Study on the Motor Function of Actomyosin by Engineered Acto-Si Chimera Proteins

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Abstract

The sliding force of actomyosin is generated by cyclic interactions of the motor domain of myosin with filamentous actin utilizing the energy released by ATP hydrolysis. To unveil the mechanism of muscle contraction it is prerequisite to know the structural change of actin and myosin upon ATP hydrolysis. Direct evidence on the detailed structure of the attached S1 during active contraction (at other chemical states during ATP hydrolysis) has been difficult to obtain because of short life and weak affinities of such intermediates. Protein crystallography provided the essential molecular anatomy for understanding the problem of the mechanism of muscle contraction. However crystals of the actomyosin complex and hence its atomic structure are not available yet. The atomic structures of the motor proteins have been determined for a variety of myosin motors under a variety of chemical conditions, including chicken skeletal myosin II (S1), *Dictyostelium* myosin II (S1), as well as actin complexed with DNase I or profiling that allowed researchers to build a plausible model of the conformational changes but it is not known whether these conformational changes of isolated motor domains reflect those when bound with actin filaments.

Based on this structural information, models of the actomyosin rigor complex have been constructed by docking the atomic structures of actin and S1 into the structures obtained by electron microscopic observation of the complex, which showed that the motor domain binds to subdomain 1 (SD-1) of the actin subunit using several hydrophobic, stereospecific interactions.

We therefore decided to stabilize the intermediate actomyosin complex structure by engineering acto-S1 chimera proteins (CP) carrying the entire actin inserted in loop 2 of the motor domain of *Dictyostelium* myosin II with 24 or 18 residues glycine based linkers.

My thesis contains five chapters,

Chapter -1 contains introduction in which I have discussed about the general background of this research, the past research on the mechanism of acto-myosin interaction, the motivation and the purpose of our research.

In the chapter-2, I have discussed about the construction of genes to express the acto-S1 chimera proteins, expression and purification procedure of acto-S1 chimera proteins and verification of the biochemical functions of the actin and S1 part of the chimera protein individually by different types of techniques like pelleting assay, ATPase assay, SDS-PAGE and

western blotting technique to check the effect of copolymerization function with skeletal actin and self polymerization function of chimera proteins, ATP hydrolysis activities of chimera homopolymer filaments and copolymer filaments with skeletal actin, molecular weight of the heavy chain and regulatory & essential light chain, and expression of the chimera protein respectively.

To construct the plasmids to express the acto-S1 chimera protein we introduced the actin gene into the loop 2 of myosin heavy chain gene by using two different lengths of glycine based linker (24 &18 residues). Finally the acto -S1 gene was inserted into the pTIKLOE vector with actin promoter and myosin terminator.

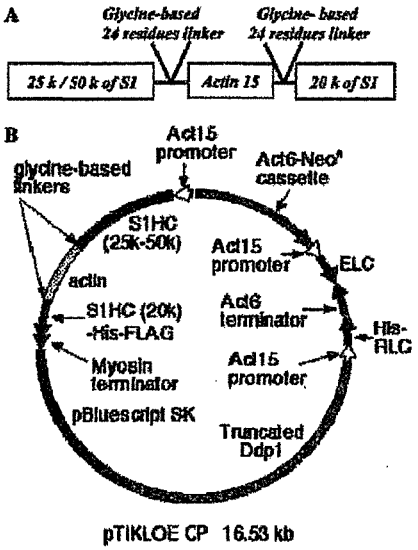

Fig. 1. Schematic structure of the acto-S1 chimera protein. (A) The acto-S1 chimera protein consists of actin and myosin S1 that has the lever arm. The glycine-based 24 residue-linkers were inserted between the actin and myosin S1 chains. (B) Construction of the plasmid for expressing the acto-S1 chimera protein.

The acto-S1 chimera proteins expressed in *Dictyostelium* cells was purified and was also verified the presence of actin and S1 part individually by western blotting .The chimera proteins were capable of self polymerization as well as copolymerization with skeletal actin and exhibited rigor like structure. The Mg-ATPase rate of chimera protein with 24 residues linker (CP24) homopolymer was 0.29 s^{-1} and CP24-skeletal actin copolymer was 1.06s^{-1}, which is slightly lower than the V_{max} of *Dictyostelium* S1. The molecular weight of the chimera protein is 170 kDa and the molecular weight of the essential and regulatory light chains are 18 and 14 kDa respectively that was verified by SDS-PAGE. We also observed the chimera homopolymer and copolymer with skeletal actin filaments in the electron microscope. Both the chimera proteins (CP24, CP18) showed arrowhead structure like natural acto-S1 rigor filaments in the absence of ATP.

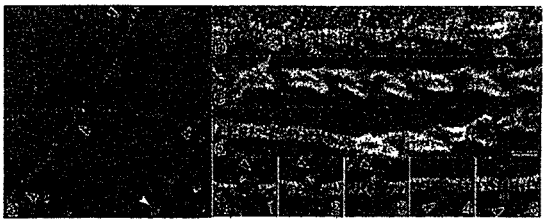

Fig 2: Figure-2.7. Electron microscopic images of CP24 chimera protein.. (a) General view of CP24 chimera protein (arrowheads) copolymerized with skeletal actin. Five chimera particles are shown including the one on the top of actin

(white arrowhead). (b) Control image of skeletal actin filament decorated with chicken chymotryptic S1 under rigor condition. (c) Self-polymerized filament of CP24 chimera protein showing arrowhead structure. (d) Block-copolymer filament of CP24 chimera protein and skeletal actin. (e) Images of individual CP24 molecule incorporated in an actin filament.

In the chapter-3, I have discussed about the fluorescence spectroscopic analysis of acto-S1 chimera proteins copolymerized with pyrene labeled actin in the presence of ATP, AMP-PNP and ADP. I also analyzed only pyrene labeled filament actin solution in the presence of ATP,AMP-PNP and ADP. I also discussed about the possible effect of hypermobile water on the fluorescence intensity.

In the case of pyrene actin and acto-S1 chimera protein copolymer (6 to 1 ratio) a large increase of fluorescence intensity was occurred in the presence of ATP , whereas no change of fluorescence intensity was occurred for only pyrene actin filament in the presence of ATP. This result suggested that a large structural change of actin filaments was occurred by S1 motor domain in the presence of ATP at 6 to 1 molar ratio (pyrene actin to acto-S1 chimera protein) for both 24 and 18 residues linker containing Chimera protein. But the structural change of actin filaments occurs at 15 to 1 molar ratio (pyrene actin to acto-S1 chimera protein) was almost half of 6 to 1 molar ratio. Our result suggested that the structural change of actin filaments by myosin motor domain is occurred in the presence of ATP which is known as weak binding state and such a change is propagated significantly beyond the actin subunits that are in contact with myosin motor domain. and also the structural change of actin filament is cooperative . We also observed that the fluorescence spectral change occurred in the presence of AMP-PNP by S1 motor domain of chimera which is a structural analogue of ATP and we did not observe any fluorescence spectral change in the presence of ADP. To understand whether or not the fluorescence intensity is increased by the structural change of actin filament by S1 part of the chimera protein upon ATP hydrolysis, we repeated the measurement of fluorescence spectrum after the ATP addition. It was observed that the fluorescence intensity was almost same after and before addition of ATP when all the ATP was consumed. It was also observed that the fluorescence intensity was increased again after the addition of ATP again which is the direct evidence of the structural change of actin filaments by myosin motor domain upon ATP hydrolysis.

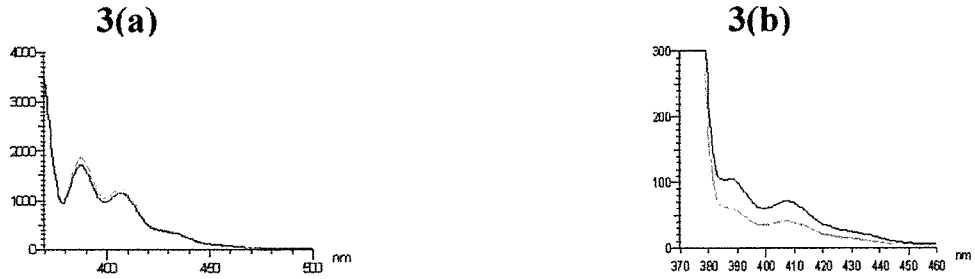

Fig-3(a) Fluorescence intensity of pyrene F-actin solution (final 0.2mg/ml) . The red line shows the intensity before addition of ATP and the black line shows the intensity after addition of ATP.

Fig-3(b) Fluorescence intensity of pyrene–actin and acto-S1 chimera (24 residues linker) copolymer (6 to1 ratio) solution. The red line shows the intensity before addition of ATP and the black line shows the intensity after addition of ATP.

In the chapter-4, I have discussed about the motility function of the acto-S1 chimera protein homopolymer filaments (CP24 & CP18) on the skeletal myosin coated glass surface.

We examined the motility function of the chimera protein homopolymer filaments on a skeletal myosin coated surface to understand whether or not the Subdomain-1 is the essential domain for the force generation. Productive interactions between surface-bound skeletal myosin and SD-1 of the actin portion of the chimera filament should be sterically hindered by the S1 portion of the chimera protein but surprisingly, the chimera protein homopolymer filaments moved at nearly

normal speeds on myosin coated surface. The sliding speed of CP24 and CP18 homopolymer filaments were 2.9±0.6 and 4.1± 0.8μm/s (mean±SD), respectively on skeletal myosin coated glass at 27°C.

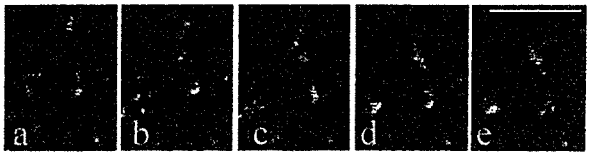

Fig.4: Sliding movement of chimera protein homopolymer filaments.The time interval is 0.2 s. The white bar indicates 5 μm.

In the presence of caged-ATP instead of ATP both the filaments of CP18 and CP24 were attached stably on the surface coated with skeletal myosin. After illumination of UV from mercury lamp through objective lens of the microscope both types of filaments showed sliding movements for several minutes until the liberated ATP molecules were consumed. This cycle was reproducible more than ten times without marked change in the sliding movement. Statistical thermodynamic considerations suggest that the S1 portion of chimera protein mostly resides on SD-1 of the actin portion even in the presence of ATP. So, this sliding movement of the chimera homopolymer filaments on skeletal myosin suggested an intriguing possibility that force sliding interactions between actin and myosin does not involve SD-1 of actin molecules . The 3D model of S1-bound F-actin under rigor condition by Lorenz exhibits two helical grooves between the ridges made of bound-S1 assembly. We speculate that, during sliding motion, SD-3 is the likely site along actin filaments to interact with the motor domain of skeletal myosin, since SD-2 and SD-4 are less accessible below SD-1 and SD-3, respectively.

In the chapter-5, I have discussed about the conclusion of my whole thesis.

As a tool we have engineered the complete acto-S1 chimera protein which has a lever arm with regulatory and essential light chains. The actin was inserted into the loop2 of S1and was linked by glycine based linkers (24 and 18 residues). Then we verified the biochemical functions of the actin and S1 part of the chimera protein individually.We found that the acto-S1 chimera protein was biochemically functional. Finally we observed the chimera protein filament and skeletal actin-chimera protein copolymer filament by electron microscopy. Also we could develop a high expression system of chimera protein (3.0-4.0 mg from 15- 25g of cells) and the amount of the chimera protein was sufficient for the fluorescence spectroscopic measurements.

By using the acto-S1 chimera proteins with 24 or 18 residues linkers we observed significant spectral changes both in the profile and the intensity in the presence of either ATP or AMP-PNP which is a structural analogue of ATP that indicate cooperative large structural change of actin filaments. On the other hand not detectable change of fluorescence spectrum was observed in the presence of ADP. The results suggest that about 6 actin molecules may be affected by one myosin subfragment 1 during ATP hydrolysis. Though at this moment we cannot say strongly however as our result suggests that the hypermobile water suppress the fluorescence intensity it is possible to explain the structural change of actin filament by myosin motor domain in the presence of nucleotides relating with the hypermobile water.

Both types of chimera homopolymer filaments (Chimera proteins with 18 residues liker and 24 residues linker) moved smoothly in not much different manner from that of skeletal actin filaments, except for slight stumbling. As the S1 part of the chimera protein already binds with the subdomain-1 of actin, the other subdomains such as subdomain-3 may interact with myosin head for motion generation . This motility function of the chimera homopolymer filaments cannot be described by the tight coupling theory of muscle contraction but it is more possible to describe by loose coupling theory of muscle contraction.

論文審査結果の要旨

第１章ではこの研究の意義について述べている。タンパク質の機能発現のメカニズムを詳細に理解することは、医療分野にとどまらず、生体物質をモデルとした材料設計にも有用であり、それらのメカニズムを利用した生体材料の開発等、広い分野で応用が期待される。本論文は運動機能を有するアクトミオシン系の動作機構に注目し、F-アクチンの動的構造変化が筋収縮のメカニズムに重要な機能を果たしているのか知るため、遺伝子工学的にキメラタンパクを開発し、その生化学的特性、アクチン側の動的な構造変化を蛍光分光法で調べ、さらに運動アッセイ法により運動に必要な結合サイトの検証実験を行いまとめたものである。

第２章では、ミオシンと相互作用するアクチン側の動的構造変化を調べるためのツールとして、遺伝子工学的にミオシンサブフラグメント１(S1)にあるアクチンと結合するループ２部にアクチンの全シーケンスを挿入した遺伝子を作成し、粘菌でタンパクを発現させ精製することができた。このアクト S1 キメラタンパクの生化学特性を調べた結果、キメラのアクチン部は天然の骨格筋アクチンと共重合しフィラメントを形成するだけでなく、キメラのみで重合し、天然のアクチンと S1 のライゴール構造とほとんど区別のつかない構造になることを電子顕微鏡観察により明らかにした。これらの２種のフィラメントは天然のアクト S1 と同等の加水分解機能を有していることも確かめた。構造と生化学的機能の両面から、共有結合でつながれたアクトミオシン複合体を実現できた意義は大きい。

第３章では、作成したアクト S1 キメラと蛍光ラベルした骨格筋アクチンとの共重合フィラメントを作成し、各種ヌクレオチドとの結合時の蛍光スペクトルを測定した。また ATP を添加した後の蛍光スペクトルのダイナミックな変化も測定した。その結果、ヌクレオチドのない場合と ADP と結合した場合では、蛍光スペクトルに顕著な変化は見られなかったが、AMP-PNP と ATP を結合した場合は 380nm から 500nm にわたって明らかな蛍光強度の増大を観測した。とくに ATP 添加の後のスペクトルは急激な蛍光強度増大を示し、ATP が消費されるまでそれが続き、ATP 消費後はもとのレベルにもどることを確認した。強度の変化量がキメラの混合比をふやすとともに急激に上昇したことは協同的なアクチンフィラメントの構造変化が起こっていることを示した証拠であり、その意義は大きい。

第４章では、キメラタンパクのみで重合したフィラメントの運動アッセイ実験をまとめている。キメラタンパクのアクチン部のサブドメイン１はミオシン S1 とのもっとも重要な結合部位と考えられており、すでに両者はペプチドリンカーで結合されているために、その結合サイトはほとんどふさがれている。にもかかわらず、骨格筋ミオシンをコートしたガラス板上でこのキメラフィラメントは天然のアクチンに近い速度ですべり運動をした。このことは力を発生するために重要な結合サイトと考えられてきたアクチンのサブドメイン１が実は運動機能そのものには直接かかわっていないことを強く示唆するもので、本実験は新たに運動機構を再構築することの必要性を示したもので非常に重要な発見であり、意義は高い。

第５章では総括として、アクトミオシン系解析に非常に有効なツールであるキメラタンパクを作成したこと、それによりアクチンの協同的な構造変化が起こっていることを示し、運動機能を新しい観点から再考すべきことを述べている。以上より、本論文は理学的・工学的に有用な知見を提示している。

よって，本論文は博士(工学)の学位論文として合格と認める。