Protein Systems for Measuring 24 Hours - Basic Design of a Circadian Clock

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Lives on Earth live skillfully by adapting to a 24-hour day/night cycle. We, human beings, carry wristwatches and use them in our daily lives, but animals, plants, and bacteria that do not have wristwatches also have clocks in their cells that program their daily lives. This clock, called a circadian clock (biological clock), is so accurate that it hardly deviates even if the temperature changes. This is very strange, considering that temperature greatly affects biological activity. Of course, we also have a circadian clock that precisely regulates various bodily functions and sleep.

The question of how life remembers the Earth's rotation period in its cells and generates temperature-independent oscillations over 24 hours has fascinated researchers in a wide range of fields. In 1992, we developed an experimental system in cyanobacteria using bioluminescence and found three clock genes, *kaiA, kaiB*, and *kaiC* (*kai* named after rotation), and showed that the expression of *kaiC* was similar to the clock model based on gene expression proposed in higher organisms. However, we gradually came to believe that this model could not explain the stable 24-hour period length and sought to elucidate the biochemical function of the KaiC protein, which was thought to play a key role in solving this mystery. As a result, in 2005, we discovered that a temperature-independent 24-hour rhythm is generated just by mixing three Kai proteins and ATP in a test tube and succeeded in reconstructing the circadian clock *in vitro*. (Figure 1). Surprisingly, the rhythm of proteins are very stable, and the period is temperature-compensated, and it has the same properties as the circadian clock of living cells and individuals. This discovery was a Copernican turn for studying circadian clocks, which were thought to be a function of living cells, and had an enormous impact on investigating clocks in higher organisms. This achievement has also attracted the attention of researchers in the fields of chemistry and physics since it revealed an entirely new function of proteins in "ticking time.

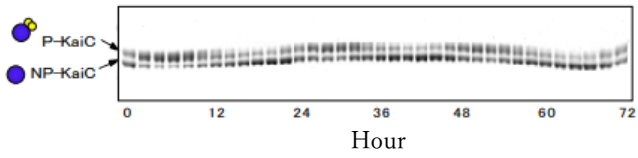

Fig. 1. Circadian Clock by Protein
KaiC repeats phosphorylation autonomously *in vitro* in an approximately 24-hour cycle. Purified KaiA and KaiC were mixed *in vitro* with ATP and kept at 30° C. A small amount was taken every 2 hours to stop the reaction, and the state of KaiC was examined by electrophoresis; the KaiC protein was detected in two bands, the upper phosphorylated form, and the lower dephosphorylated form. The figure clearly shows that the phosphorylation state oscillated in a 24-hour cycle. This period length was temperature compensated, and the mutant protein exhibited the same period as rhythm of the mutant cell.

How does the Kai protein count 24 hours? The leading player, KaiC, is composed of two ATP-degrading enzymes (ATPases); ATP is the "currency of the cell" and is the direct energy source molecule for most biological activities. Life uses various ATPases to break down ATP to obtain energy for vital activities. When we measured the activity of KaiC quantitatively, we once again surprised by the results (Fig. 2). First of all, the activity was extremely low, degrading only 10-15 ATP per day. This activity is so low (almost zero level) that it seems to be an enzyme that has lost its function. Still, upon closer examination, this activity is not affected by temperature and is highly stable. When further examined in a mutant KaiC with an altered period, this activity was proportional to the speed (frequency) of the circadian clock. This means that the activity of the ATPase determines the characteristics of the circadian clock (period length and its temperature conpensation). More importantly, this characteristic is found even in KaiC incubated alone, which does not generate rhythms. This indicates that KaiC does measure period length by generating rhythms but rather memorizes 24 hours as an intrinsic property within KaiC structure.

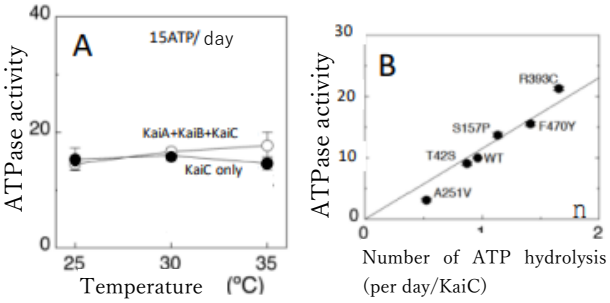

Figure 2. ATPase activity of KaiC. Only KaiC was placed with ATP, and ATPase activity was measured. No phosphorylation rhythm occurred in the case of KaiC alone. (A) The ATPase activity of KaiC is very low but precisely temperature compensated. (B) Furthermore, this activity is proportional to the frequency (speed) of the circadian clock. The ATPase activity of the wild type and the five periodic mutants is plotted against their frequency (reciprocal of the number of cycles).

How does KaiC's ATPase realize this? This is the most fundamental question of the circadian clock, yet it is not well understood. However, with the amount of energy used, it is impossible to explain the 24-hour oscillation with the sequential combination of chemical reactions, and a completely different mechanism seems to be required. Recent studies have examined the possibility that the ATP-degradation energy from CI-ATPase causes distortions in its molecular structure and mechanically suppresses its own ATP-degradation activity. This process would establish stable negative feedback regulation (Figure 3). If this is realized, a spring-like mechanism would be maintained in the internal strain of the protein, and it will be possible to function as a simple harmonic

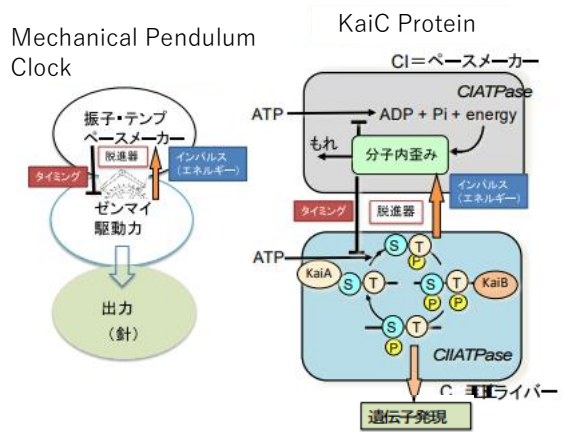

Figure 3: Mechanical Pendulum Clock and KaiC Protein Designs
Both are composed of very similar designs, although the entities are quite different. See text for details.

oscillator which period is independent of temperature or amplitude, like a balance in a mechanical wristwatch. However, harmonic oscillation of CI alone would, sooner or later, stop anyway and would not be able to transmit period to outside.

Therefore, it is important that another ATPase (CII) is used to couple it with a pacemaker (CI-ATPase) to sustain CI oscillations. KaiC's CIIATPase collaborates with KaiA and KaiB to sequentially repeat phosphorylation and dephosphorylation of two adjacent amino acids of CII. The cycle of this process is naturally affected by temperature and various environmental factors. But if the pacemaker of CI gates its progression, it will cycle as CI-oscillation. And suppose CI can receive a very small amount of mechanical energy from CII somewhere in this cycle. In that case, CI can acquire energy in the same cycle that could sustain the CI oscillation. In this way, the two cycles can continue to stable oscillations of the same period, and CII can also obtain a stable period and control various metabolic activities in a 24-hour cycle. In other words, it has combined two different types of oscillations with different properties and has a good combination of the two. In fact, this configuration is similar to the design of mechanical watches, and the coupling mechanism is called an escapement mechanism. The escapement connects the isochronous pendulum discovered by Galileo to a rotating tool with a faster periodic spring or weight. The mechanism developed by Huygens, Hooke, and many subsequent watchmakers restricts the direction of action to one direction and the duration of action to a very short period, allowing the pacemaker (pendulum or balance) is designed to continue free harmonic oscillation by reliably controlling the spring-driven rotating mechanism while ensuring that very little energy is gained.

Is such a mechanism possible within KaiC? It will take some time to reveal the atomic structure, since the movements will likely be a slight movement at the atomic level. But among many mutants of KaiC isolated, some mutants showed the abnormalities in this function. In this mutant, the cycle is affected by temperature, and the cycle becomes 8-10 hours, and the phosphorylation cycle seems to rotate freely, with no temporal resting period as found in the wild type. The presence of these mutants expects to have a clock mechanism similar to that of mechanical clocks in proteins.