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Mechanical Modulation of Inhibitory Pausing State and ATP binding of V1-ATPase

Chapter 2
Two Pauses of V1-ATPase and Mechanical Modulation of long pause state

The biochemical ATPase assay of V1 suggested that all active V1 molecules, after a while, completely lose their activities. To prevent the interference from this inactive state to the modulation experiment, characterization of this pausing state was necessary. Therefore, before continuing with angular modulation of ATP binding affinity, first, these pauses were studied. During the rotation assay of single V1 molecules, the existence of this irreversible final pause was confirmed and called as 'long pause' hereafter. In addition, a new pause was discovered, which frequently interrupted V1's rotation. This pause was reversible and second-order therefore was called as 'short pause'. Short pause was not predicted from biochemical assay, so it is a new finding of this study.

Later, the angular positions and kinetic constants of these pauses were characterized. Angular positions of pauses were determined to be same as that of ATP binding and hydrolysis, which makes the rotation scheme of V1:ATPase more complicated than that of F1:ATPase. Finally, the long pause state of V1-ATPase was mechanically modulated to see whether V1 will resume rotation or not with external energy input. With magnetic field, the magnetic bead which was attached to shaft of V1:ATPase was modulated. The bead was rotated to a certain angle, and stalled there for a specific period of time, and then released. Upon this type of stall-and-release experiment, two general behaviors were observed: V1 either went back to original angle and continued to stay in long pause state, which was called as 'Failure in Reactivation' or resumed rotation, which was called as 'Reactivation'. The probability of 'Reactivation' was calculated and, its stall angle dependency was determined. Reactivation was observed starting from +70°, reaching its maximum value at +110°. Including ADP in the chamber buffer suppressed the reactivation probability drastically, implying that long pause state is the ADP-inhibited state of V1. This part of my study showed that chemical reactions of V1 can be efficiently modulated by rotation of the shaft.

Chapter 3
Mechanical Modulation of ATP-binding Affinity of V1-ATPase

After characterization of pauses of V1:ATPase, I continued with the main objective, which is to study the angular modulation of ATP binding of V1:ATPase. Stall-and-release experiments were performed at non-saturating ATP, where we observed clear ATP binding dwells. Upon release from the magnetic field, two behaviors were observed: One is going back to original binding angle, called as 'off' event, and the other is stepping to next binding angle, called as 'on' event. 'off' event implies that at the time of release from the magnetic field, V1 was not bound ATP therefore could not generate torque needed for stepping to the next angle. After many trials, the probability of 'off' event (Poff) among whole number of trials was calculated. Poff increased depending on stall angle and stall time. From the stall time vs Poff graph, rate constants of ATP binding and release were determined, both of which exponentially increased with stall angle. Torque generated by ATP binding was estimated from the slope of koff as 4 pNnm, which is almost 100% of the whole torque. ATP binding generated larger torque in F1:ATPase, almost 13 pNnm, which implies that ATP binding is not the primary torque generating step in V1:ATPase. ATP binding affinity was successfully modulated, and torque by ATP binding was calculated in this chapter.

Chapter 4
General Conclusion and Future Work

This study showed, for the first time, the mechanical modulation of chemical reactions in V1:ATPase. The chemical reactions that were analyzed were long pause state and ATP binding of V1:ATPase. Meanwhile a second-scale, reversible 'short pause' was discovered during V1 rotation. Even though its angular position and kinetic constant were determined, these findings did not give a clue about the physiological role of short pause. So, further research is necessary for this pause as discussed in 'Future Work' section.

Modulation experiment suggested that long pause represents the ADP-inhibited state of V1, which is an inhibitory state caused by not releasing the hydrolysis product, ADP.

Finally, I think that this study will pave way for new ideas in both science and applied technology.
構造の相等性を持つ回転分子モーターであり、ATPの加水分解で得られる化学エネルギーを回転運動という形で力学エネルギーに直接変換することができる興味深い性質を持つ。その構造的相等性から、V-ATPaseはF型-ATP合成酵素と同様の作動機構で、エネルギー変換を行っていると考えられていたが、昨今の分子操作法によって、その作動機構は大きく異なることが明らかになりつつある。本論文では、分子操作法を用いて、その作動機構の詳細な解明に取り組んでいる。

第一章では、V-ATPaseに関する一般的な知見を紹介している。
具体的には、1) V-ATPaseの生体内での機能および生理学的な重要性。2) V-ATPaseの立体構造。3) 回転分子モーターとしてのV-ATPaseの回転機構に関するものである。

第二章では、新たに発見された2種類の不活性化メカニズム（回転運動の停止状態）を紹介している。
新たに発見されたV-ATPaseの2種類の不活性化状態は、F型ATP合成酵素のADP阻害状態と同等のものであると考えられるが、不活性化状態に陥るもしくは不活性化状態から活性化するメカニズムはF型ATP合成酵素と大きく異なることが示されている。

第三章では、ATPの結合過程で出力される回転トルクの大きさを分子操作法によって定量的に計測している。
ATPの加水分解は、ATPの結合、分解、生成物であるADPおよび無機リン酸の解離の4つの反応過程から構成され、F型ATP合成酵素では、それぞれの反応過程で出力される回転トルクの大きさが計測されている。その計測では、ATPの結合過程で出力される回転トルクが全回転トルクの半分以上を占めており、つまりはF型ATP合成酵素の回転運動は主にATPの結合によって駆動されていることが明らかにされていた。一方、本論文の計測では、V-ATPaseは、ATPの結合過程で4pNの回転トルクしか出力しないことが示され、この値は、F型ATP合成酵素と比較すると非常に小さいことがわかった。すなわち、V-ATPaseではATPの結合以外の反応過程が主となり回転運動が駆動されているのである。これは、近年明らかにされたV-ATPaseの立体構造を基にした結晶学的な予想とも整合性がとれる結果である。

第四章では、F型ATP合成酵素との比較からV-ATPaseの回転機構について議論している。
Boyerの回転触媒説では、基質の結合および生成物の解離によって大きな回転トルクが発生し、回転運動が駆動されると考えられていた。この説はF型ATP合成酵素では実証されたが、V-ATPaseはこの説の限界ではないことが明かし、つまりは、本論文によって、V-ATPaseはF型ATP合成酵素とは全く異なる作動原理に基づいて回転していることが明らかにされたのである。今後、他の反応過程の出力する回転トルクの大きさも計測することができれば、両者の違いがより明確になるであろう。それらの知見はそのうえに、ATPの加水分解を駆動力とする分子モーターの普遍的な作動原理を導き出すことが可能になると考えられる。
よって本論文は博士論文として価値あるものと認める。