MOLECULAR MECHANISMS GOVERNING ENSEMBLE BEHAVIOUR OF MYOSIN II MOTORS UNDER UNLOADED CONDITIONS

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by

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Dedicated to

My Loved Ones

CERTIFICATE

This is to certify that the thesis entitled "**Molecular Mechanisms Governing Ensemble Behaviour of Myosin II Motors Under Unloaded Conditions**", being submitted by **Ms. Khushboo Rastogi** to the Indian Institute of Technology Delhi for the award of degree of **Doctor of Philosophy**, is a record of bonafide research work carried out by her, which has been prepared under our supervision and guidance of conformity with the rules and regulations of "Indian Institute of Technology Delhi". The research reports and the results presented in this thesis have not been submitted in part or full to any other University/ Institute for the award of any degree or diploma.

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ABSTRACT

Myosin II is a low duty ensemble motor that carries out vital physiological functions such as muscle contraction. *In vivo,* an ensemble of myosin molecules functions asynchronously to generate muscle contraction or tension development. Understanding the molecular/allosteric rules that govern the co-ordination and performance of an ensemble of myosin II molecules has been a longstanding puzzle for the research community. Though unloaded shortening and isometric tension have been well studied *in vivo,* it has been challenging to quantify the ensemble properties of Myosin II motor.

In this work, we present the first detailed analysis of processive sliding and breakage of actin filaments at various heavy meromyosin (HMM) densities, ATP concentrations and inorganic phosphate in IVMA. We observed that with addition of ATP solution, the actin filaments fragmented stochastically; we then determined mean length and velocity of surviving actin filaments post breakage. Using density of HMM molecules and length of actin, we estimated the number of HMM molecules per actin filament (N) that participate in processive sliding of actin.

Only those filaments that were able to interact with a minimum number of HMM molecules could sustain processive sliding. N was observed to be a function of ATP concentration, added inorganic phosphate and active binding sites on actin. Only those filaments that were able to interact with a minimum number of HMM molecules could sustain processive sliding. At 2 mM ATP, 88 ± 24 molecules and at 0.1 mM ATP, 54 ± 22 molecules are required for continuous sliding of actin in IVMA. Similarly, for added Pi (10 mM), $N = 72 \pm 30$ at 2 mM ATP and did not show any variation with change in HMM density. Processive sliding of actin filament was

observed only when N lay within the above range at each ATP concentration. These estimates helped arrive at a minimum lower limit (N_{min}) and a maximum upper limit (N_{max}) to the number of HMM molecules that can interact with a smooth sliding actin filament in IVMA under our experimental conditions. When $N < N_{min}$, the actin filament diffused away from the surface and processivity was lost, probably because at least one tightly bound myosin molecule is required to anchor the actin filament to the surface. When $N > N_{max}$, the filament underwent breakage eventually and could not sustain processive sliding. We postulate this maximum upper limit arises due to increased number of strongly bound myosin heads.

We also widened our perspectives to the understanding of sliding of regulated native thin filaments (NTF) and established a similar limit on N. Unlike unregulated actin, we observed no actin breakage of NTFs irrespective of any experimental condition.

We have thus also determined for the first time the number of simultaneously attached myosin molecules (N_a) to be ~1-2 at a given time despite changes in the experimental environment. Based on our experimental findings, we concluded that processive sliding of the actin filament depends on the relative values of the detachment and attachment rates. We concluded that the unloaded shortening velocity of the actin filaments is a function of ATP only (or ATP and Pi in experiments with external addition of Pi) and is independent of pCa and myosin density.

We believe these inferences from our experimental data will greatly aid in reaching a better understanding of the mechanisms underlying myosin motility in an ensemble. The results may also prove useful in the design of novel molecular motility-based devices in nanotechnology.

सार

मायोसिन द्वितीय एक कम कर्तव्य वाला सामूहिक मोटर होता है जो मांसपेशियों के संकुचन जैसे महत्वपूर्ण शारीरिक कार्य करता है । शरीर में, मायोसिन अणुओं का एक टुकड़ा असामान्य रूप से मांसपेशियों के संकुचन या तनाव पैदा करने के लिए कार्य करता है। आणविक / सबोस्टोरिक नियमों को समझना जो मायोसिन द्वितीय अणुओं के सामूह के समन्वयन और प्रदर्शन को नियंत्रित करते हैं, यह अनुसंधान समुदाय के लिए एक पुरानी पहेली है। यद्यपि अनलोडेड शॉर्टिंग और आइसोमेट्रिक तनाव का शरीर में अच्छी तरह से अध्ययन किया गया है, यह मायोसिन द्वितीय मोटर के सामूहिक गुणों को मापने के लिए चुनौतीपूर्ण रहा है।

इस काम में, हम विभिन्न भारी मेरोमोओसिन (एचएमएम) घनत्व, एटीपी सांद्रता और ईवीएमए में फॉस्फेट पर प्रोटीसिव फिसलने और एक्टिन फिलामेंट्स के टूटने का पहला विस्तृत विश्लेषण प्रस्तुत करते हैं। हमने पाया कि एटीपी समाधान के साथ, एक्टिन तंतु प्रसंभात्य खंडित होते हैं। हमने तो लंबा और जीवित एक्टिन तंतु टूटने के बाद,उसके वेग की गति और लंबाई निर्धारित करी है। एचएमएम अणुओं के घनत्व और एक्टिन की लंबाई का उपयोग करके, हमने अनुमान लगाया है कि प्रति एक्टिन फिलामेंट,एचएमएम अणुओं (एन) की संख्या एक्टिन की प्रोसेसली फिसलने में भाग लेती है।

केवल उन तंतुओं जो एचएमएम अणुओं की न्यूनतम संख्या के साथ बातचीत करने में सक्षम थे, प्रोसेसिव फिसलने को बचा सकते हैं। एन एटीपी एकाग्रता का एक समारोह माना गया था, अकार्बनिक फॉस्फेट जोड़ा गया और एक्टिन पर सक्रिय बाध्यकारी साइटें। केवल उन तंतुओं जो एचएमएम अणुओं की न्यूनतम संख्या के साथ बातचीत करने में सक्षम थे, प्रोसेसिव फिसलने को बचा सकते हैं। 2 एमएम एटीपी में, 88 ± 24 अणुओं और 0.1 एमएम एटीपी पर, IVMA में एक्टिन की निरंतर स्लाइडिंग के लिए 54 ± 22 अणु आवश्यक हैं। इसी तरह, पीई (10 मिमी) के लिए, एन = 72 ± 30 2 एमएम एटीपी में और एचएमएम घनत्व में परिवर्तन के साथ कोई भिन्नता नहीं दिखा। एटीन रेशा की प्रक्रियात्मक स्लाइडिंग केवल तब देखी गई थी जब एन प्रत्येक एटीपी एकाग्रता में ऊपर की सीमा के भीतर था। इन अनुमानों ने न्यूनतम प्रयोगशाला (एनएमएन) और अधिकतम ऊपरी सीमा (एनएमएक्स) में एचएमएम अणुओं की संख्या तक पहुंचने में मदद की जो कि हमारे प्रयोगात्मक स्थितियों में एक चिकनी फिसलने एक्टिन फिलामेंट के साथ IVMA में बातचीत कर सकते हैं। जब एन <Nmin, एक्टिन रेशा सतह से दूर फैल गया था और प्रसंस्करण खो गया था, शायद क्योंकि कम से कम एक कसकर बंधन myosin अणु सतह के लिए एक्टिन फिलामेंट एंकर करने के लिए आवश्यक है। जब एन> एनएमएक्स, फिलामेंट अंततः टूटता हुआ था और प्रोसेसली फिसलने को नहीं बचा सकता था। हम मानते हैं कि इस अधिकतम ऊपरी सीमा को मजबूती से बाध्य मायोसिन सिर की बढ़ी हुई संख्या के कारण उठता है।

हमने अपने परिप्रेक्ष्य को विनियामक देशी पतली तंतुओं (एनटीएफ) की फिसलने की समझ को चौड़ा कर दिया और एन पर एक समान सीमा की स्थापना की। अनियमित एक्टिन के विपरीत, हमने किसी भी प्रयोगात्मक हालत के बावजूद एनटीएफ की कोई क्रियात्मक क्षति नहीं देखी।

इस प्रकार हमने प्रायोगिक माहौल में परिवर्तन के बावजूद पहली बार एक साथ समय पर 1/2 होना करने के लिए एक साथ जुड़े मायोसिन अणुओं (एनए) की संख्या निर्धारित की है। हमारे प्रयोगात्मक निष्कर्षों के आधार पर, हमने निष्कर्ष निकाला कि एक्टिन फिलामेंट की प्रोसेसिव स्लिसिंग टुकड़ी और लगाव दर के सापेक्ष मूल्यों पर निर्भर करता है। हमने निष्कर्ष निकाला है कि एक्टिन फिलामेंट्स की उतार-चढ़ाव के वेग एटीपी (या एटीपी और पीआई के बाह्य जोड़ के प्रयोग के साथ प्रयोग में है) और पीसीए और मायोसिन घनत्व से स्वतंत्र है।

हम मानते हैं कि इन प्रयोगों से हमारे प्रयोगात्मक आंकड़ों से बहुत अधिक सहायता मिलेगी जो कि मेसिन गतिशीलता के आधार पर एक पहनावे में बेहतर ढंग से समझने में सहायता करेगी। परिणाम नैनो में उपन्यास आणविक गतिशीलता-आधारित उपकरणों के डिजाइन में भी उपयोगी साबित हो सकते हैं।

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ABBREVIATIONS

ADP	-	Adenosine Diphosphate
ATP	-	Adenosine Triphosphate
ßME	-	2-β Mercapto Ethanol
BSA	-	Bovine serum albumin
EMCCD	-	Electron Multiplying Charge Coupled Device
ELC	-	Essential Light Chain
Ca^{2+}	-	Calcium (divalent ions)
CrP	-	Creatine Phosphate
CPK	-	Creatine Phosphokinase
d	-	Working stroke of myosin II motor
DTT	-	Dithiothreitol
EDTA	-	Ethylene Diamine Tetraacetic Acid
EGTA	-	Ethylene Glycol Tetraacetic Acid
HMM	-	Heavy Meromyosin
IVMA	-	*In Vitro* Motility Assay
KCl	-	Potassium chloride
k_{-ADP}	-	Rate constant for ADP release
k_{+ATP}	-	Second order rate constant of ATP binding
kDa	-	Kilo Dalton
K_m	-	Apparent binding constant for [MgATP]
k_{-Pi}	-	Rate constant for Pi release

KPi	-	Potassium phosphate buffer
LMM	-	Light Meromyosin
$MgCl_2$	-	Magnesium chloride
mM	-	Millimolar
MOPS-3	-	(N-mopholino)propanesulfonic acid
n	-	Hill Coefficient
NaN_3	-	Sodium Azide
N.A	-	Numerical aperture
NTF	-	Native thin filaments
pCa	-	Negative logarithm (base 10) of Ca^{2+} ion concentration
PMSF	-	Phenylmethylsulfonyl fluoride
Pi	-	Inorganic Phosphate
RLC	-	Regulatory Light Chain
S1	-	Sub-fragment 1
S2	-	Sub-fragment 2
sCMOS	-	scientific Complementary Metal Oxide Semiconductor
S.D	-	Standard Deviation
S.E	-	Standard Error
SDS PAGE	-	Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
TAME	-	N-alpha-p-tosyl-L-arginine methyl ester hydrochloride
TPCK	-	N-alpha-tosyl-L-phenylalanine chloromethane ketone
Tm	-	Tropomyosin
Tn C	-	Troponin C

Tn I	-	Troponin I
Tn T	-	Troponin T
T_{on}	-	Time of attachment
Tris-Cl	-	Tris hydrochloride
TRITC	-	Tetramethyl Rhodamine Isothiocyanate
V_F	-	Velocity of thin filament sliding in the IVMA
V_{max}	-	Velocity of thin filament sliding at saturating [MgATP]