Fluorescence Spectroscopy II

3883-Pos

The Conformational Cycle of Mitochondrial Hsp70

Martin Sikor[1], Koyeli Mapa[1], Volodymyr Kudryavtsev[1], Karin Waegemann[1], Stanislav Kalinin[2], Claus A.M. Seidel[2], Walter Neupert[1], Dejana Mokranjac[1], Don C. Lamb[1].
[1]LMU Munich, Munich, Germany, [2]Universitaet Duesseldorf, Duesseldorf, Germany.

Mitochondrial Heat Shock Protein 70 (mtHsp70) is involved in the import of proteins into mitochondria and in protein folding in the mitochondrial matrix. The protein consists of two functional domains: the N-terminal nucleotide binding domain and the C-terminal substrate binding domain with a lid to hold the substrate peptide.

We have investigated the conformation of mtHsp70 by cloning mutants that allowed specific labeling to observe the interdomain distance and the opening state of the peptide binding lid. We have performed single-pair Förster resonance energy transfer (spFRET) experiments to investigate the distribution of conformations in mtHsp70 molecules under different conditions. The FRET efficiency is a very sensitive measure for the distance between donor and acceptor on the scale of 2-10 nm and thus provides information over the conformation of mtHsp70 during its conformational cycle. To extract quantitative information about the homogeneity of the observed states we used probability distribution analysis on the burst analysis data and performed spFRET TIRF experiments on immobilized molecules to obtain longer time traces.

Experiments were performed with different nucleotides as well as in the absence or presence of peptide substrates and cochaperons. Our results show a surprisingly heterogenous conformational distribution for ADP bound mtHsp70, opposed by a more homogenous state with an open lid and close domain in the ATP bound state. Substrate binding in the presence of the cochaperone Mdj1 results in the undocking of the domains and closure of the lid holding the peptide.

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Actin Nucleation and Polymerization Investigated using Fluorescence Fluctuation Spectroscopy

Nikolaus Naredi-Rainer[1], Alvaro H. Crevenna[2], Matthias Höller[1], Roland Wedlich-Söldner[2], Don C. Lamb[1].
[1]LMU Munich, Munich, Germany, [2]MPI Biochemistry, Martinsried, Germany.

Actin filaments are cytoskeletal polymers that are fundamental for several biological processes including cellular motility, cytokinesis, and mechanical resistance. The nucleation mechanism, i.e. the formation of the stable tetramer via dimers and trimers, whether for spontaneous or assisted nucleation (e.g. via formin or ARP2/3) has been inferred mainly on the basis of kinetic modeling fitting only polymer measurements (i.e. pyrene fluorescence assays). Fluorescence fluctuation spectroscopy is a technique that allows determination of the diverse fluorescence species and their dynamics. By following polymerization over 12 hrs, we observed the simultaneous decrease in monomer concentration with the concomitant appearance of polymer. Both parameters are used to determine a more reliable kinetic mechanism. Moreover, the decrease of monomer to a steady state indicates the critical concentration for the conditions used. Using this approach, we tested the influence of phalloidin, pH and actin concentration on the nucleation and polymerization processes. This is the first direct experimental evidence of the spontaneous nucleation mechanism, which is not only applicable for actin but also a general method to investigate any protein that undergoes polymerization or aggregation.

3885-Pos

Alex Single Molecule Three Color in TIRF Microscopy

Jinwoo Lee, Sungchul Hong Hohng.
Seoul National University, Seoul, Korea, Republic of.

Single-molecule three-color FRET is powerful in studying complex molecular interactions. Existing techniques, however, have been limitedly used for immobilized molecules, and never realized in TIRF microscopy. The main reason of this limited utilization is poor photostability of FRET probes selected for single-molecule three-color FRET.

In this work, we realized single-molecule three-color FRET in TIRF microscope for the first time. By using Cy3, Cy5, and Cy7 as FRET probes, we could use the conventional oxygen scavenger system with Trolox to efficiently reduce photobleaching of all fluorophores. Well-separated emission peaks of three fluorophores made data analysis more reliable. To monitor three-domain motion in real time, we synchronized the data acquisition of EM-CCD with the fast switching of 532-nm and 633-nm lasers. To demonstrate the capability of the setup, we observed the conformational dynamics of the Holliday junction and that of RNA 4-way junction from the hairpin ribozyme.

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Structural Rearrangements of the Motor Protein Prestin Revealed by Fluorescence Resonance Energy Transfer

Kristin Rule Gleitsman[1,2], Michihiro Tateyama[1], **Yoshihiro Kubo**[1].
[1]Division of Biophysics and Neurobiology, National Institute for Physiological Sciences, Okazaki, Japan, [2]Department of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, USA.

Prestin is a membrane protein expressed in the outer hair cells (OHCs) in the cochlea that is essential for hearing. This unique motor protein transduces a change in membrane potential into a considerable mechanical force, which leads to a cell length change in the OHC. The non-linear capacitance in cells expressing prestin is recognized to reflect the voltage-dependent conformational change of prestin, whose precise nature remains unknown. In the present work, we aimed to detect the conformational changes of prestin by a FRET-based technique. We heterologously expressed prestin labeled with fluorophores at the C- or N-terminus in HEK 293T cells, and monitored FRET changes upon depolarization-inducing high KCl application. We detected a significant decrease in intersubunit FRET both between the C- termini and between the C-and N-termini. A similar FRET decrease was observed when membrane potential was directly and precisely controlled by simultaneous patch clamp. Changes in FRET were suppressed by either of two treatments known to abolish non-linear capacitance, V499G/Y501H mutation and sodium salicylate. Our results are consistent with significant movements in the C-terminal domain of prestin upon change in membrane potential, providing the first dynamic information on its molecular rearrangements.

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Binding Kinetics and Fraction of Immobile Enzymes Bound to Cellulose Fibrils Studied Through Confocal Laser Scanning Fluorescence Microscopy and FRAP

Jacob C. Bolewski, Jose M. Moran-Mirabal, Larry P. Walker.
Cornell University, Ithaca, NY, USA.

Biofuels and bioproducts derived from cellulosic biomass represent great potential renewable and environmentally friendly technologies. Key to converting cellulosic biomass into soluble sugars is the depolymerization of the cellulose macromolecules by enzymes called cellulases. These enzymes depolymerize the cellulose chain by binding to the exposed cellulose surface and cleaving β-glucosidic bonds. Although much work has been done studying the dynamics of these enzymes in bulk solution, little is known about how these enzymes operate at the micron to nanoscale. To this end, our lab has fluorescently labeled three of these enzymes (Thermobifida fusca Cel9A, Cel5A and Cel6B) to study their binding and catalytic behavior through a variety of spectroscopic techniques. The work presented aims at quantifying the binding and unbinding kinetics, and the fraction of immobile enzyme bound to the cellulose substrate through scanning confocal microscopy and FRAP (fluorescence recovery after photobleaching).

Sonicated BMCC (bacterial microcrystalline cellulose) was patterned on glass surfaces through ''molecular combing'' to produce oriented cellulose bundles and mats. The patterned cellulose was incubated with fluorescent cellulases at saturating conditions (2nM) for approximately three hours. Cellulose aggregates were imaged with a confocal laser scanning microscope. FRAP experiments were performed on both mats and fibril bundles at various temperatures to elucidate the kinetics of binding/unbinding, and to estimate the immobile fraction of cellulases on the cellulose surface. Results from this study showed that the binding/unbinding kinetics and the immobile fraction for each enzyme differ according to the cellulase mode of hydrolysis (random versus processive) and varied significantly with temperature. This study helps to further the understanding of the molecular basis of cellulose hydrolysis and could potentially aid in the development of more efficient enzymes suitable for industrial applications.