Centre de Biochimie Structurale, Montpellier, France*
[2] *Centre de Recherche en Cancérologie de Montpellier, Montpellier, France.*

Nuclear receptors (NR) play key roles in a multiple cellular functions and are implicated in a large number of human pathologies. As such they represent major targets of drug development programs, and many of their ligands are used therapeutically. Quantitative information about their ligand-dependent interactions in live cells would greatly enhance our understanding of their function and our ability to modulate their activity.

The goal of this research is to quantitatively characterize, in live cells, the interactions between nuclear receptors (NR) and their coregulator partners with the aim of more clearly defining their functional mechanisms. We use two photon two colour fluorescence cross correlation spectroscopy (TPTCFCCS). This approach allows for combining 2-photon 3D microscopy with correlation spectroscopy. By using interacting protein pairs expressed as fusions of two different colored fluorescent proteins, it is possible to measure quantitatively the degree of interaction between the NR and their coregulator partners in live cells. Our study focuses on the NR implicated in estrogen signalling, homodimers of human estrogen receptors (ER) α and β, and their major transcriptional regulatory partners, in particular TIF2 and RIP140, in Cos-7 cells. We first constructed cerulean-ERα, cerulean-ERβ, cherry-RIP140 and cherry-TIF2, and tested their activity in transiently transfected cells in the presence or absence of agonist (estradiol, E2) or antagonist (ICI). We next analyzed quantitatively their interactions by TPTCFCCS. We also investigated the formation of ERα/ERβ heterodimers.

The presence of agonist leads to nearly 100% interaction between ERα-TIF2 and ERβ-TIF2, while antagonist totally inhibits the formation of these complexes. Similar results were obtained for ER-RIP140 interactions.

962-Plat Molecular Thermometers For Temperature Measurements In Biological Systems

Felix H.C. Wong, Daniel S. Banks, Asmahan Abu-Arish, Cécile Fradin

McMaster University, Hamilton, ON, Canada.