Mitotic KinesinEg5-ADP-Pi analogues ternary complexes which mimic different transient states in ATPase cycle

ATP Driven Motor Protein Research Group
Fofou Yonta Tostani [1)], Islam MD Alrazi[1)]
Supervisor Shinsaku Maruta[1)2)]

1) Soka university Graduate School of Science and Engineering, Department of Biosciences, Maruta Laboratory (Laboratory of Bionanotechnology)
2) Soka university, Faculty of Science and Engineering, Department of Science and Engineering for Sustainable Innovation

Keywords: Mitotic kinesin, ATPase, Motor protein, Transient state, Characterization

1. Background and Aim of Study

Mitotic kinesin Eg5 is one species of kinesin, ATP driven motor proteins that plays a physiological role by the formation of bipolar mitotic spindles during cell mitosis and are highly expressed in cancer cells. It is known that conventional kinesin moves along microtubules with processive motility. On the other hand, kinesin Eg5 has been shown as a non-processive motor protein. However, the mechanism in detail is still obscure. Interestingly, another ATP driven motor protein share the well conserved motor domain with kinesin. Therefore, the two ATP driven motor proteins share the common energy transducing mechanism. We have previously shown that in the presence of MgADP and phosphate (Pi) analogues (AlF_4^- , BeFn), myosin and the conventional kinesin forms stable myosin(kinesin)-ADP-phosphate analogues ternary complexes which mimic the different transient steps along the ATP kinetic pathway. In this study, we are aimed at Characterizing Mitotic Kinesin Eg5-ADP-Pi Analogues Ternary Complexes Which Mimic Different Transient States in ATPase Cycle to clarify the non-processive energy transducing mechanism.

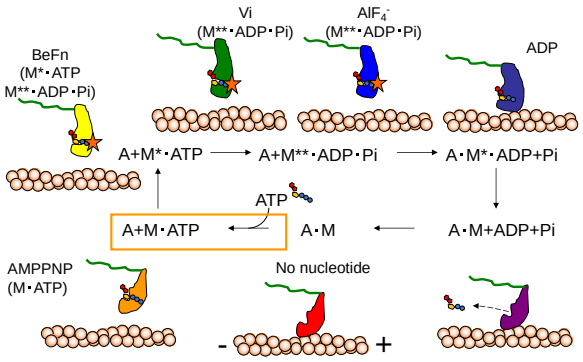

Fig. 1 Transient states in the myosin ATPase cycle

2. Materials and Methods

Preparation of kinesin Eg5 The motor domain of Eg5 was prepared according to the following method, which is a modification of the previously reported method (1). The cDNA of the motor domain of wild-type (WT) mouse Eg5 (WT, amino residue 1-367) was amplified by polymerase chain reaction and ligated into the pET21a vector. The Eg5 WT expression plasmids were used to transform Escherichia coli BL21 (DE3). Eg5 WT was purified by using a Co-NTA column.

ATPase assay The ATPase assay was performed according to the methods we have previously reported (1).

The generated Pi from ATP hydrolysis was quantitatively analyzed with the Bio-Mol phosphate detecting reagent.

Fluorescence Measurement and Formation of Kinesin Eg5-Mant-ADP-Fluorometal Ternary Complexes

Fluorescence was measured at 25°C with a F-2500 spectrofluorometer (Hitachi). The change in fluorescence intensity of 0.3 μ M Mant-ADP in the presence of 1.5 μ M Eg5 on addition of nucleotides or P, analogues (1 mM AlF_4^-, 1 mM BeF_n, or 1 mM Vi) was monitored in a solution of 20 mM NaCl, 30 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$. The excitation and emission wavelengths were 360 and 445 nm, respectively.

2. RESULTS and DISCUSSION

2-1. Monitoring the formation of kinesin Eg5-ADP-Pi analogues ternary complexes.

To monitor the binding of ADP to the catalytic site of kinesin eg5 accompanied by formation of Eg5-ADP-Pi analogues ternary complexes, we employed fluorescent ADP analogues, Mant-ADP (Excitation 360 nm, Emission 445). The excitation spectrum

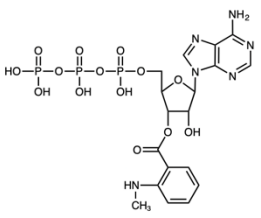

Fig. 2 Structure of Mant-ATP

of Mant-ADP is well overlap with emission spectrum of the tryptophane residue (Excitation 290 nm, Emission 320 nm). located at near the catalytic site. Therefore, we can monitor the binding of Mant-ADP to the catalytic site and formation of the ternary complex by measuring the Fluorescent Resonance Energy Transfer (FRET). To the solution in the presence of Mant-ADP, kinesin Eg5 was added. As shown in Fig.3, the fluorescence intensity (Excitation 290 nm, Emission 445 nm) was enhanced by around 300 %. Subsequently
addition of AlF_4^- as a Pi analogue induced significantly further increase of fluorescent intensity.

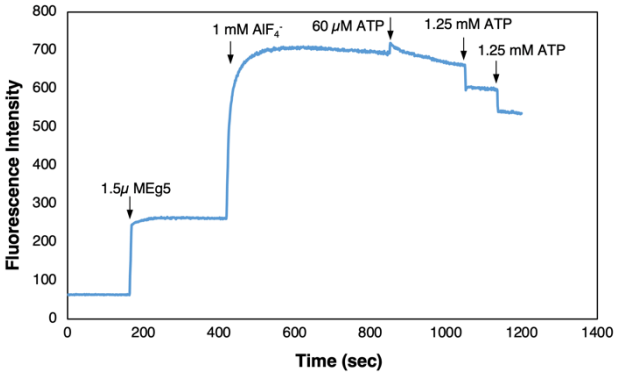

Fig. 3 Monitoring the formation of the Eg5-ADP-AlF_4^- ternary complex by FRET

The drastically fluorescent change after addition of Pi analogues indicates the tryptophan residue at position 127 in loop 5 moves closer to catalytic site accompanied by the formation of Eg5-ADP-AlF_4^- ternary complex. On the other hand, in the case of addition of another Pi analogue BeFn, fluorescent intensity was slightly decreased. Therefore, the results suggest that the conformation of Eg5-ADP-BeFn is apparently different from that of Eg5-ADP- AlF_4^-.

2-2. Inhibition of ATPase Activity of Eg5 by Pi analogues.

The formations of the ternary complexes of Eg5-ADP-Pi analogues were monitored by measuring the inhibition of ATPase activity. As shown in Fig. 4, ATPase activity of kinesin Eg5 was strongly inhibited in the presence of ADP and phosphate analogues in a time-dependent manner. The BeFn ternary complex formed slightly faster than that of AlF_4^-. Some inhibition was also observed in the presence of ADP but not Pi analogues due to the competing of ATP with ADP initially added and production of ADP by the hydrolysis of ATP during the Eg5 ATPase reaction. Previously, we have shown that the MgATPase activity of myosin is inhibited by A1F_4- and BeFn in a similar time-dependent manner (2). Therefore, The ATPase inhibition apparently indicating the formation of the Eg5-ADP-Pi analogue ternary complexes. The results suggested that the ADP of the ternary complexes is tightly trapped in the catalytic site with the phosphate analogues.

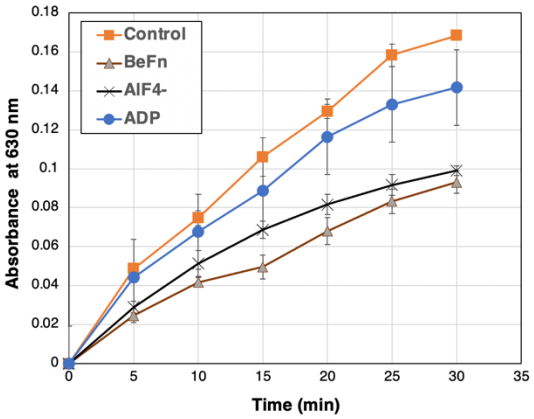

Fig. 4 Inhibition of Kinesin Eg5 ATPase activity by Pi analogues.

2-3. Stability of the ternary complexes.

The stability of the ternary complexes was monitored by the Time course of ADP release from Eg5-ADP-Pi analogue ternary complexes using dialysis apparatus. The ternary complexes were stable. Even after for 4 days dialysis, more than 60% ADP still retained in the catalytic site for AlF_4^- complex.

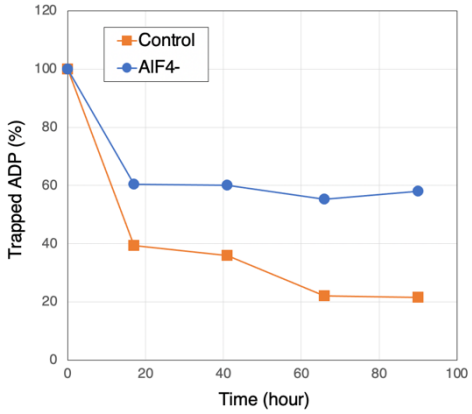

Fig. 5 Time course of ADP release from the Eg5-ADP-AlF4- complex.

CONCLUSION

In the presence of MgADP, Kinesin Eg5 forms stable ternary complexes (Eg5-ADP-Pi analoues) with phosphate analogues AlF_4^-, BeFn. The complexes exhibit different conformation at near the catalytic sites suggesting the ternary complexes mimic different transient states each other in the kinesin Eg5 ATPase cycle.

Future Plans

1. Structural analysis of the ternary complexes by Small angle solution X-ray scattering.
2. Quantitative analysis of the Al and Be in the ternary complexes.

References

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