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ATP-driven rotation of VoV1 from *Thermus thermophilus*

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Vacuolar-type H⁺-ATPases (V-ATPases, VoV1), which are commonly found in most eukaryotic cells, function as a proton pump with consumption of ATP. The homologs of eukaryotic V-ATPase are also found in some bacteria, such as a thermophilic bacterium, *Thermus thermophilus*. We previously demonstrated the rotation of both the DF rotor in V1 and the Vo-ring in V-ATPase driven by ATP hydrolysis.

Here, we report some progresses of observation system for the rotation of holo V-ATPase with ATP hydrolysis. We isolated the Vo with histidine tag in the Vo-ring from *T. thermophilus* membranes. The Avi-tag sequence was introduced into the each A subunit in the V1 from expression system using *E. coli*. The mutant holo V-ATPase was reconstituted from the isolated V1 and Vo. By using the reconstituted V-ATPase, the frequency of rotating molecules was drastically increased. Then, we observed stepwise rotation at low ATP concentrations. The rotational speed was almost proportional to ATP concentrations between 10 and 50 μ M, indicating that binding of ATP to the VoV1 is a rate-limiting step under these conditions. By fitting the histograms of dwell times with a single exponential equation, k_{on} for ATP was estimated to be $5.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, well consistent with bulk phase data. Under the same condition, the estimated k_{on} of V1 was $1.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is about 3 times higher than that of V-ATPase. This result indicates that binding of Vo to V1 domain affects catalytic properties in V1 domain.

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Mechanism of mechanical work and efficiency enhancement in myosin filament assembly

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Recent optical trapping studies of individual motor proteins have enabled us to gain insight into the mechanism of muscle contraction. It is well known that myosin II is non-processive, and so, myosins have to assemble into a thick filament to produce force continuously as required in muscle. Thus, it is very intriguing to ask the question: How do individual myosins assemble into a thick filament to become a force generator with the maximum efficiency as observed in muscle? Therefore, we investigated displacements (d) and forces (F) produced by different lengths (molecule numbers) of skeletal muscle myosin filaments using the optical trapping nanometry. Both short (0.7 μ m) and long (1.2 μ m) filaments processively produced discrete stepwise movements averaging 5-6nm and produced the 6 and 12pN peak force, respectively. The stepping efficiency (ϵ : the ratio of forward steps to backward steps) was higher for long filaments than short filaments at any given load. Thus, the velocity and mechanical work ($F \times d \times \epsilon$) per filament length produced by long filaments were higher at any given load. The second-order rate constant for ATP binding calculated by using the mean dwell time was similar to the value for the acto-myosin ATPase. Thus, each step presumably corresponds to one ATPase cycle. From these results, we concluded that the increase in myosin molecules enhances the degree of unidirectional movement, resulting the increase in thermodynamic efficiency (mechanical work/free energy of ATP hydrolysis).

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A novel expression system for dominant negative mutant actins

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It is generally accepted that myosin generates force by tilting the neck domain. However, several studies suggested that myosin binding induces cooperative conformational changes within actin filaments, which play important roles in myosin movement. For instance, An et al (1996) identified dominant negative (DN) mutant actin alleles that impair flight of *D. melanogaster* even in the presence of three-fold excess normal actin genes. These phenotypes are consistent with the idea that the DN actins prevent presumptive myosin-induced cooperative conformational changes in actin filaments, but biochemical analyses have been hampered by difficulty in purifying DN actins from flies. Expression of recombinant actin has also met difficulties because direct epitope tagging tends to aggregate actin, and furthermore, DN actins are expected to be toxic for the host cells. Therefore, using Dictyostelium cells, we expressed and purified actin that was fused C-terminally to His-tagged thymosin beta 4, an actin monomer binding and sequestering protein, and released intact actin by cleavage with chymotrypsin immediately downstream of the native terminal Phe residue. Wild-type recombinant actin obtained this way polymerized normally as examined by sedimentation assay and electron microscopy, and moved at nearly normal speeds on surfaces coated with skeletal HMM. We are expressing each DN actin using this novel expression system. We found that G63D DN actin is unable to polymerize, and are investigating why this exerts dominant negative effect.

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Dissection of Intra-molecular Communications between the Catalytic Head and Microtubule-binding Site in the Dynein Heavy Chain

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Communications between the track-binding site and ATPase site are essential for the motile activity of motor proteins. In dynein, its ATPase site(s) is located within the head domain containing six AAA+ modules, while the microtubule (MT)-binding site called stalk head is spatially separated from the head domain by a 10-15 nm slender stalk that is predicted to be formed from an antiparallel coiled coil (CC). Therefore, the information at the ATPase site and MT-binding site of dynein must be transmitted through the long stalk CC. However, the mechanism underlying this bidirectional communications remains unclear.

Recently, it has been proposed that shifts in the alignment of two helices forming the stalk CC modulate the MT-binding affinity of the stalk head (Gibbons et al. (2005)). Here we employed a disulfide cross-linking strategy to elucidate the roles of the predicted shifts within the stalk CC in the bidirectional communications. Our results showed that when the two helices were cross-linked by a disulfide bond, the dynein displayed MT-independent "activated" ATPase activity and trapped in an ATP-insensitive "strong-binding" state. Conversely, after another type of crosslinking that is expected to cause a small shift in the alignment of the CC, the dynein exhibited MT-independent "basal" ATPase activity with an ATP-insensitive "weak-binding" state. These results strongly suggest that dynein utilizes small conformational changes within the stalk CC to couple the MT-binding and ATPase activities.