

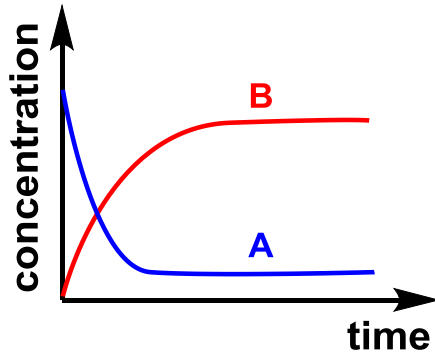


KaiC Protein Oscillation in Vitro

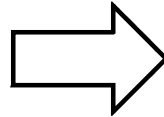
**Literature Seminar
Yamato Kanzaki (M2)
August 27th, 2016**

Contents

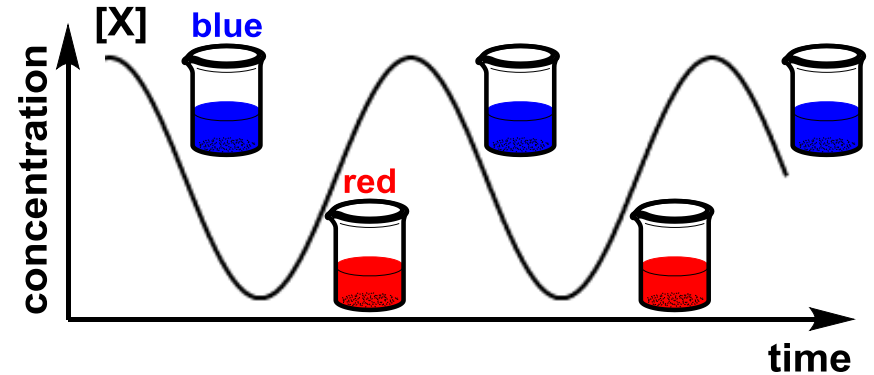
General chemical reactions



ex. $A \rightarrow B$



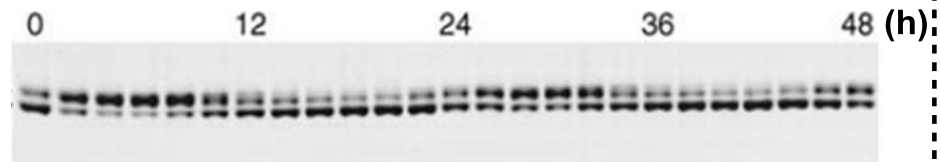
Non-equilibrium chemistry



Belousov-Zhabotinskii (BZ) reaction

(*Nature*, 1970, 225, 535. *JACS*, 1972, 94, 8649.)

mechanism? possible to design?



KaiC protein oscillation in Vitro

(*Science*, 2005, 308, 414.)

1. Introduction of Oscillation Systems

Exapmles
&
Applications

2. KaiC Protein Oscillation in Vitro

Mechanism

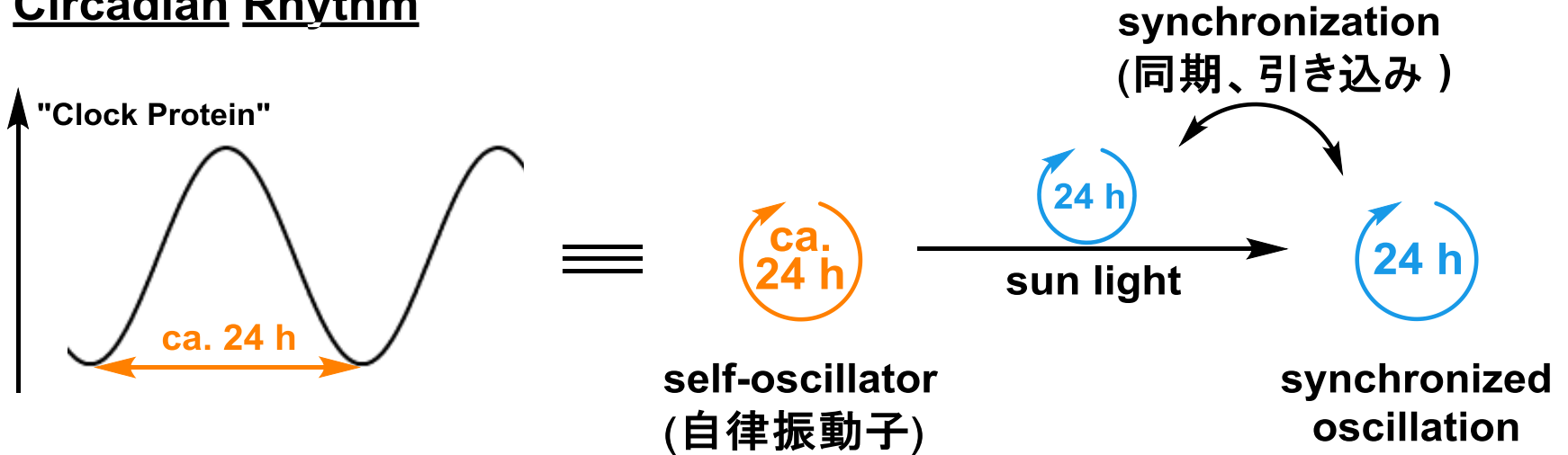
3. How would you design it?

Simulation Study
for
Designing Oscillators

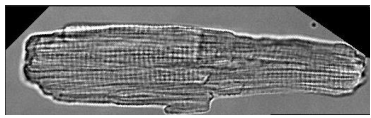
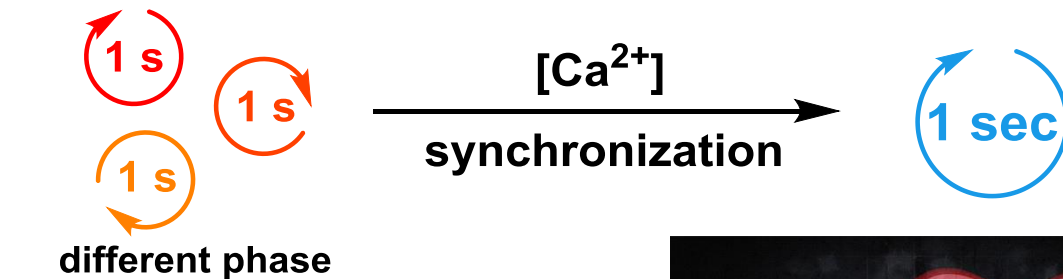
1. Introduction

Examples and Terms

1. Circadian Rhythm



2. Cardiac Rhythm

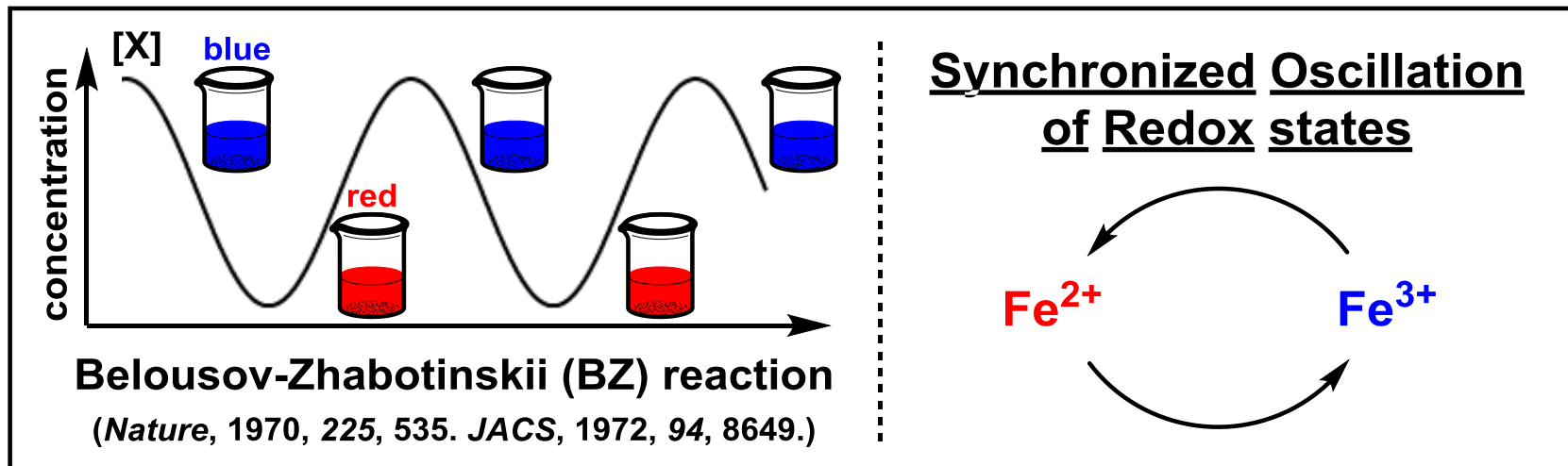


cardiac myocyte

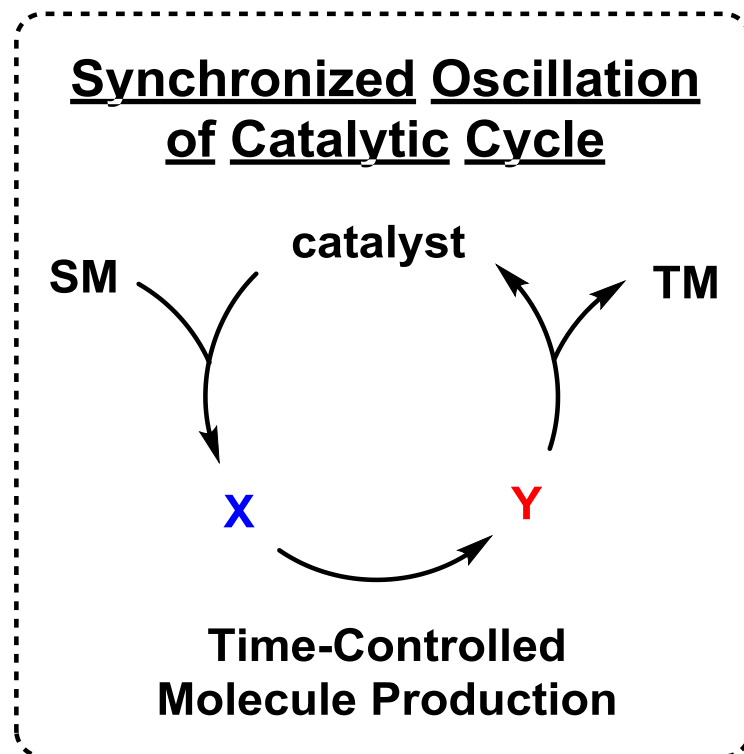
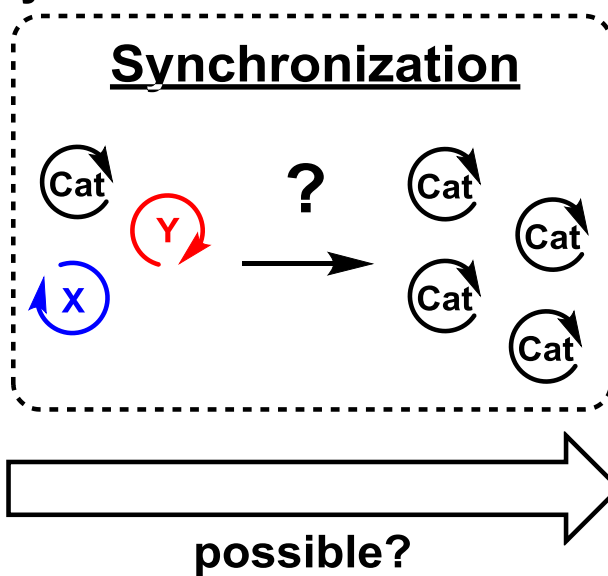
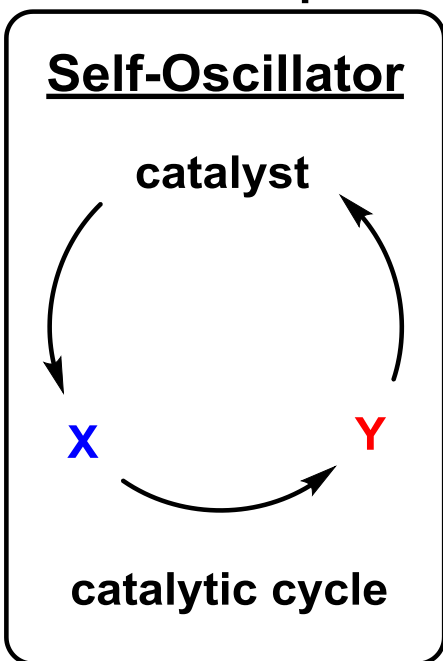


cf. NF kB oscillation
p53 oscillation
Hes 1 oscillation
Hes 7 oscillation
Peroxioredoxin oscillation
Melatonin oscillation
Insulin oscillation
...etc.

From a Viewpoint of Organic Chemistry

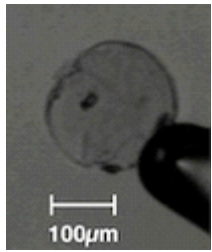


As the other possibility...

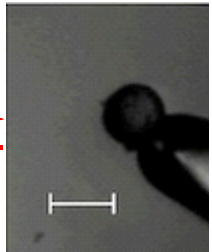


BZ reaction based self-oscillation polymer gels

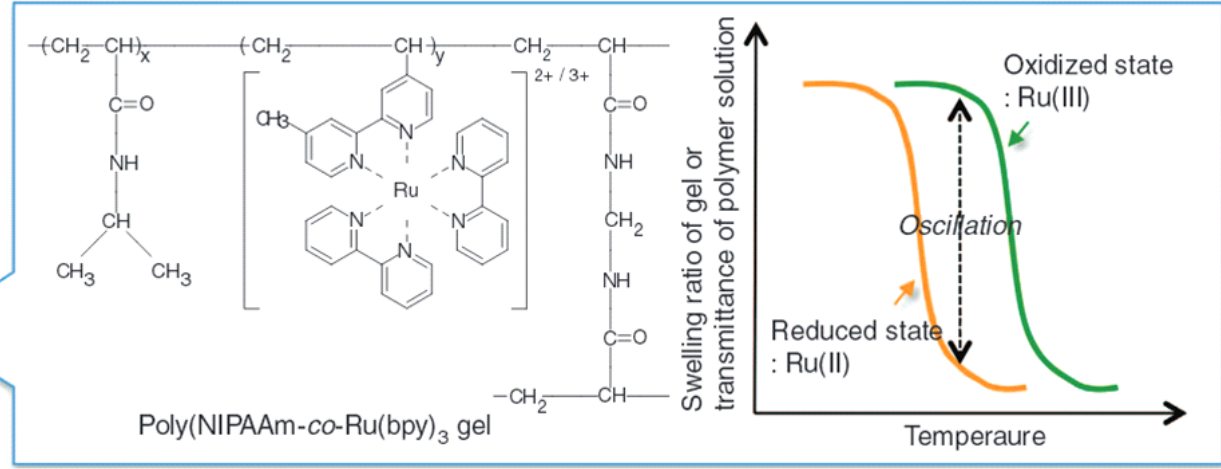
Swelling
 $Ru(bpy)_3^{3+}$
(Ox)



Deswelling
 $Ru(bpy)_3^{2+}$
(Red)



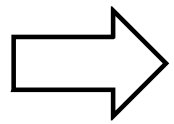
under BZ solution
(maronic acid, $NaBrO_3$, acid)



Review: Yoshida. R. et. al., *NPG Asia Materials*, 2014, 6, e170.

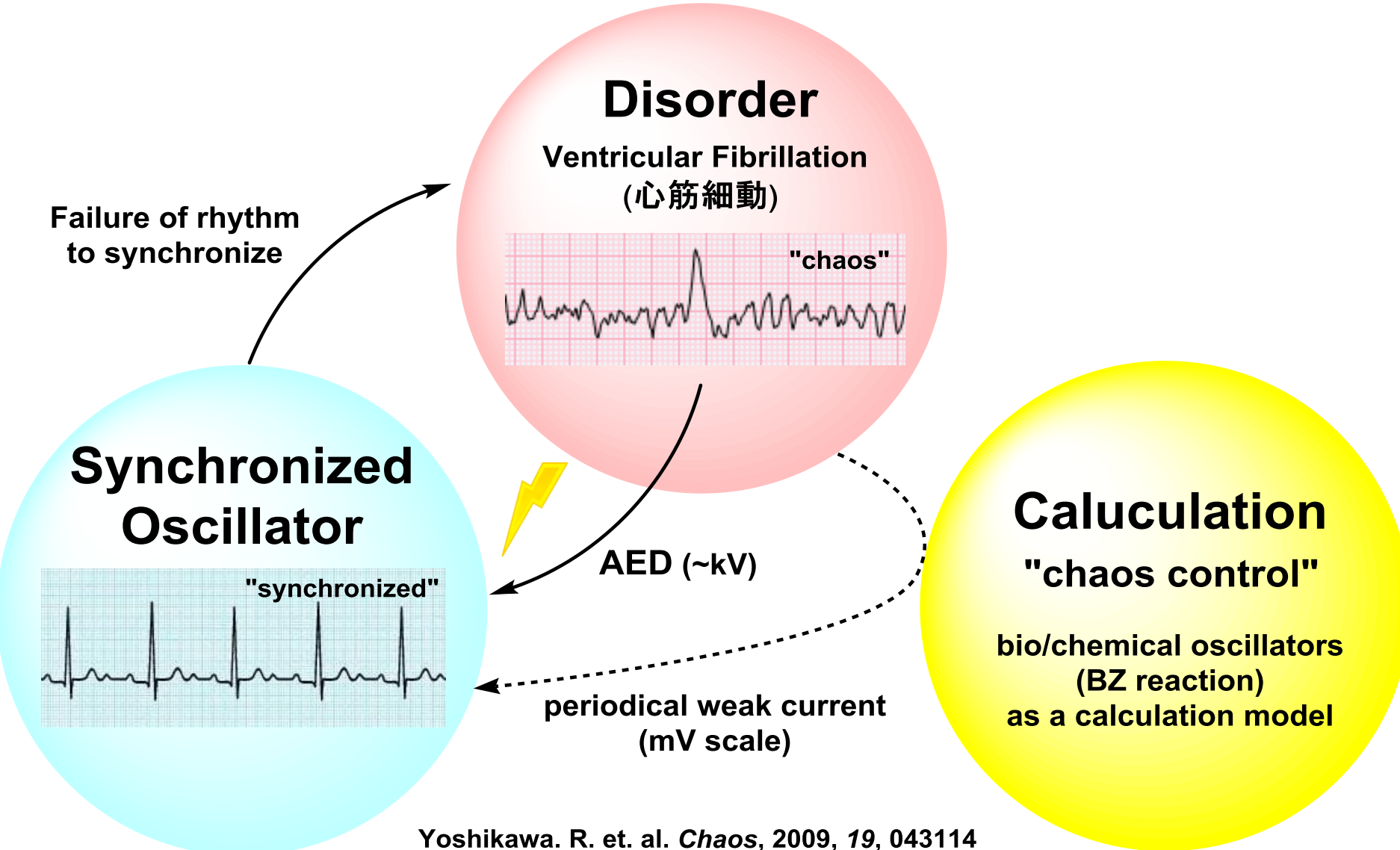
BZ reaction is the only choice to construct self-oscillation gels for now.

novel oscillation systems

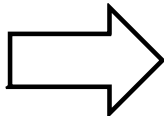


a variety of
novel (bio)materials

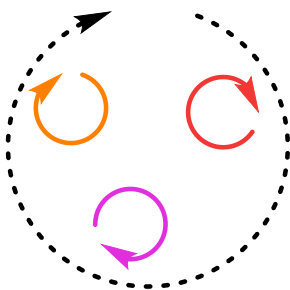
Applications: 1. Materials, 2. Calculation Model, 3. Medical Application



Yoshikawa. R. et. al. *Chaos*, 2009, 19, 043114

novel oscillation systems  novel calculation models to develop "chaos control"

Chaos System
(Disorder)



synchronization



Ordered System



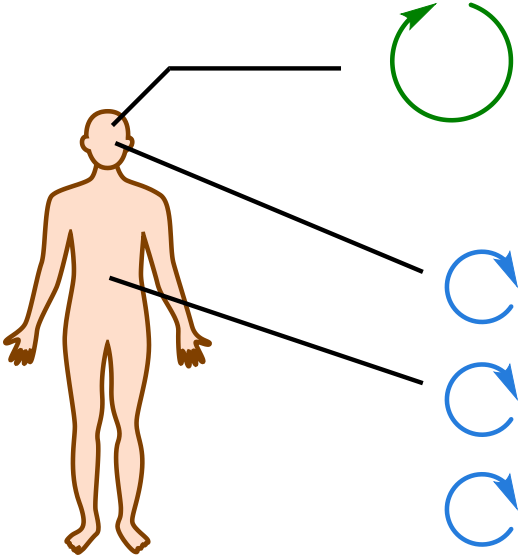
Circadian Rhythm
(core oscillator)



pineal gland (melatonin oscillation)

pancreas (insulin oscillation)

cancer, depression, etc.

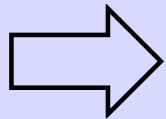


Short Summary

Broad examples and applications, but how?

Today's Topic

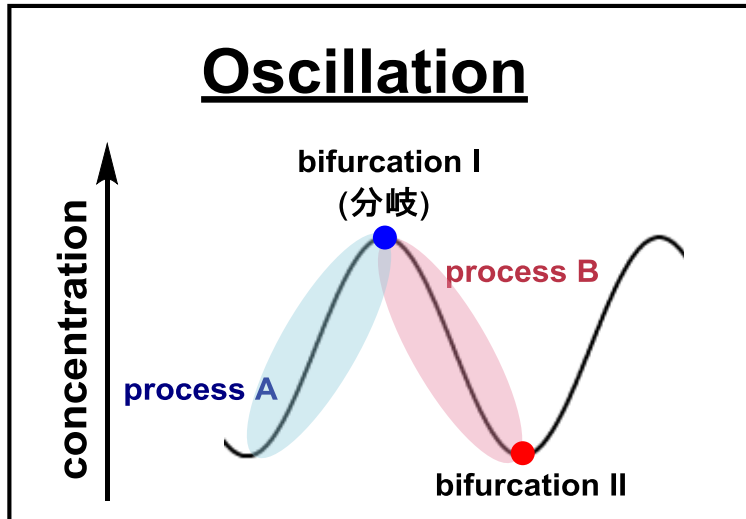
What is the required conditions for oscillation?



KaiC protein oscillation as a case study

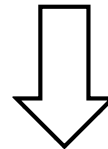
2. KaiC Protein Oscillation in Vitro

Two Types of Oscillation Systems



Requirements

- I) a continuous flow of energy
- II) two processes & two bifurcations



1) Feedback Type
(classical but complicated, see app.5)



2) Phase Transition Type
(today's topic)

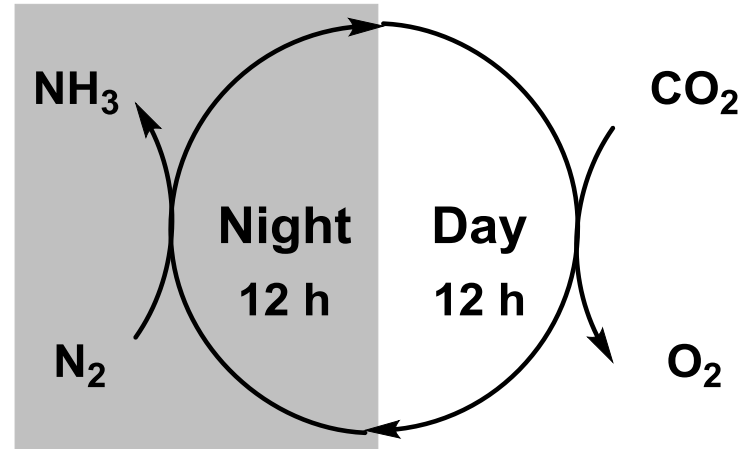


Circadian Rhythm in Cyanobacteria

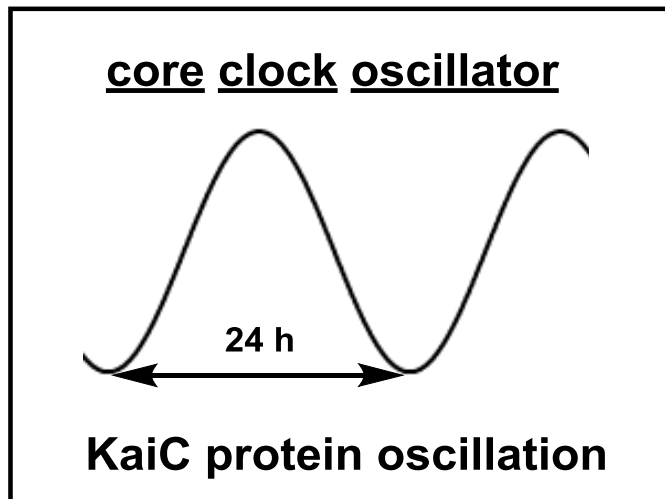
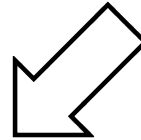


Takao Kondo
(Nagoya Univ.)

circadian rhythm in cyanobacteria

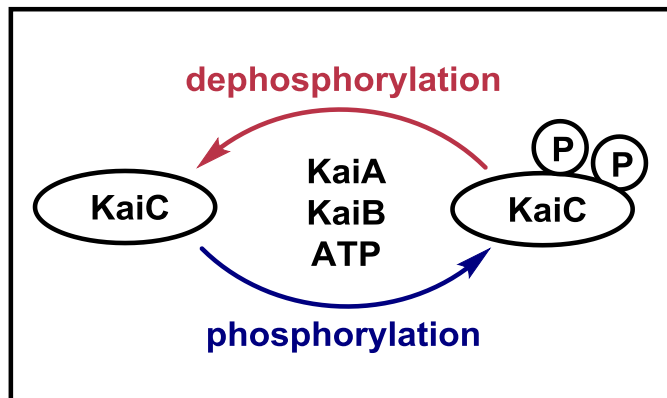
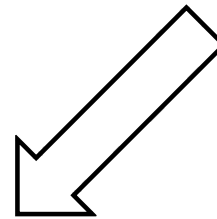
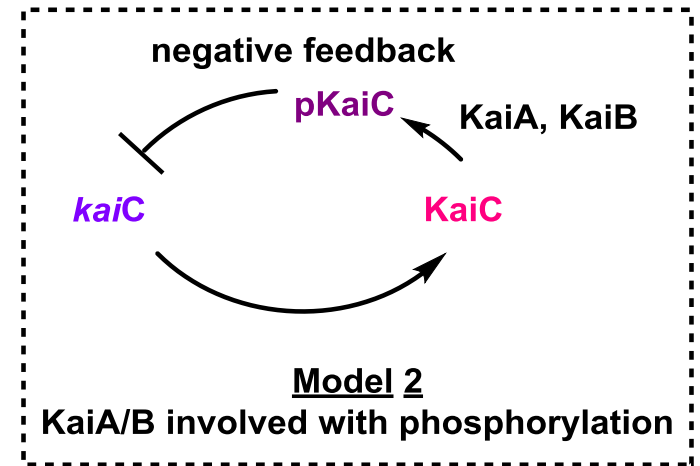
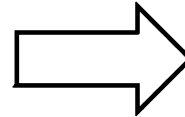
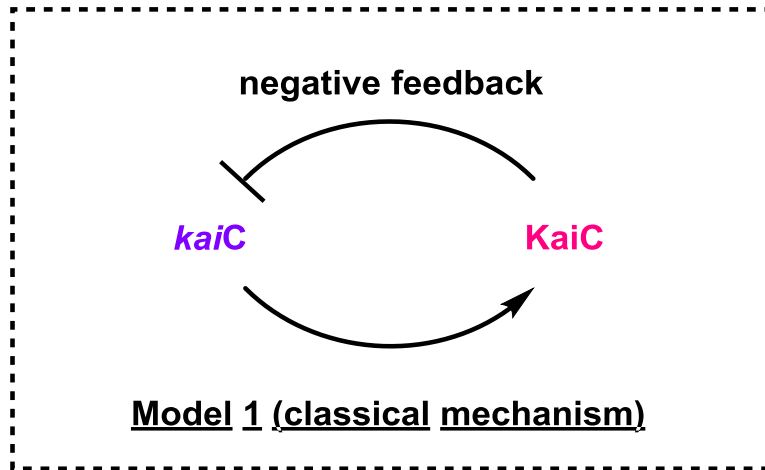


same rhythm even
under continuous light!



- Science*, 1994, 266, 1233.
- Science*, 1997, 275, 224.
- Science*, 1998, 281, 1519.
- Science*, 2000, 289, 765.
- Cell*, 2000, 101, 223.
- Science*, 2005, 307, 251.
- Science*, 2005, 308, 414.
- Mol. Cell.*, 2006, 23, 161.
- Nat. Struct. Mol. Boil.*, 2007, 14, 1084.
- Nat. Commun.*, 2013, 4, 2897.
- Science*, 2016, 349, 312.

Basic Mechanism of KaiC Protein Oscillation

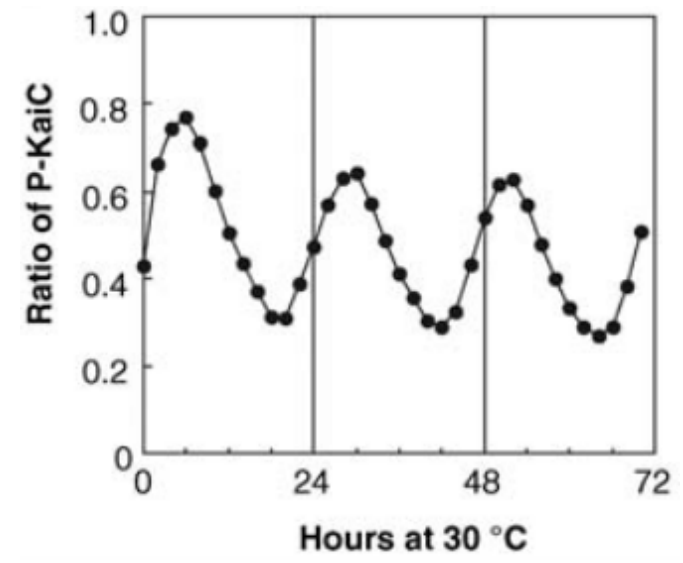
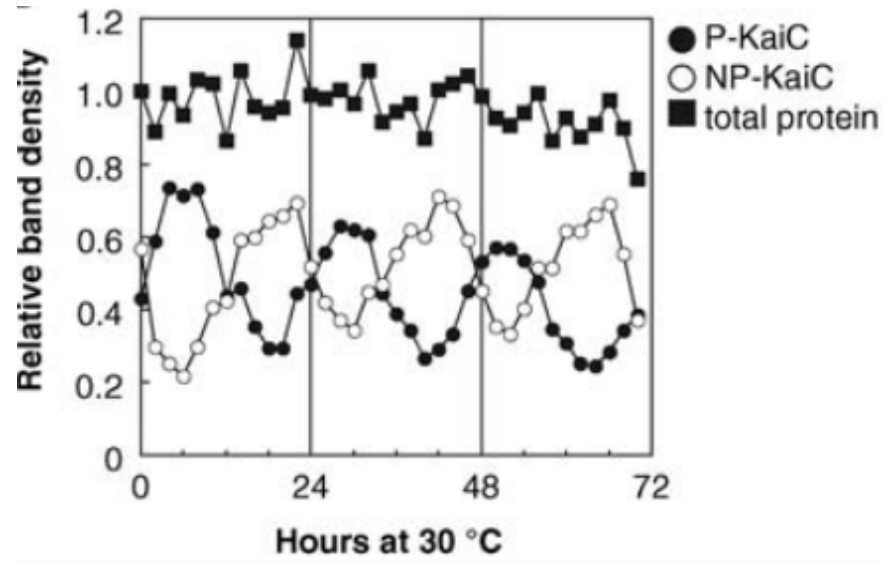
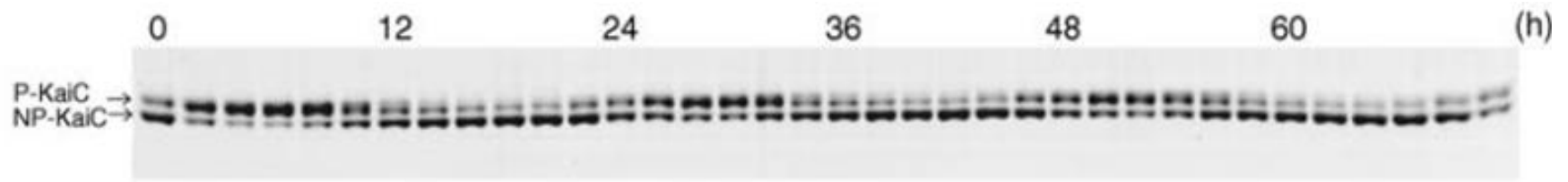
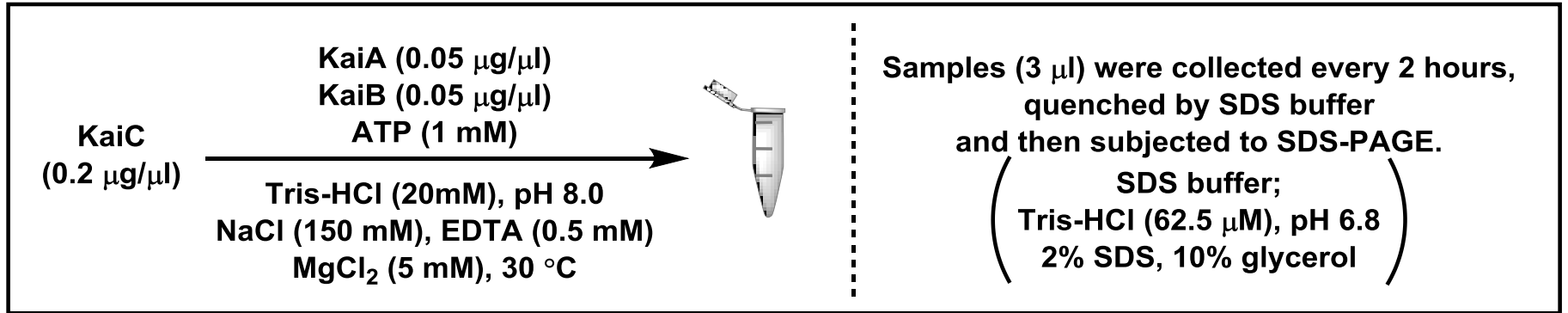


**"Reconstitution of Circadian
Oscillation of Cyanobacterial KaiC
Phosphorylation in Vitro"**

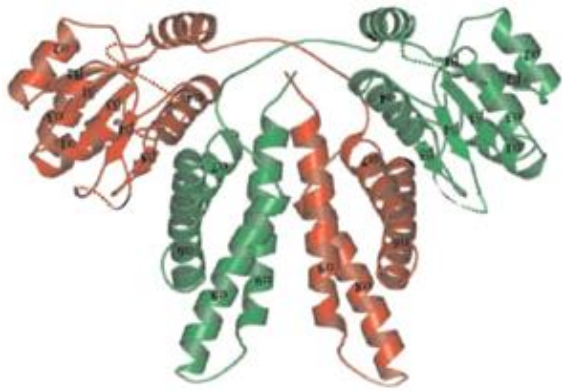
Science, 2005, 308, 414.

- 1) Mechanism of (de)phosphorylation
- 2) Role of KaiA and KaiB
- 3) Structure dynamics of KaiC

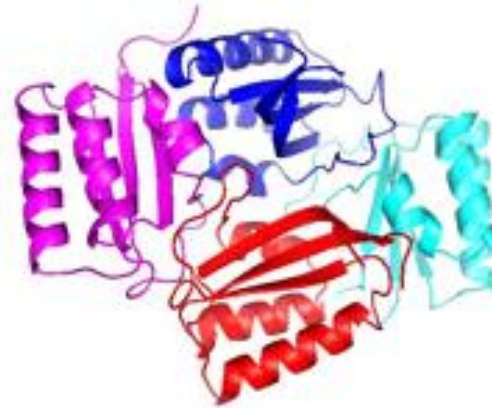
KaiC Protein Oscillation in Vitro



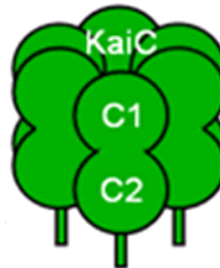
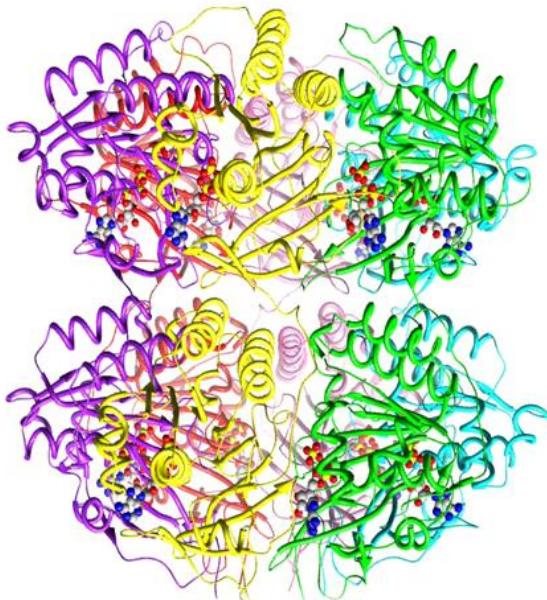
Crystal Structure of KaiA, KaiB and KaiC



KaiA dimer



KaiB tetramer



KaiC hexamer

All possible combination
of interactions are demonstrated.

KaiA-KaiB

KaiB-KaiC

KaiC-KaiA

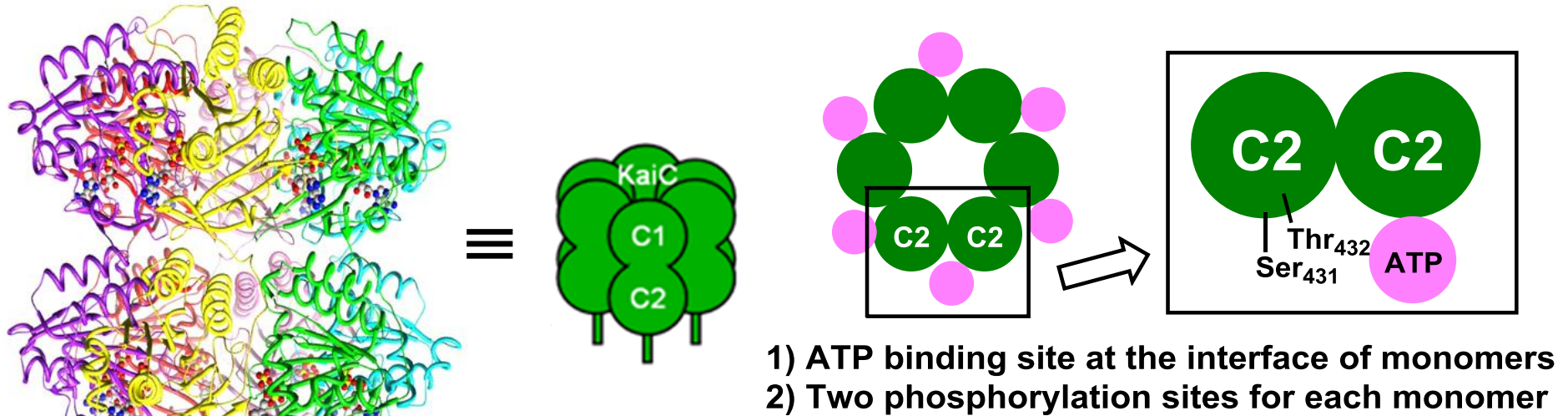
KaiA-KaiB-KaiC

EMBO J., 1999, 18, 1137.

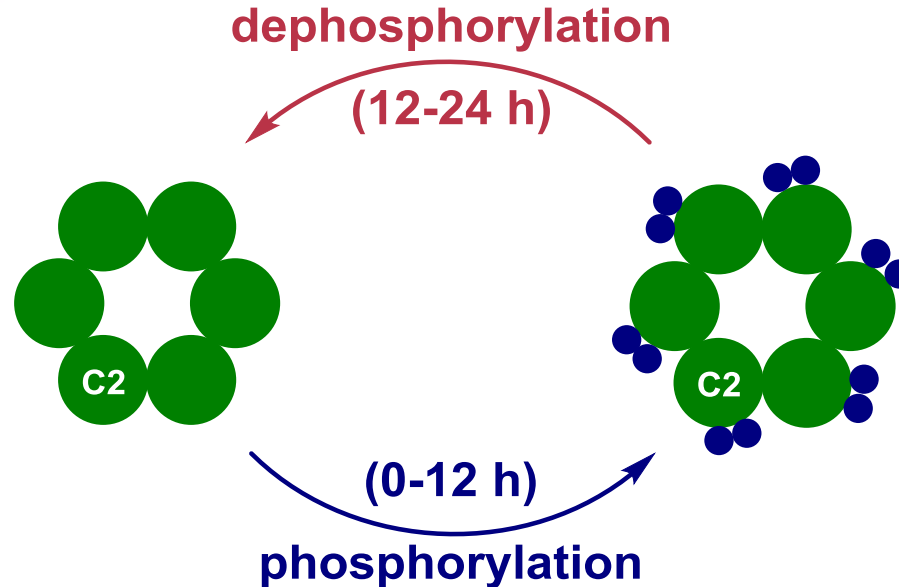
J. Biol. Chem., 2003, 278, 2388.

Egli, M. *et. al.*, *Molecular Cell*, 2004, 15, 375.

Phosphorylation Sites

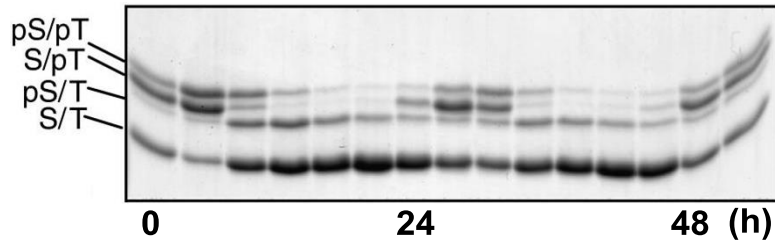


PNAS, 2004, 101, 13927.

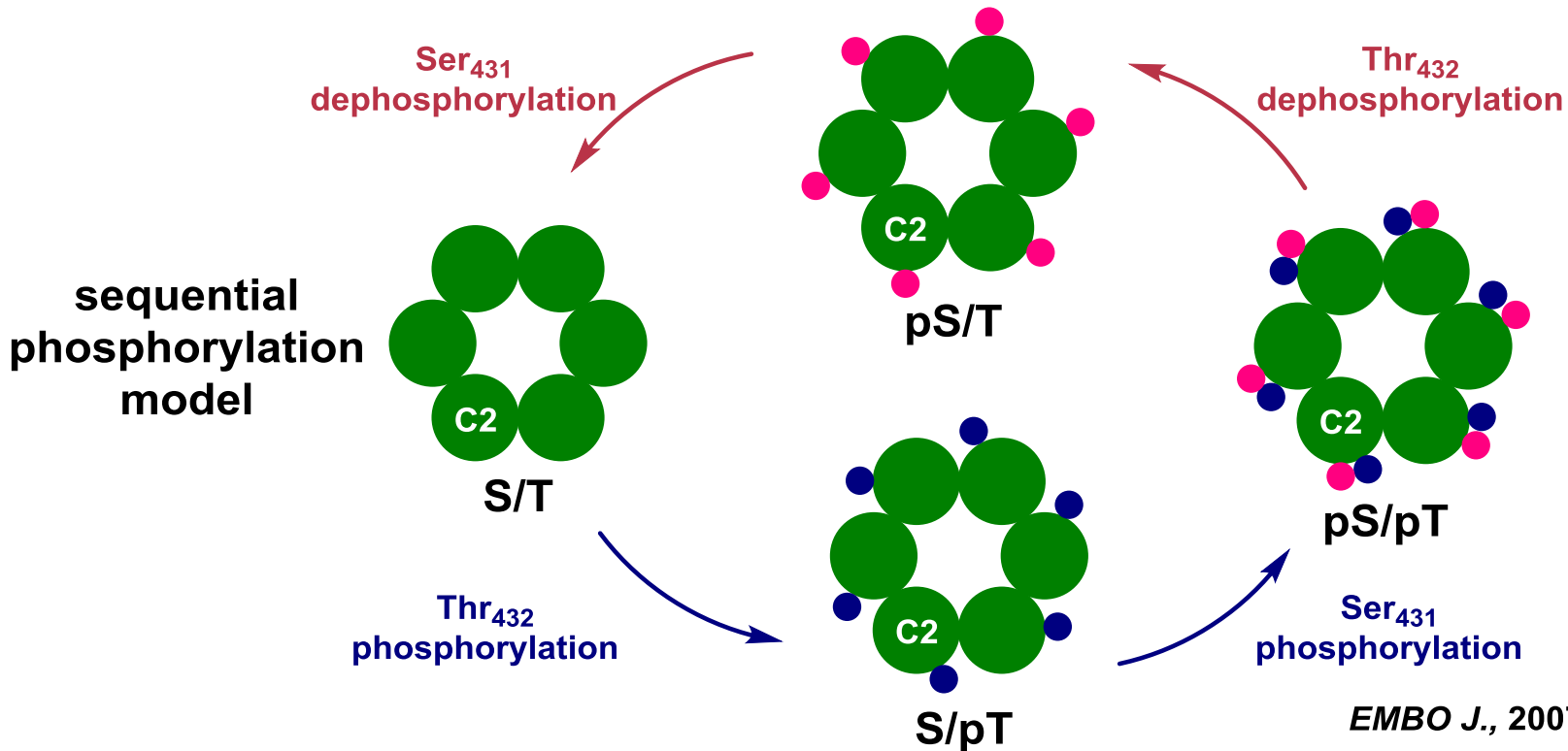
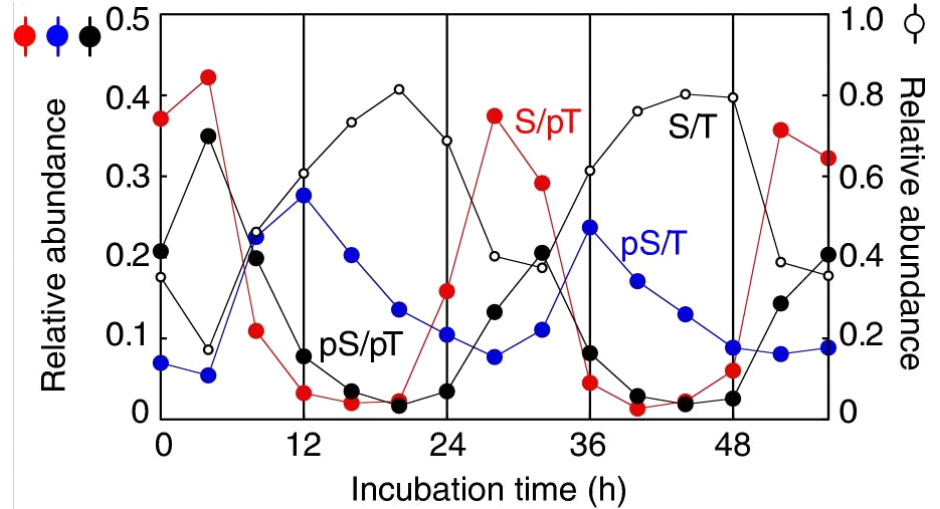


Twelve phosphorylation sites for KaiC hexamer

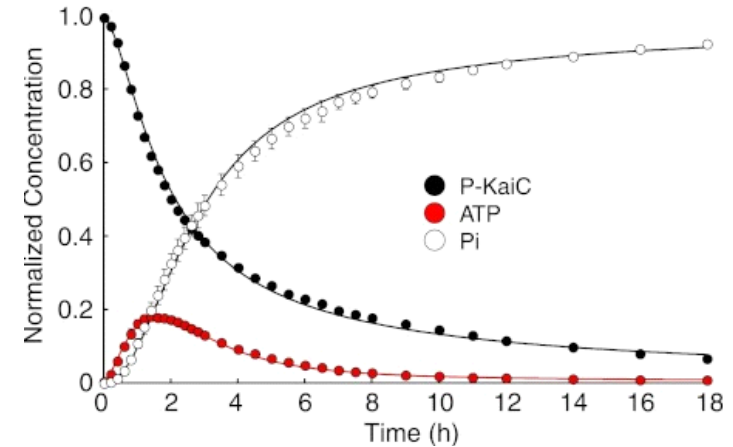
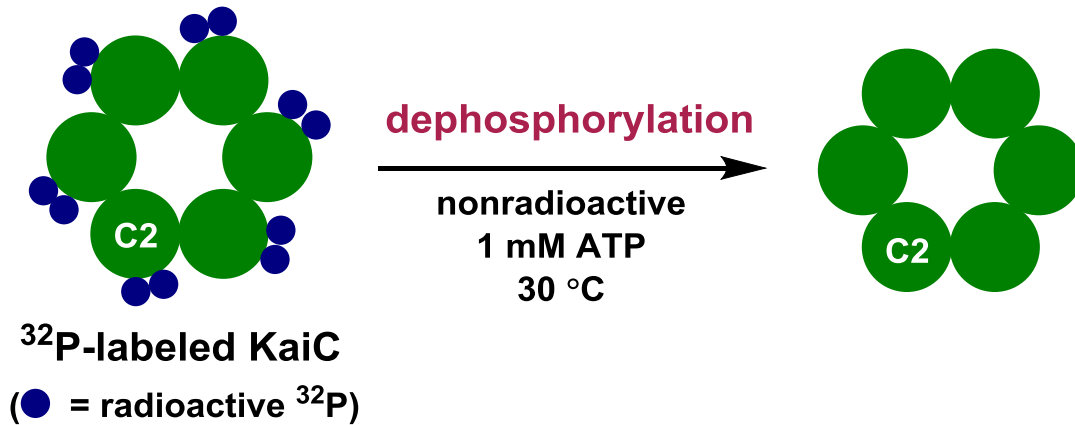
Sequential Phosphorylation and dephosphorylation



Improved SDS-PAGE shows four phosphorylation states.

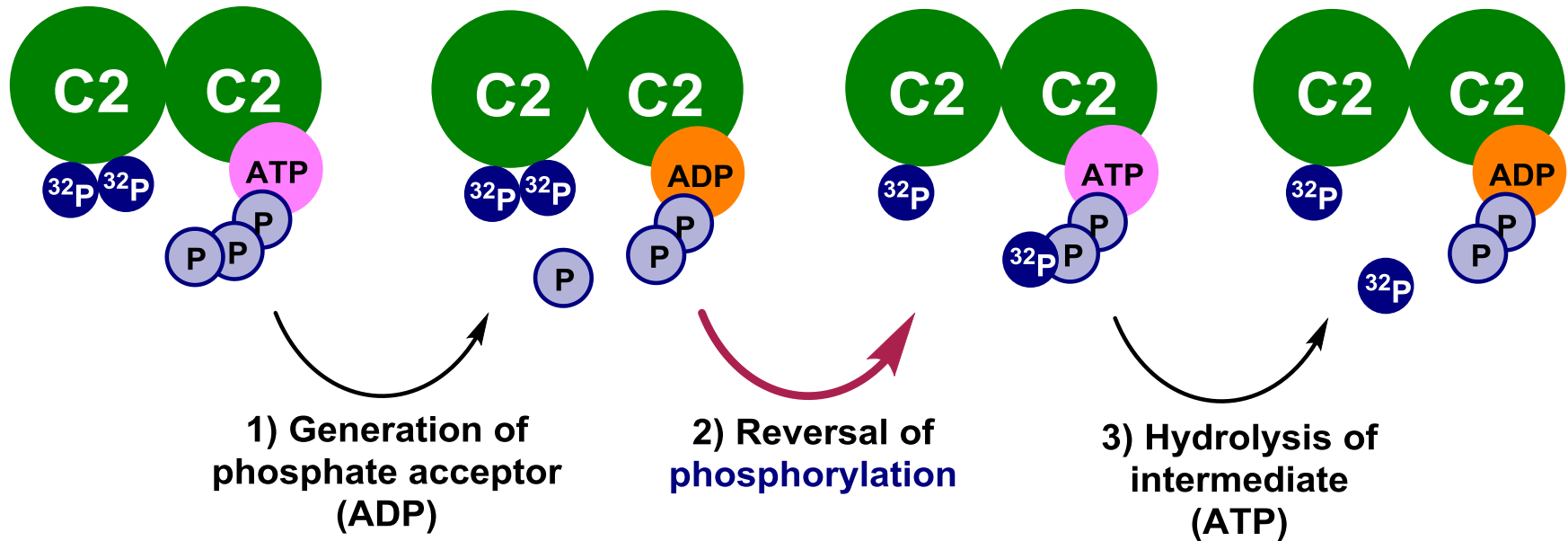


Autodephosphorylation via Formation of ATP as Intermediate



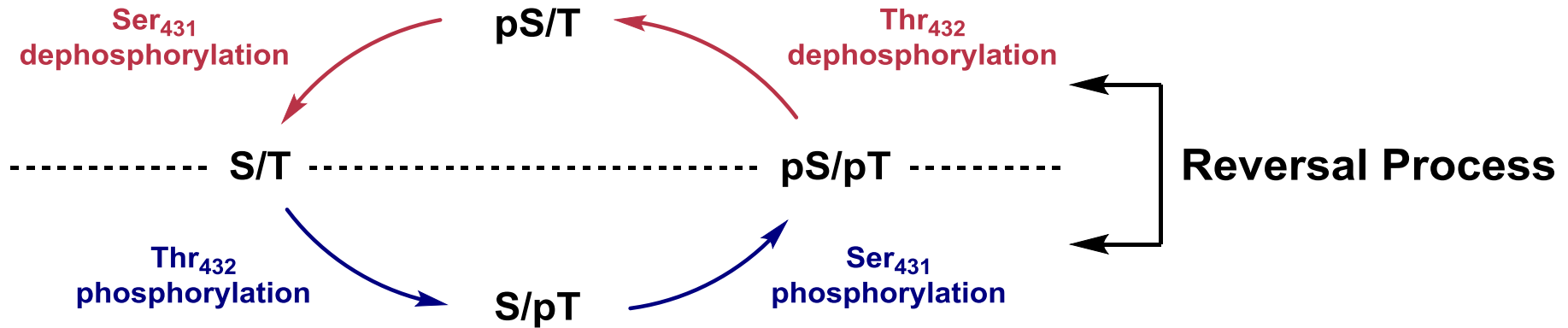
J. Bio. Chem., 2012, 287, 18030.

Transient radioactive ATP formation prior to increase of P_i levels!

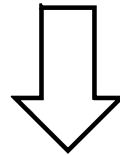


KaiC undergoes **dephosphorylation** via the reversal of **phosphorylation**.

Short Summary 1

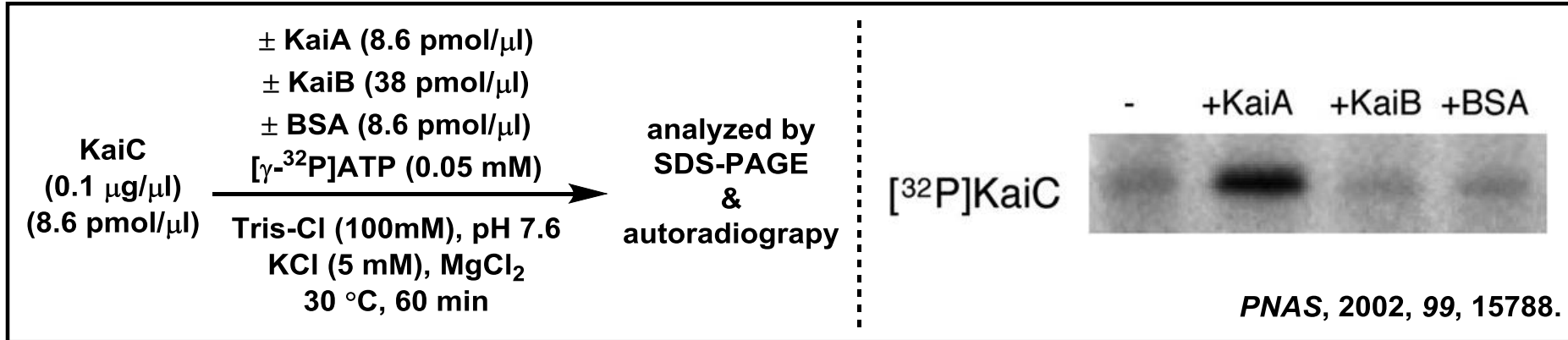


How is the phosphorylation equilibrium periodically shifted?

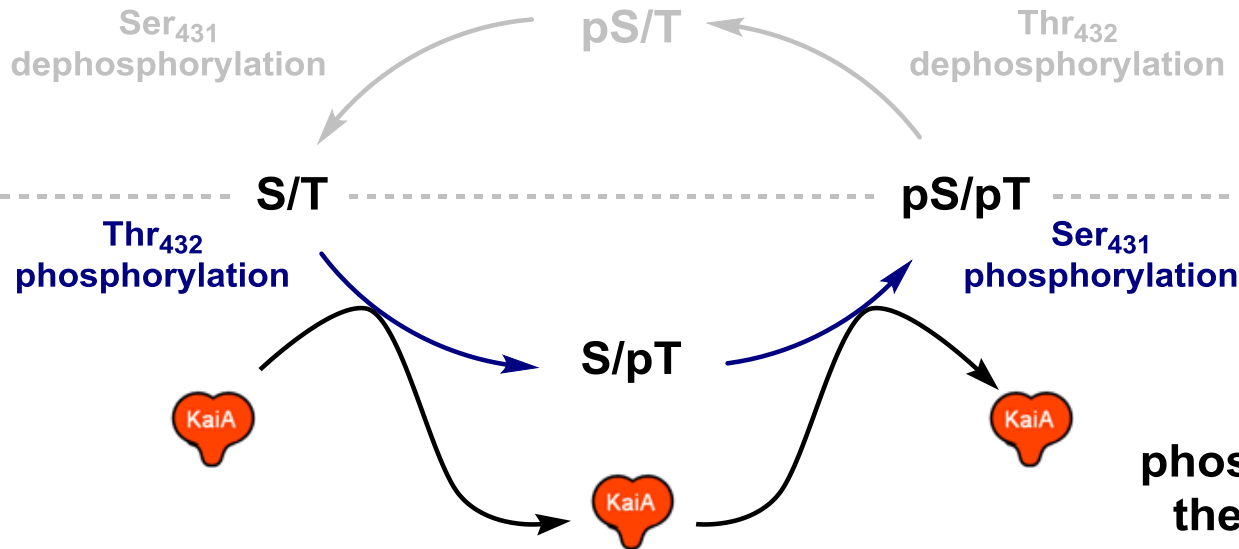


- 1) Interaction with KaiA and KaiB**
- 2) Structure dynamics of KaiC**

Role of KaiA



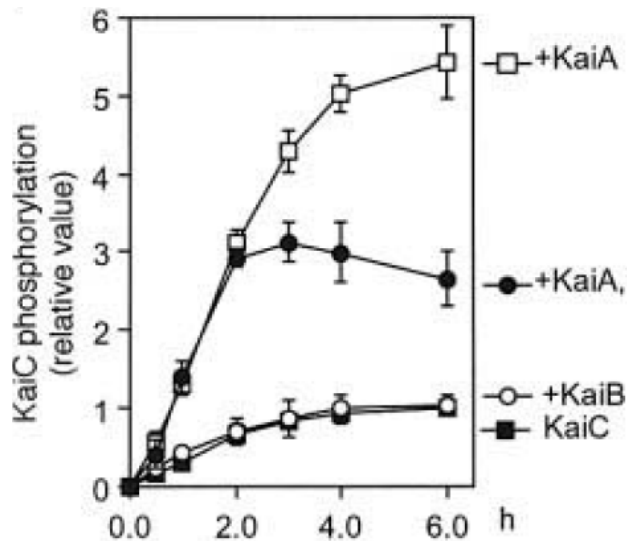
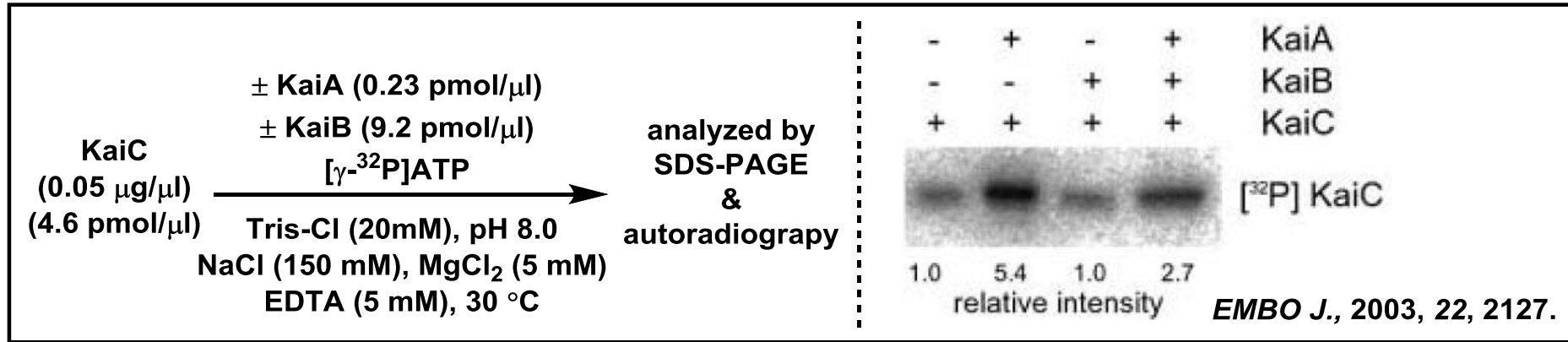
KaiA functions as phosphorylation promoter.
(KaiA shifts the equilibrium to phosphorylation phase.)



KaiA enhanced phosphorylation level even with the ratio of KaiA:KaiC = 1:20.

EMBO J., 2003, 22, 2127.

Role of KaiB

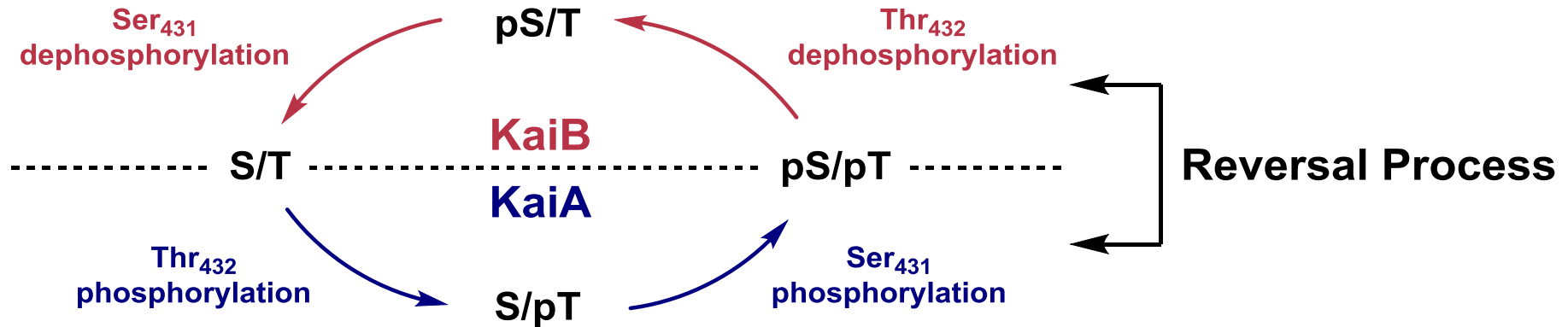


KaiA enhances phosphorylation levels.

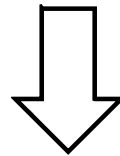
KaiB decreases phosphorylation levels.

- 1) Activation of **dephosphorylation**
or
- 2) Inhibition of **KaiA**

Short Summary 1



How is the phosphorylation equilibrium periodically shifted?

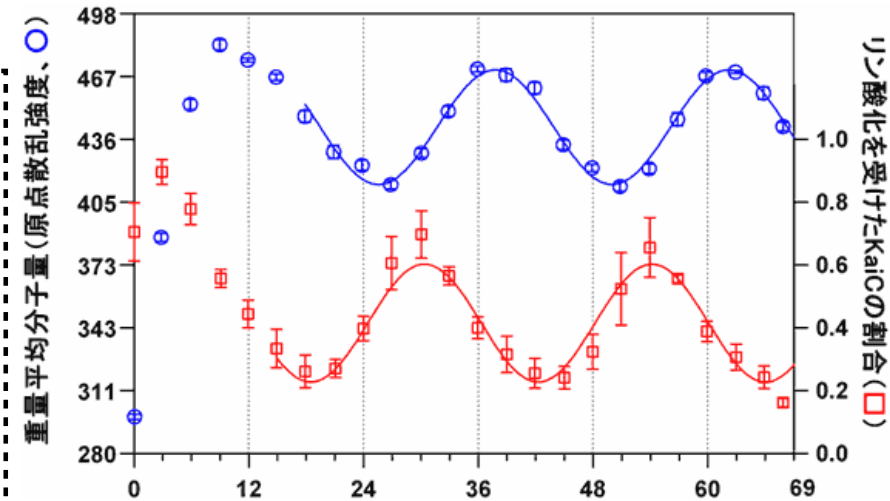
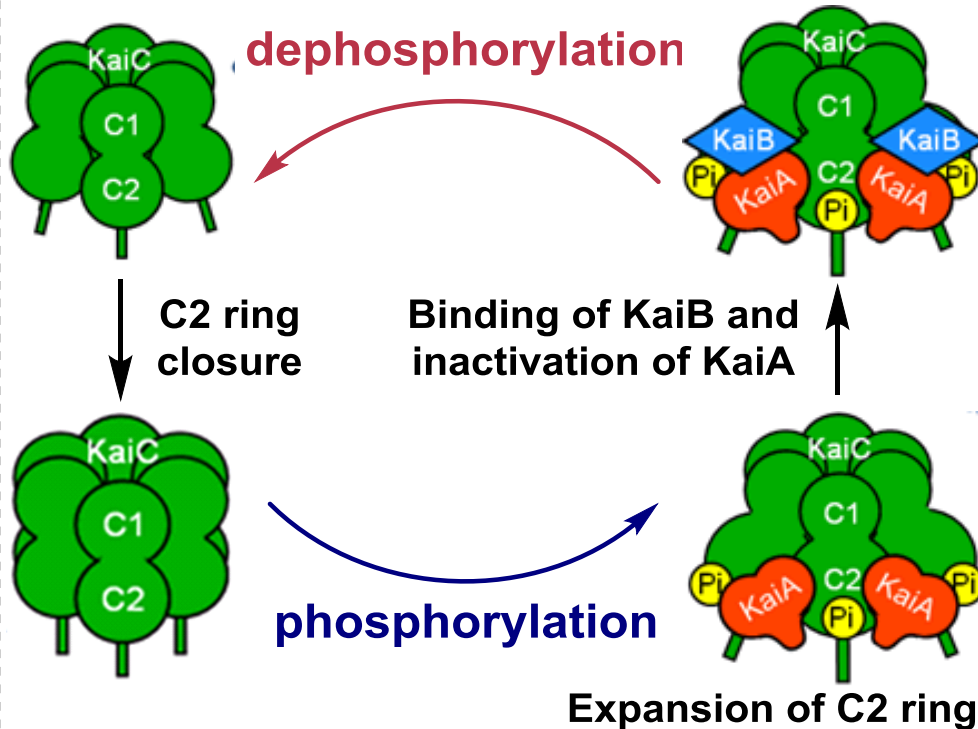


- 1) Interaction with KaiA and KaiB**
- 2) Structure dynamics of KaiC**

Structural Dynamics of KaiC and Interaction with KaiB

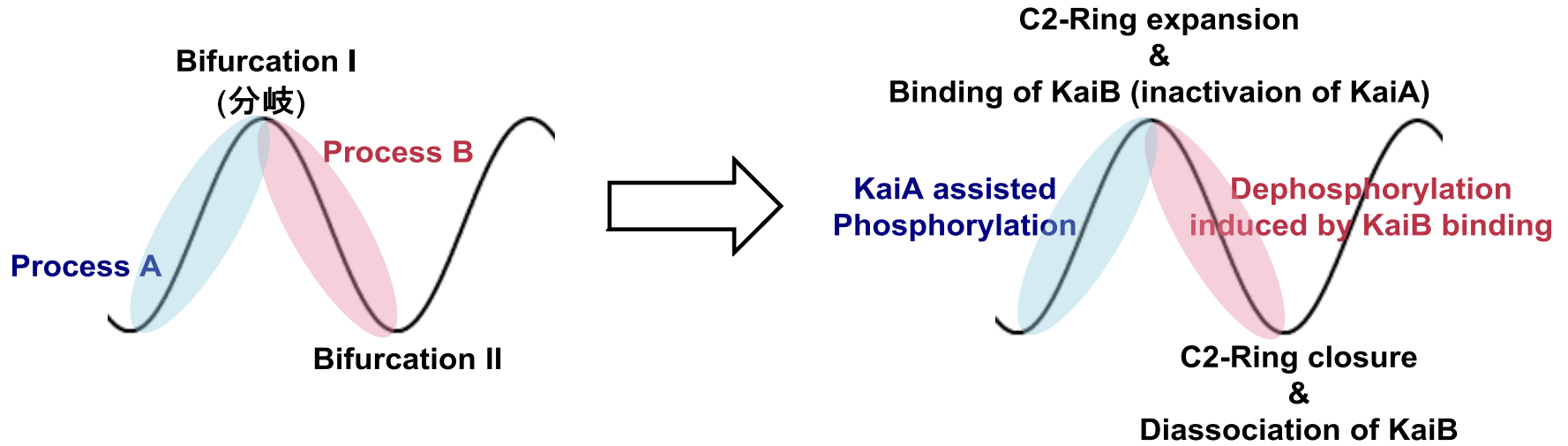
- 1) SAXS (Small Angle X-ray Scattering) shows structural transition of KaiC coupling with phosphorylation level. 2) SAXS shows assembly-disassembly dynamics coupling with phosphorylation level.

Possible Model for Oscillation

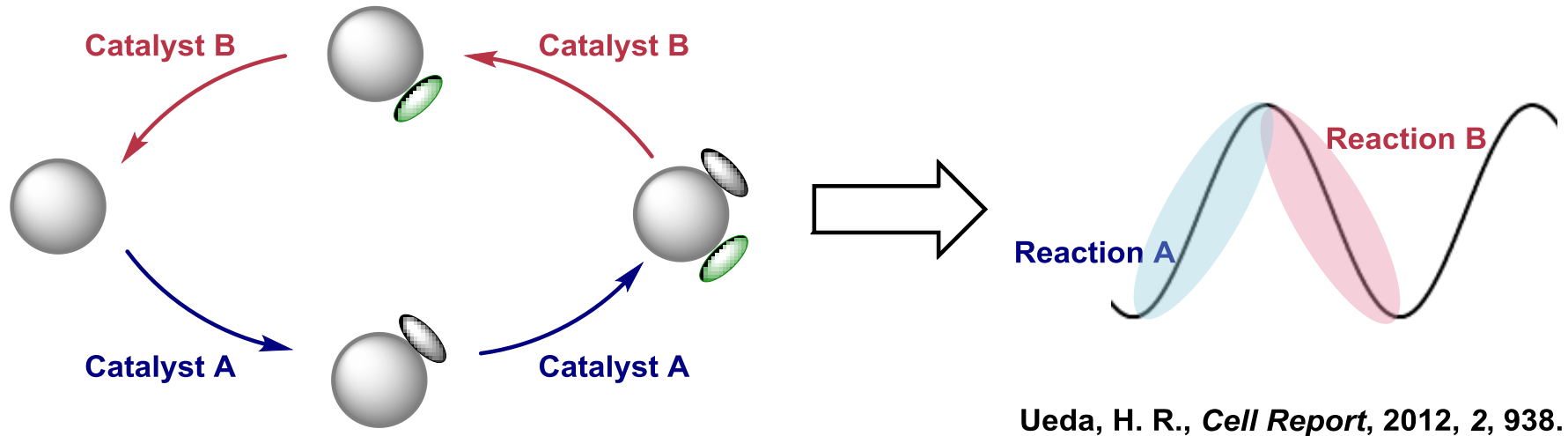


Maeda, Y. et al., *Molecular Cell*, 2008, 29, 703.
Akiyama, S. et al., *EMBO J.*, 2011, 30, 68.

Short Summary 2



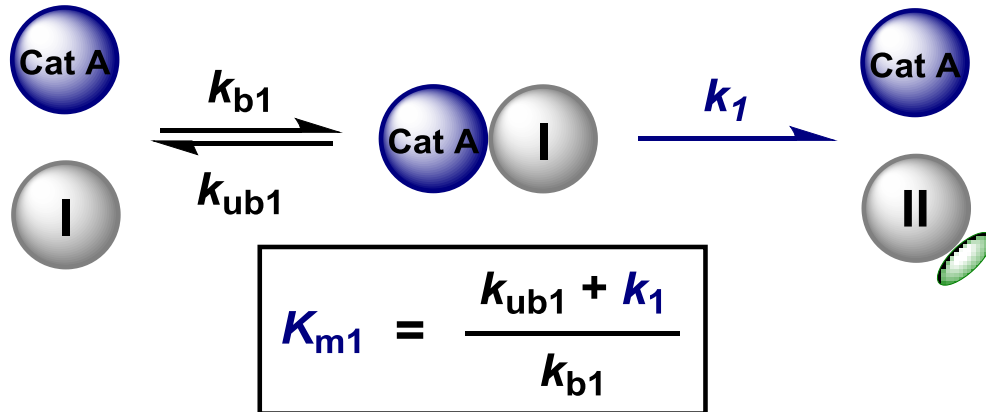
Possible to design the reaction below?



Ueda, H. R., *Cell Report*, 2012, 2, 938.

3. A Design Principle

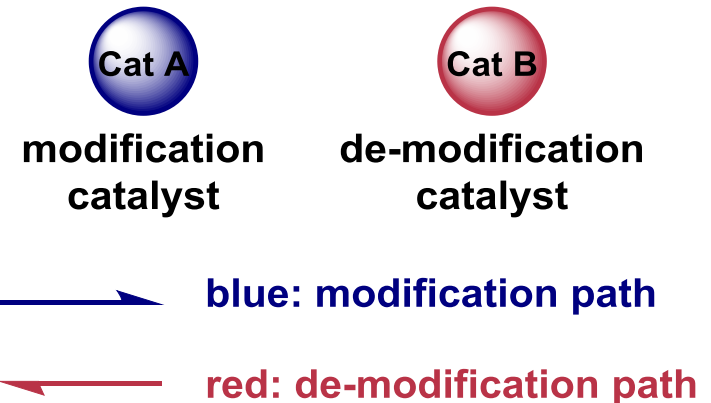
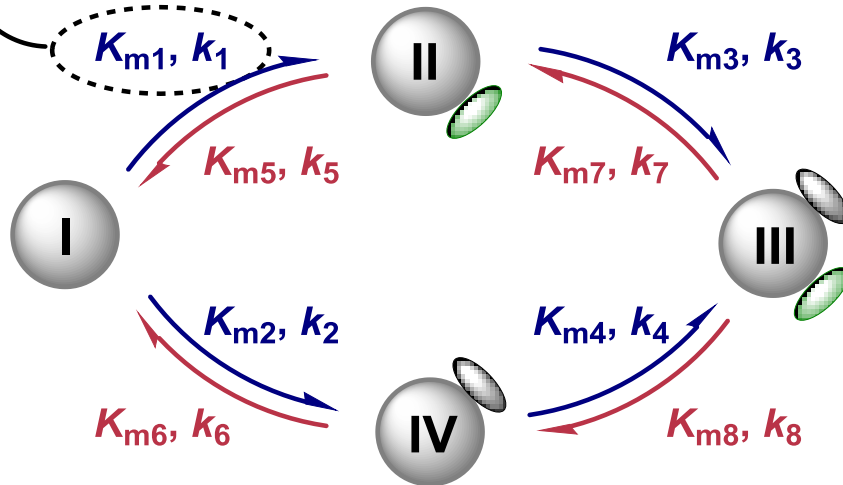
Simulation Study to Cause Oscillation



k_{1-8} : rate constant (min^{-1})

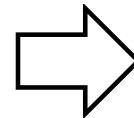
K_{m1-8} : Michaelis constant (μM)

(The lower K_m is, the stronger binding of substrate and catalyst is.)



Random 1,280,000,000 parameter sets were simulated.

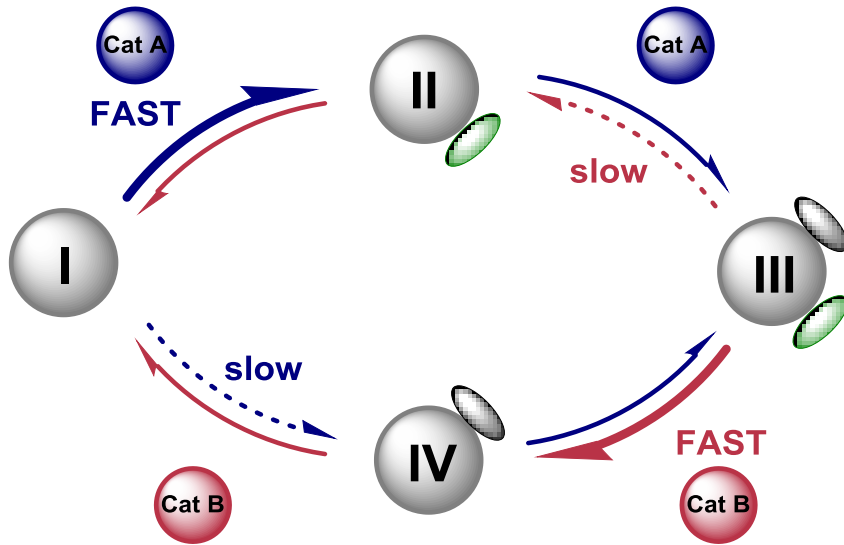
($1 < k_{1-8} < 1000$ and $0.01 < K_{m1-8} < 1000$)



0.094 % hit rate for oscillation

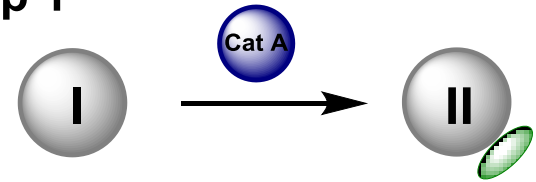
Minimum Requirements for Oscillation: k_{1-8}

Cluster 1

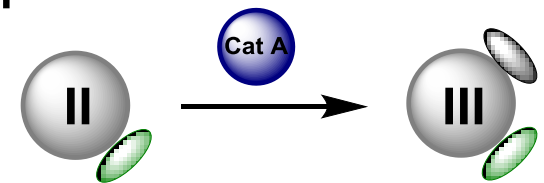


high k_1 and k_8 (two FAST reactions)
low k_2 and k_6 (two slow reactions)

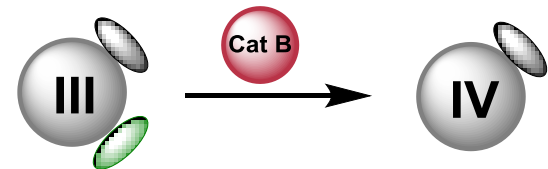
Step 1



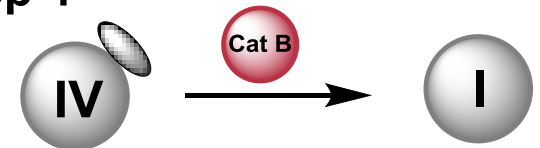
Step 2



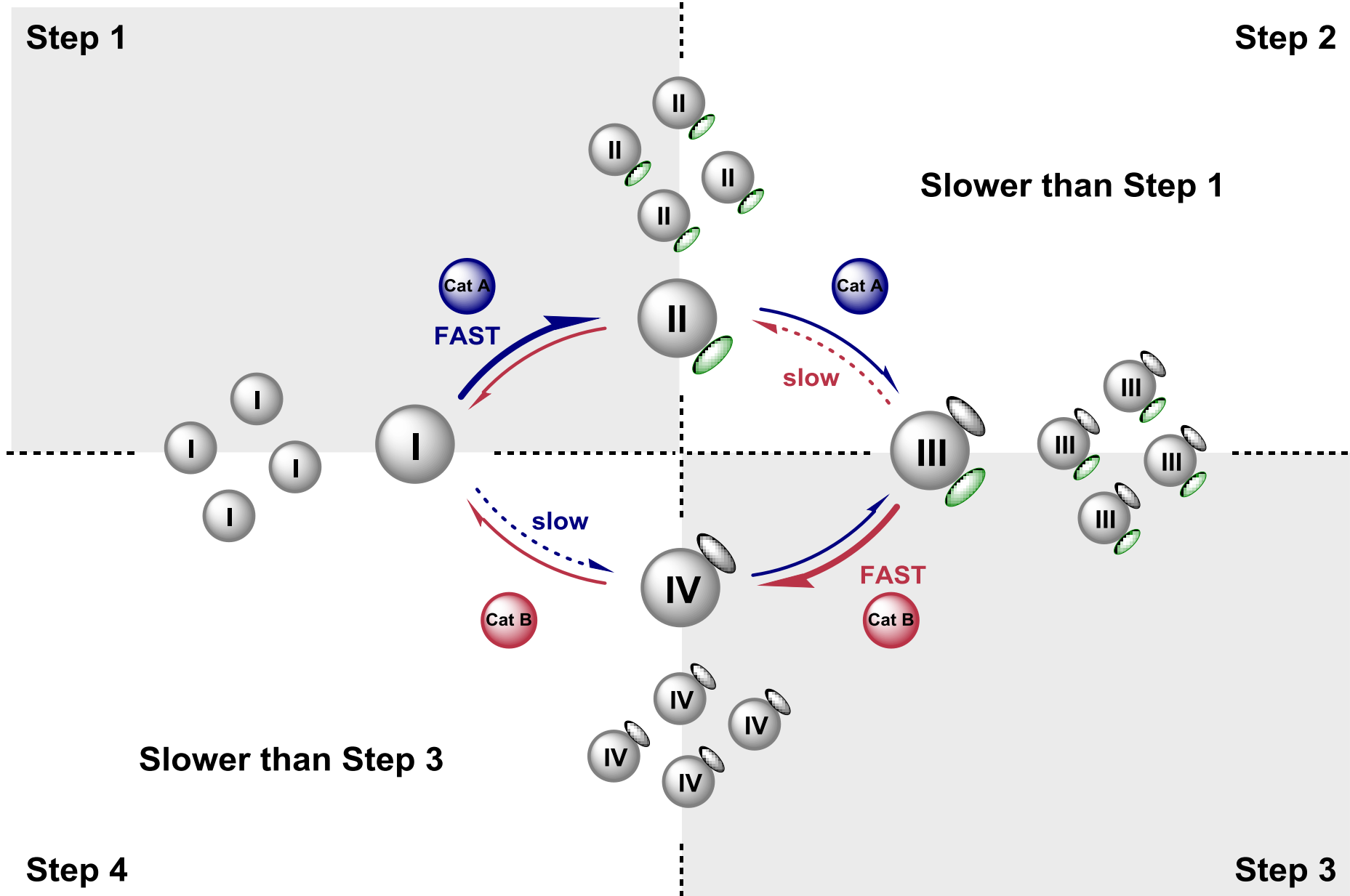
Step 3



Step 4



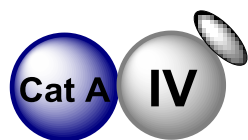
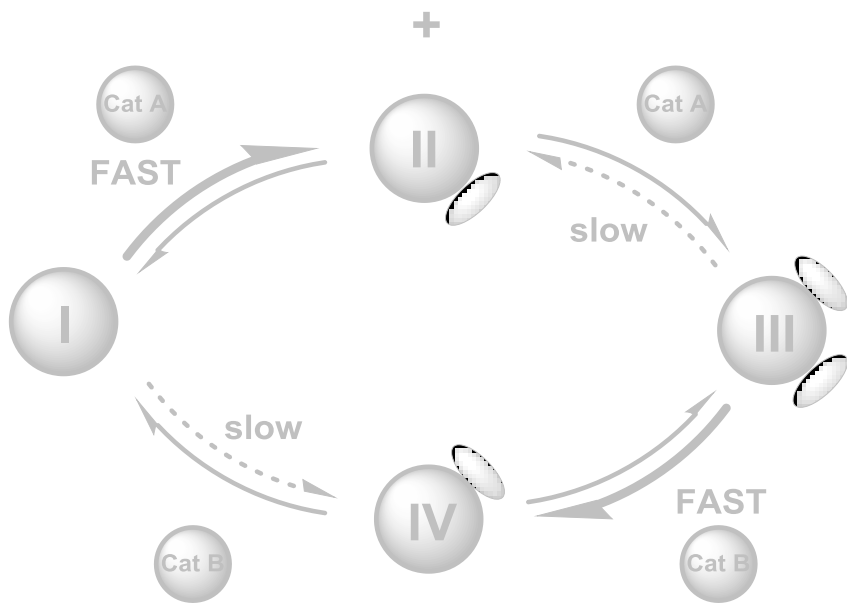
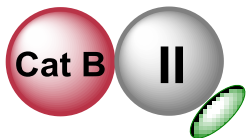
Schematic View of Simulated Oscillation



Minimum Requirements for Oscillation: K_{m1-8}

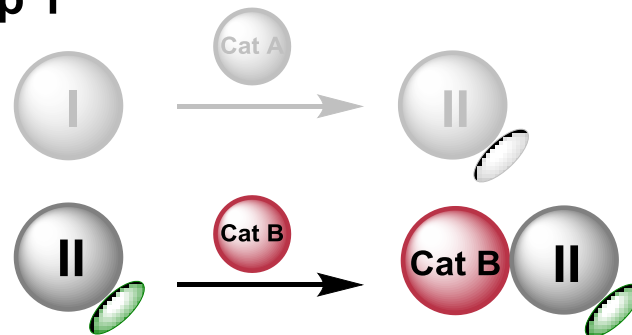
Cluster 1

strong binding
(low K_{m5})

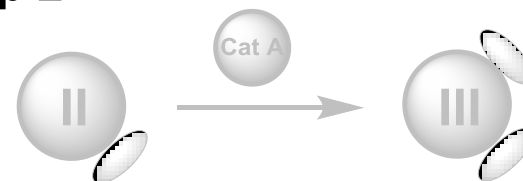


strong binding
(low K_{m8})

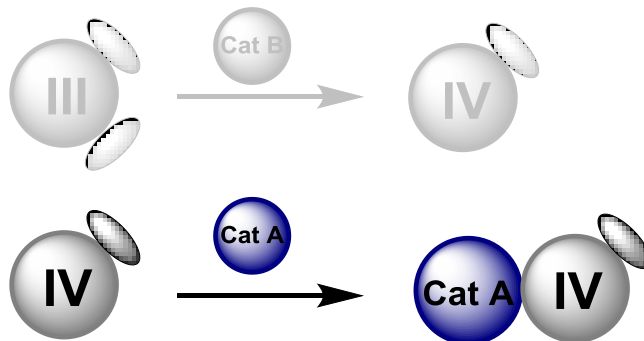
Step 1



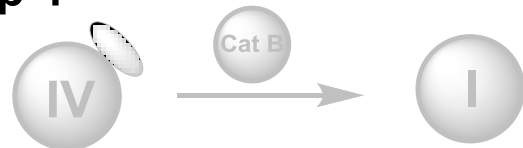
Step 2



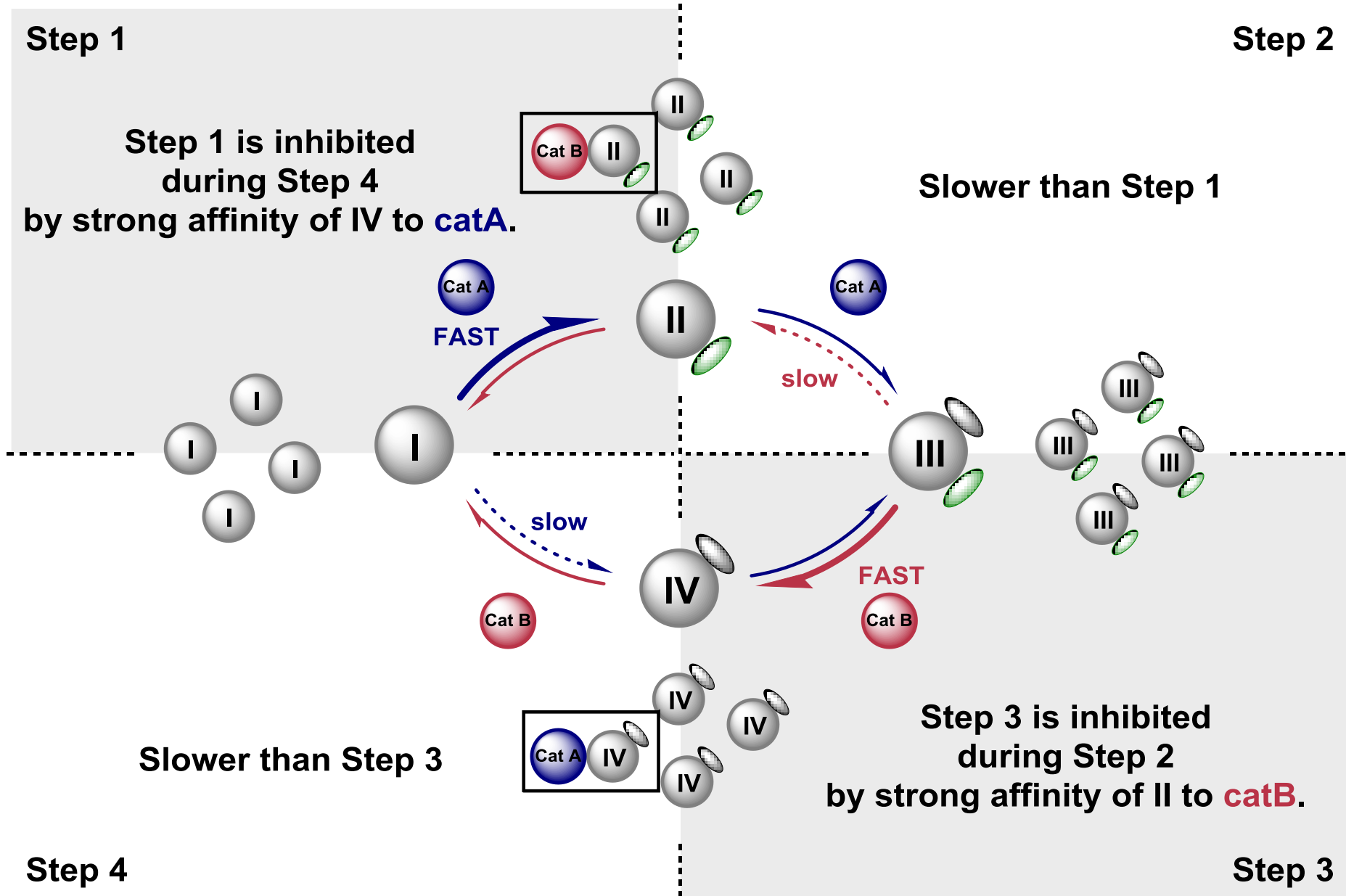
Step 3



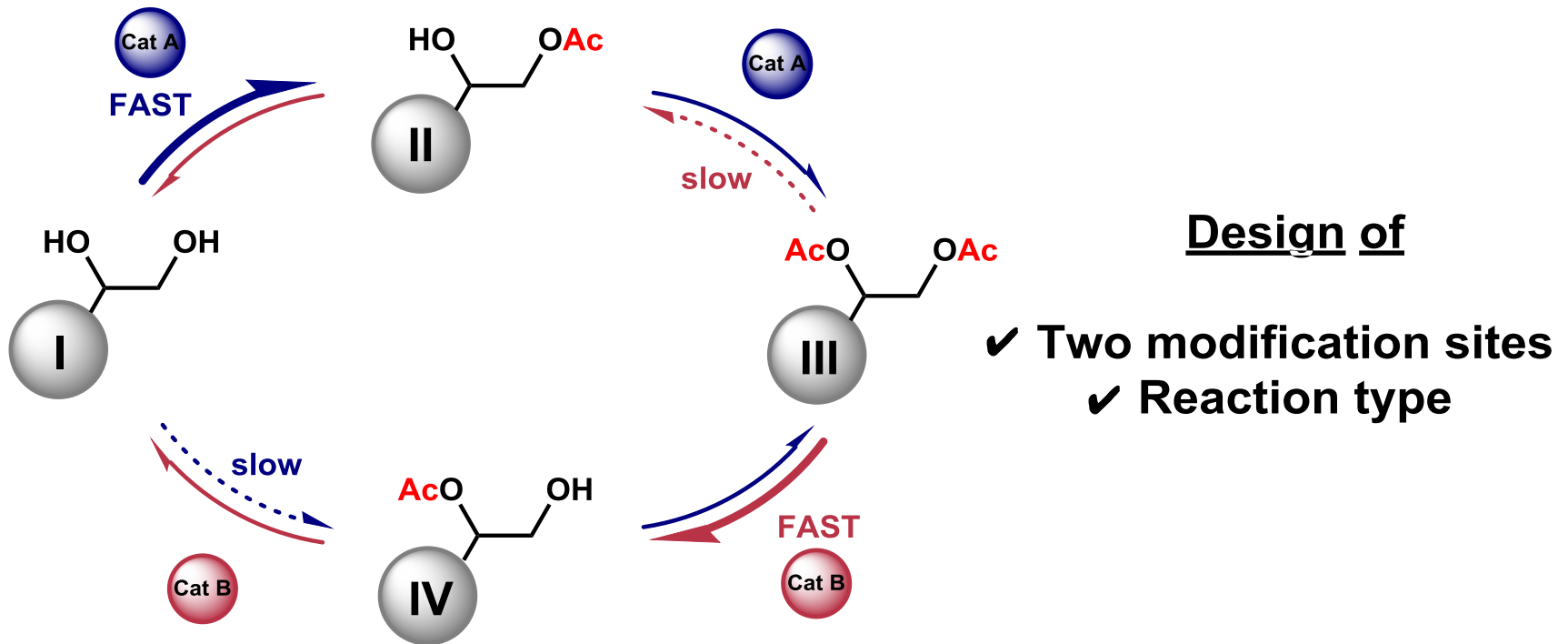
Step 4



Schematic View of Simulated Oscillation



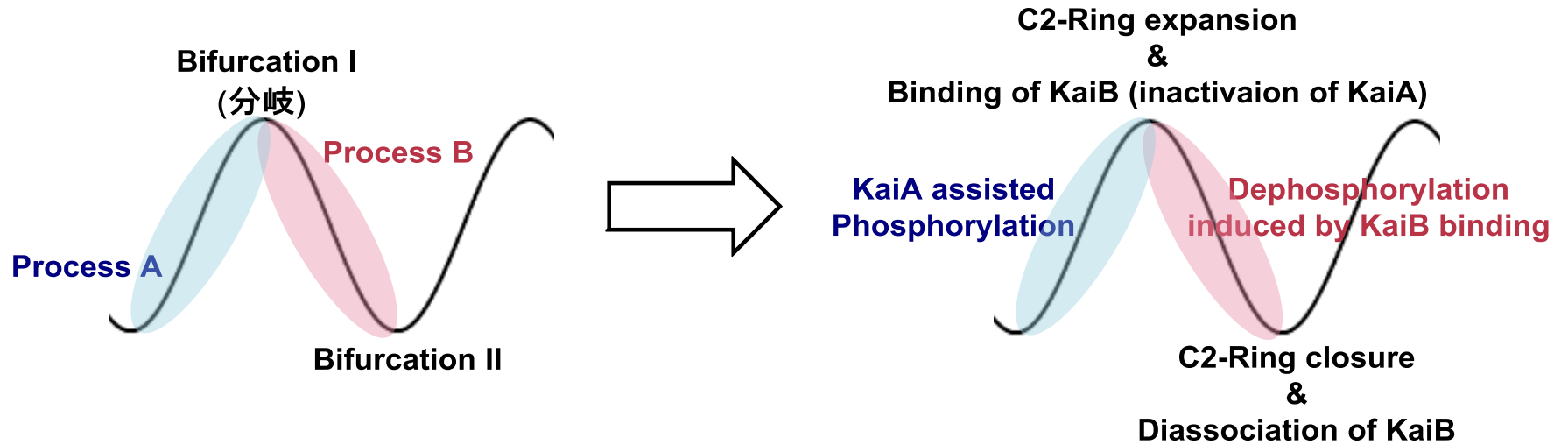
What Needed To Be Designed?



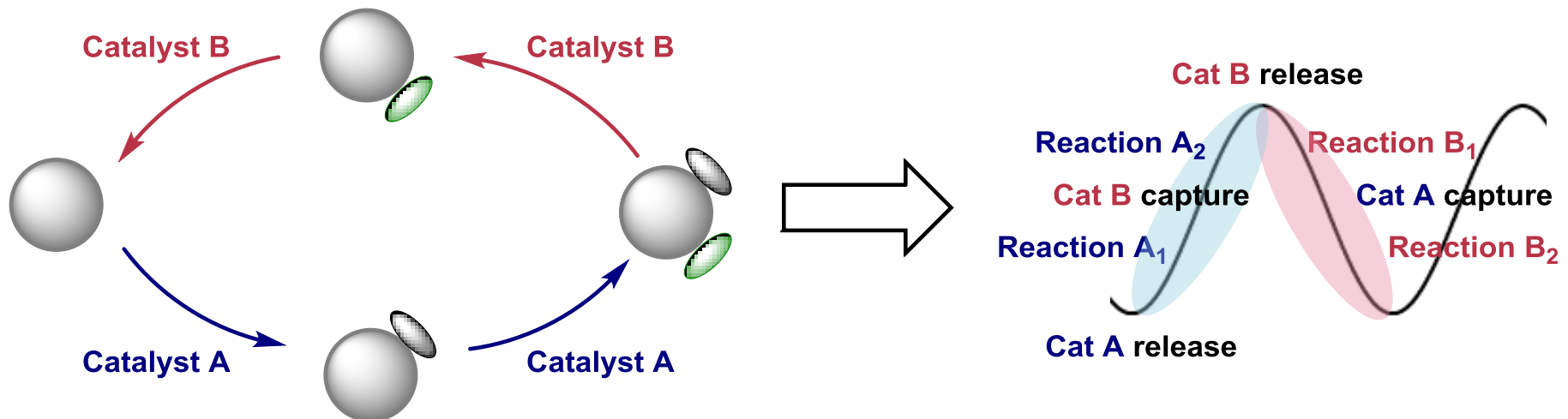
How can be the catalyst-substrate binding designed to fulfill these requirements?

Summary

2. KaiC Protein Oscillation in Vitro

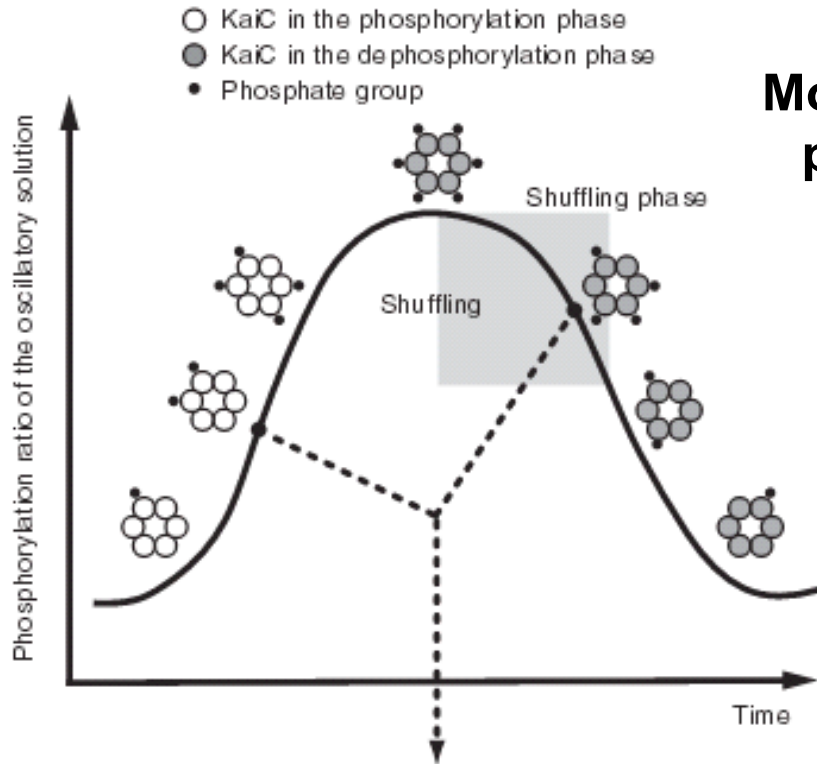


3. A Design Principle



Appendix

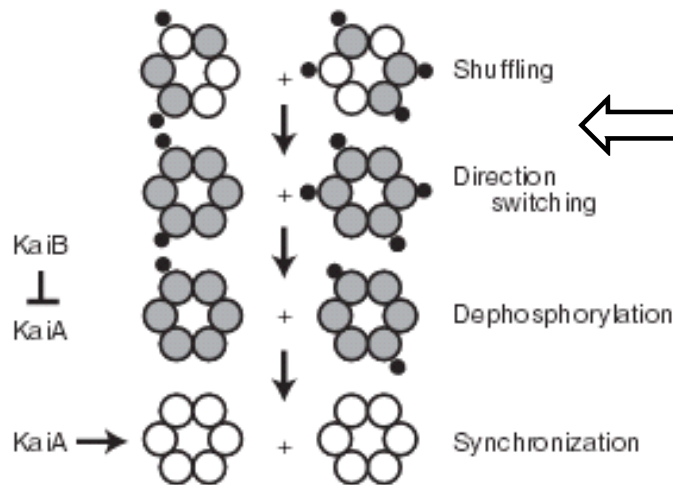
1. A Model for Synchronization of the KaiC Phosphorylation



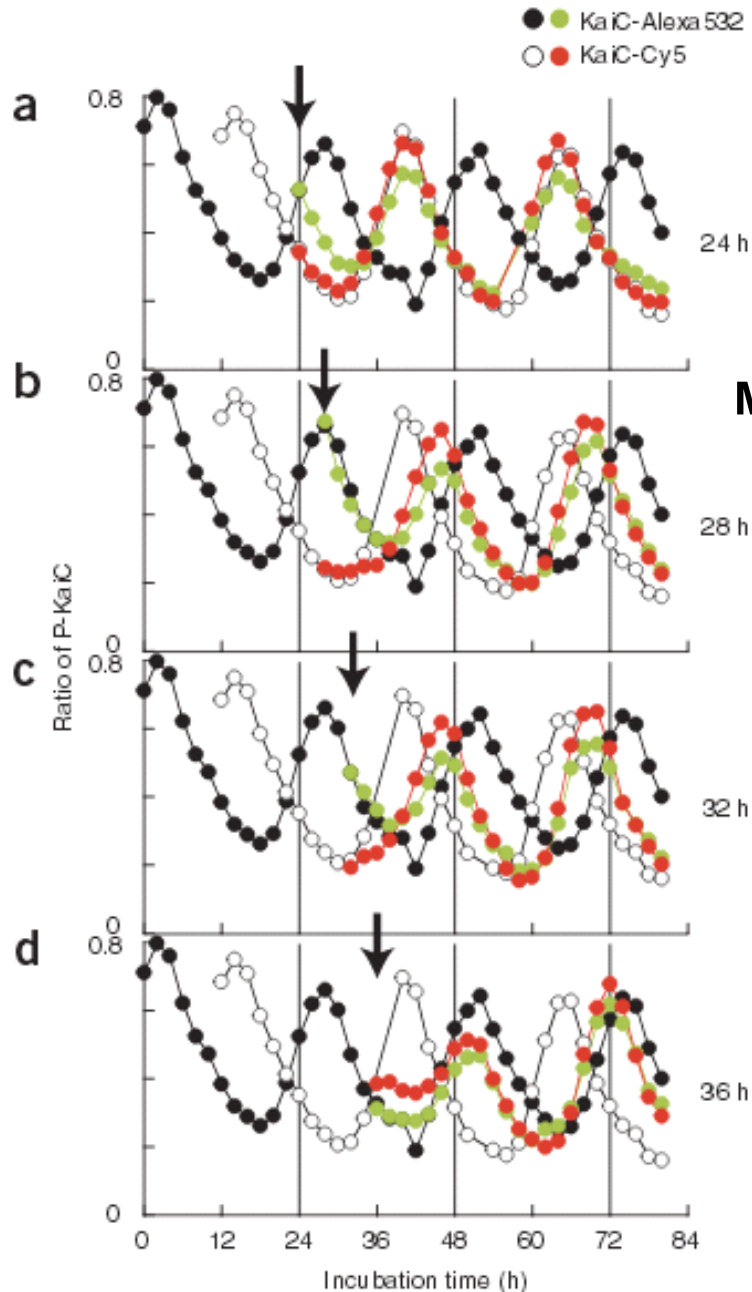
Monomer Shuffling between KaiC hexamer plays a critical role for synchronization.

↑
experiments on next slides.
↓

Synchronization occurs at dephosphorylation phase.



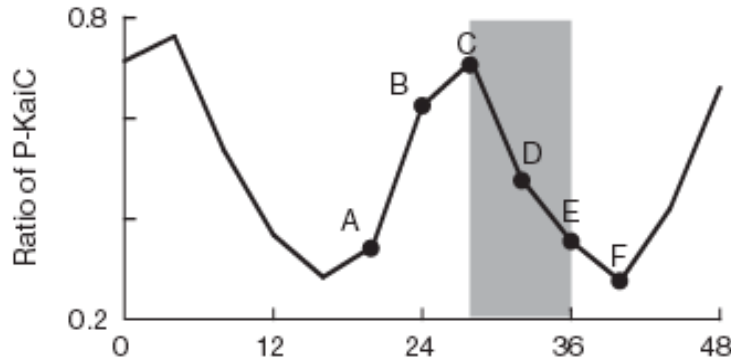
Molecular Cell, 2006, 23, 161.
Nat. Struct. Mol. Boil., 2007, 14, 1084.
Nat. Commun., 2013, 4, 2897.



Mixing different phase KaiC oscillators (black/white) resulted in rapid synchronization (green/red).

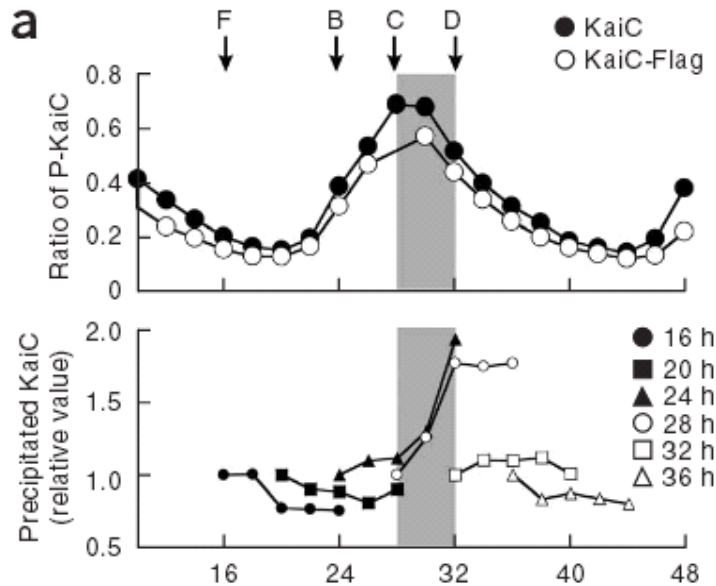
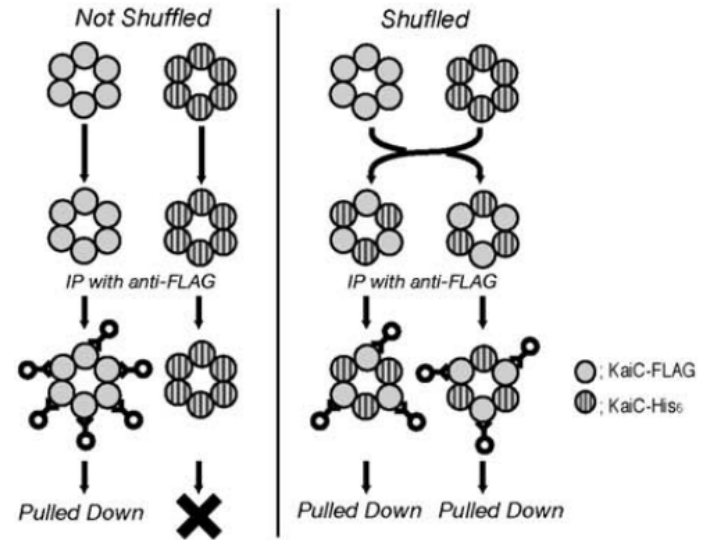
Figure 2 Synchronization of two mixed samples. Recombinant KaiC protein was conjugated to fluorochromes (Alexa Fluor 532 or Cy5). Fluorescence from the two labeled KaiCs was quantified separately to determine their phosphorylation ratios. We prepared KaiC samples with phosphorylation rhythms in opposite phases by starting incubation of KaiC–Alexa Fluor 532 (black) at 0 h and that of KaiC–Cy5 (white) at 12 h. Aliquots of these were mixed (indicated by arrows) in an equimolar ratio at 24 h (a), 28 h (b), 32 h (c) and 36 h (d). Each solution was sampled at intervals of 2 h and ratios of phosphorylated KaiC (P-KaiC) to total KaiC plotted. Green and red circles represent KaiC–Alexa Fluor 532 and KaiC–Cy5 in the mixture, respectively.

Q. When does shuffling occur?



F, A, B: phosphorylation phase
C, D, E: dephosphorylation phase

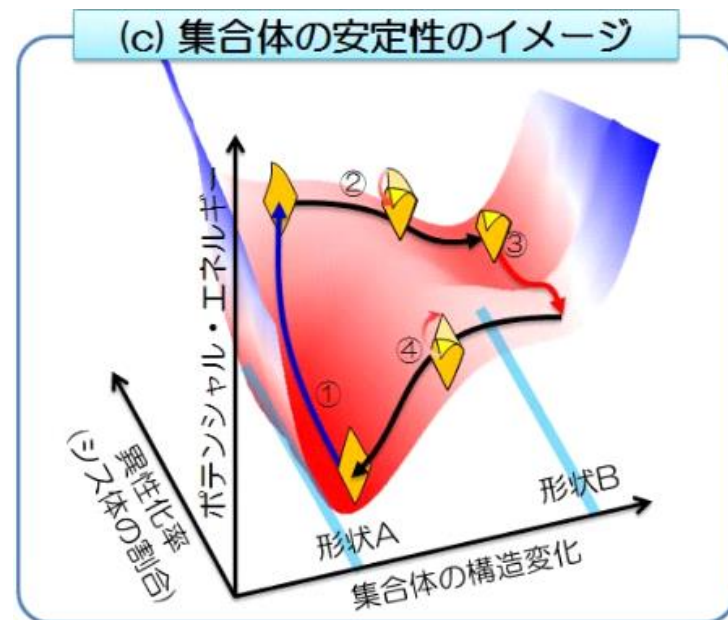
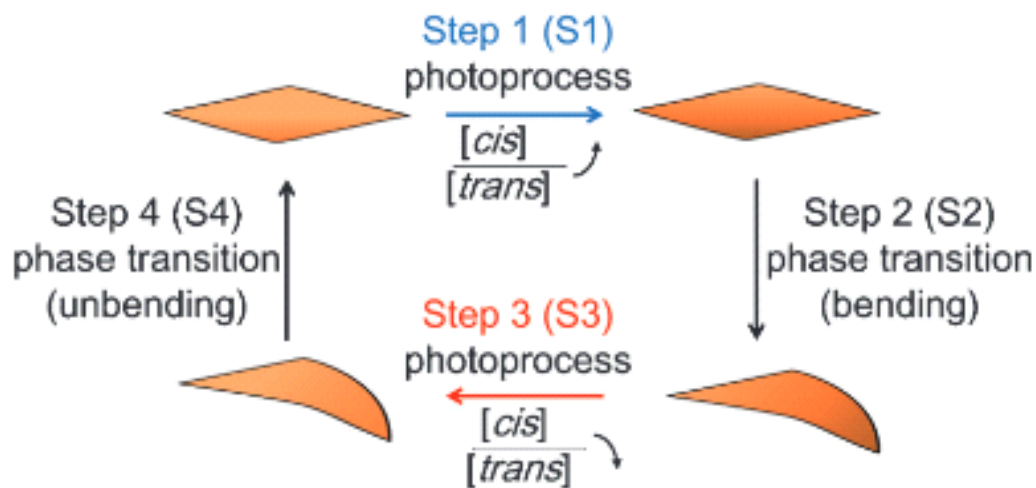
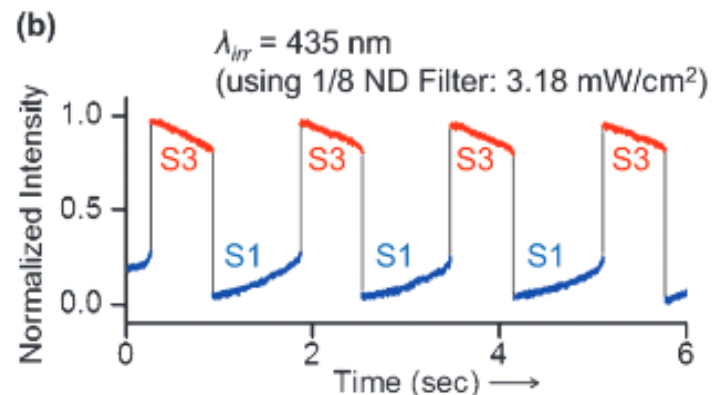
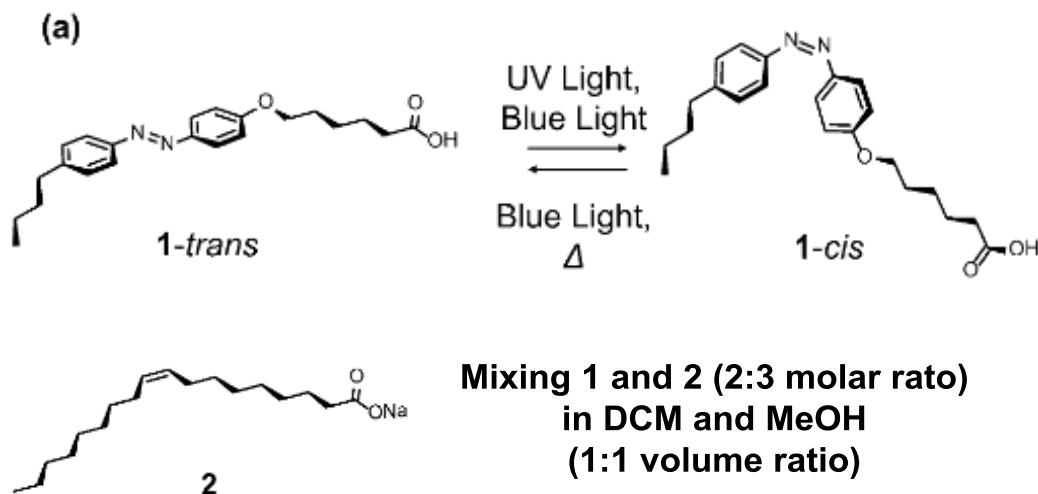
Exp. Pull down assay



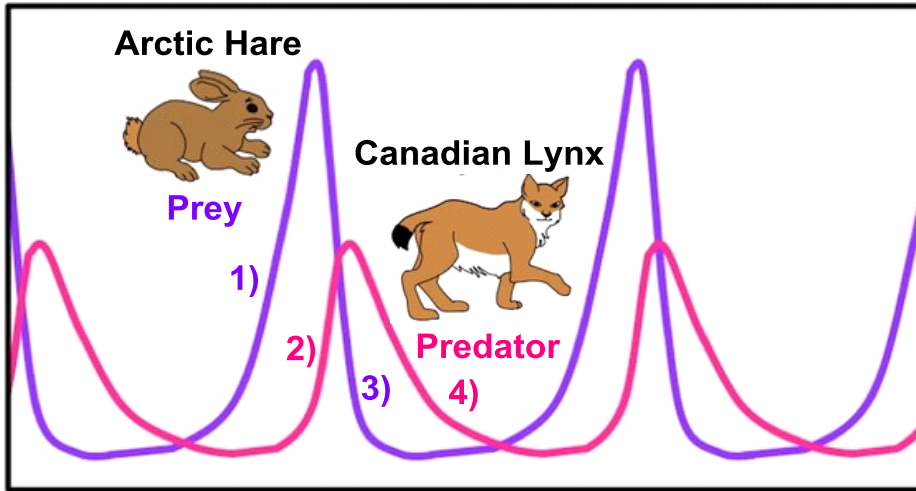
Nat. Struct. Mol. Boil., 2007, 14, 1084.

Up to 2-fold KaiC was pulled down between C and D.

Figure 4 The phase of monomer shuffling and its effect on phosphorylation rhythm. (a) Top, phosphorylation rhythms of oscillatory samples containing Flag-tagged or untagged KaiC. These two samples were mixed in equimolar ratios 16, 20, 24, 28, 32 and 36 h after the start of incubation at 30 °C. Aliquots of newly created mixtures were collected every 2 h and subjected to immunoprecipitation with Flag-specific antibody. Trapped proteins were resolved by SDS-PAGE and Coomassie stained, and the bands at the size of KaiC were quantified. Amounts of immunoprecipitated KaiC protein were normalized to the amount immunoprecipitated at the time of mixing (bottom chart). If KaiC monomers shuffled among KaiC hexamers, a greater number of KaiC hexamers (up to two-fold) would be pulled down by the Flag-specific antibody. Shaded boxes indicate shuffling phase. Duplicate experiments ($n = 2$) showed similar patterns. Phosphorylation of samples F, B, C and D approximately corresponds to those in **Figure 3**. (b) Shuffling

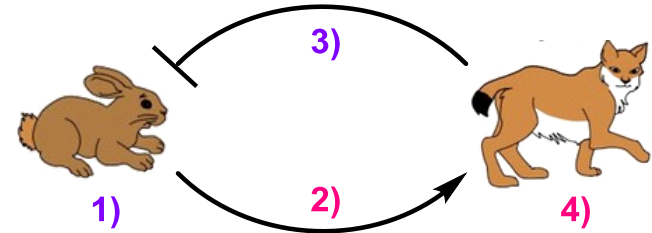


Classical Example: Predator-Prey Relationships



Lynx decreases Hare
which increases Lynx itself.

II
negative feedback

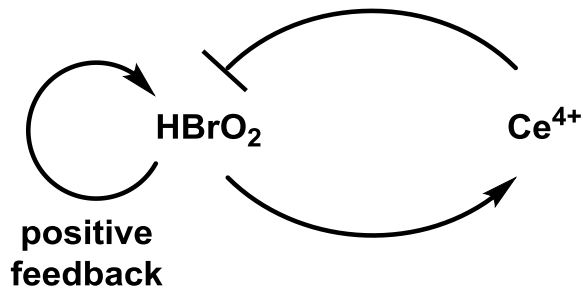


- 1) **Hare** increases as less predator.
- 2) **Lynx** increases as more food.
- 3) **Hare** decreases as more predator.
- 4) **Lynx** decreases as less food.

Chemistry

Belousov-Zhabotinskii (BZ) reaction

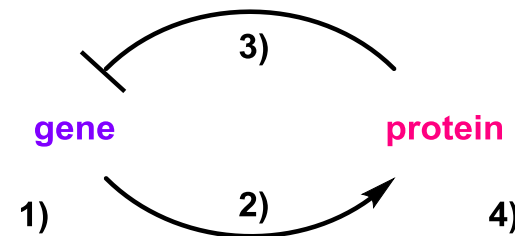
negative feedback



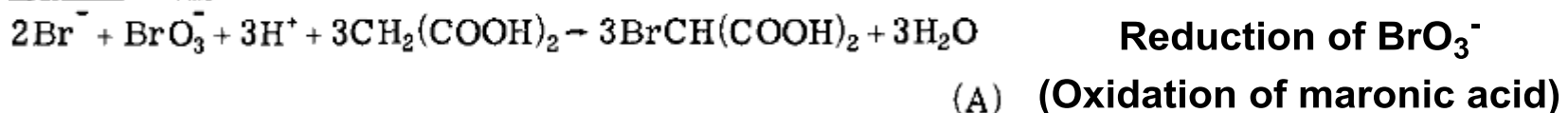
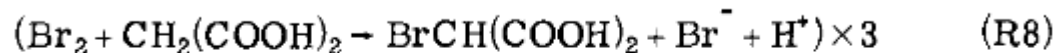
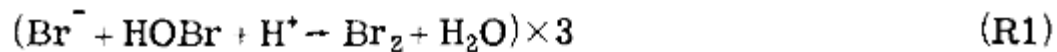
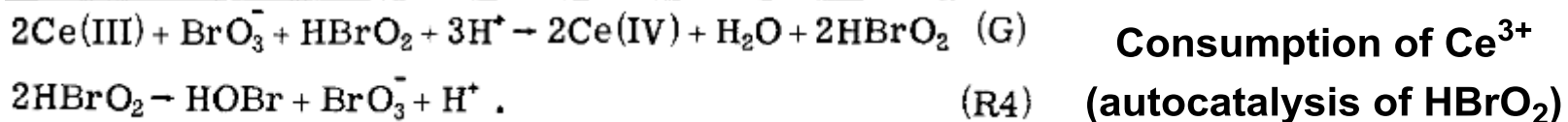
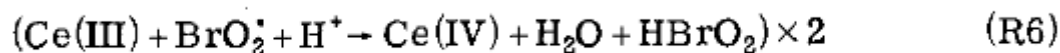
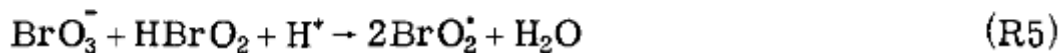
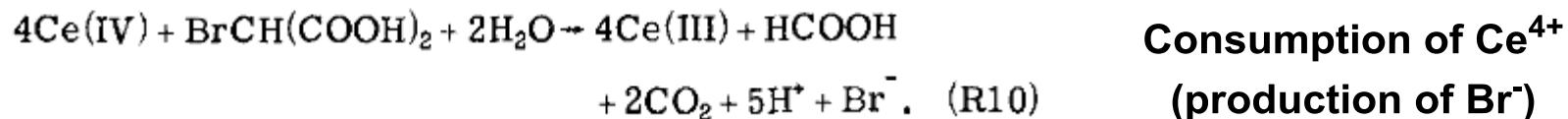
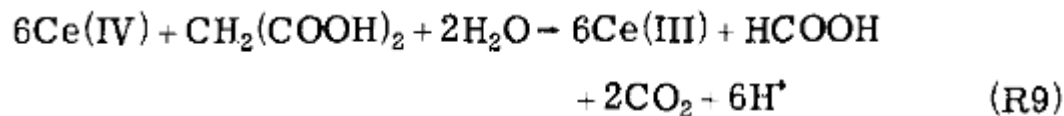
Biology

Transcriptional-Translational Oscillator (TTO)

negative feedback



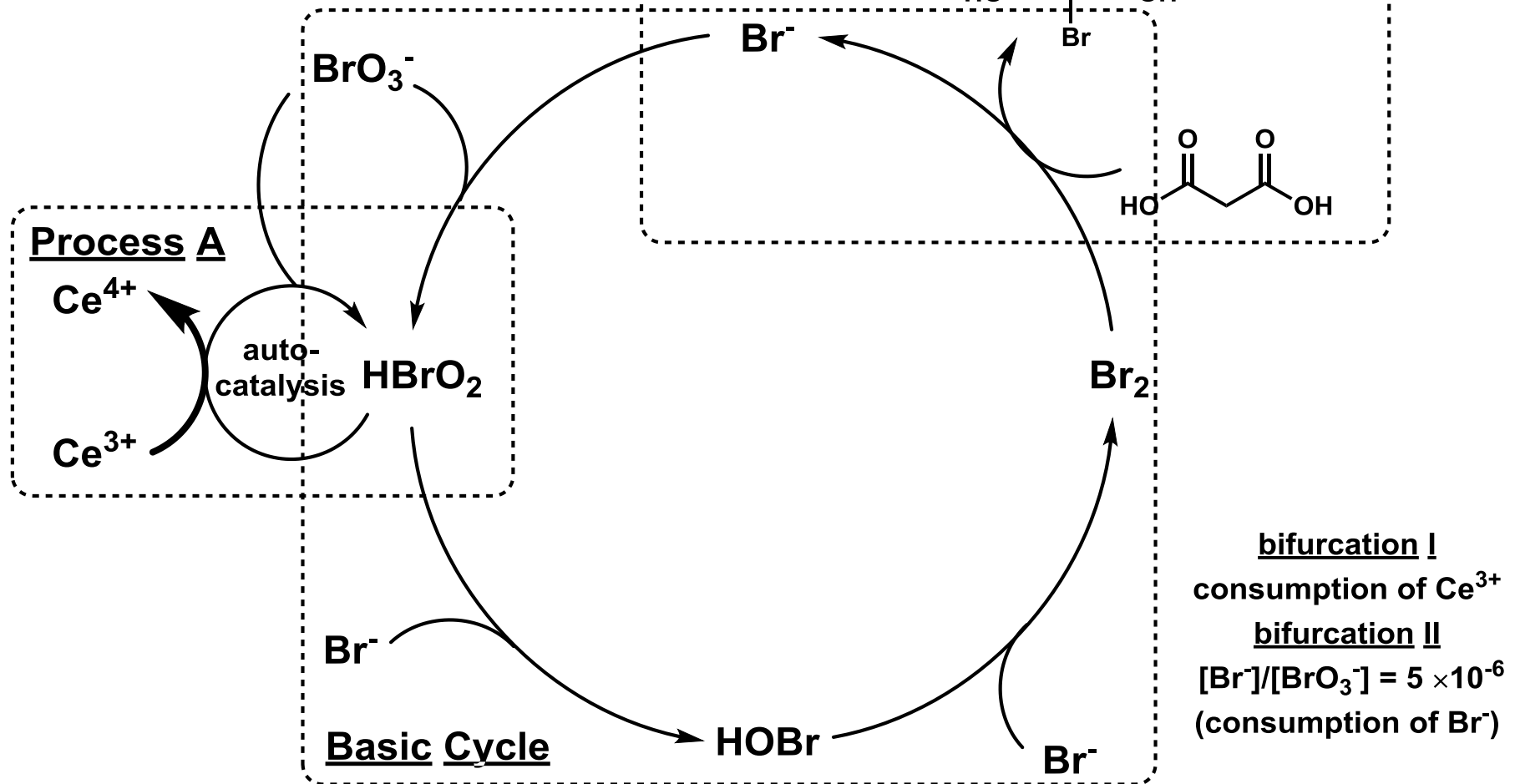
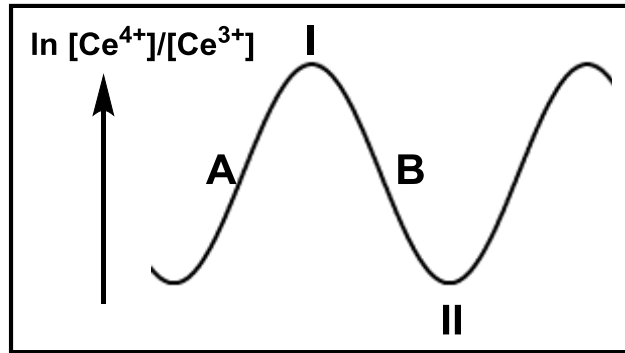
- 1) **Gene activity** increases as less protein.
- 2) **Protein** increases.
- 3) **Gene activity** decreases bound by protein.
- 4) **Protein** decreases by hydrolysis.

Basic Cycle**Process A****Process B**

FKN model: *J. Am. Chem. Soc.*, 1972, 94, 8649.

Mathmatical model based on FKN model: *J. Chem. Phys.*, 1974, 60, 1877.

7. Simplified BZ Reaction Mechanism



What Needed To Be Designed?



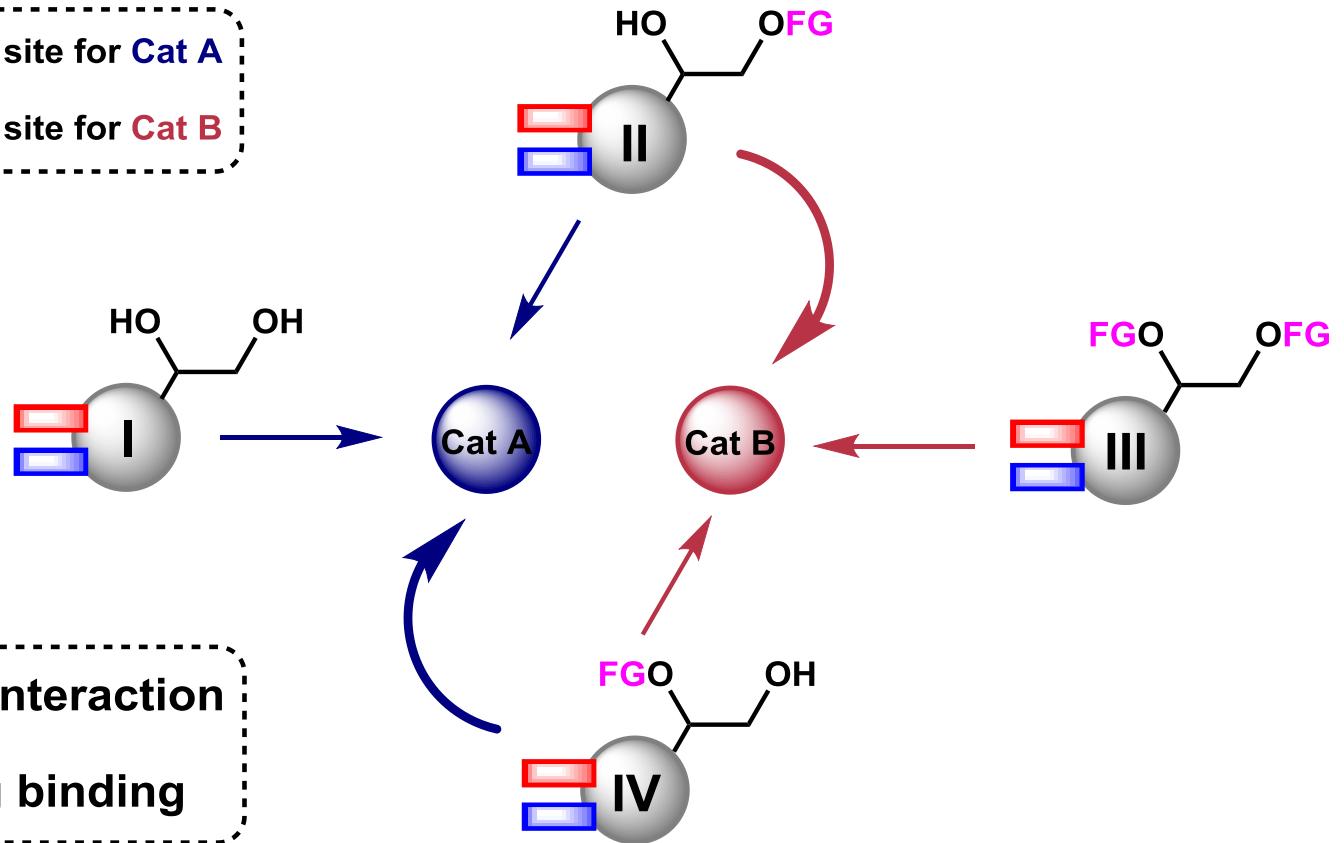
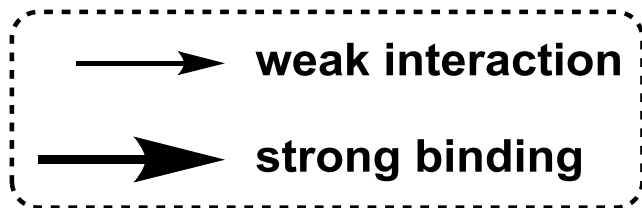
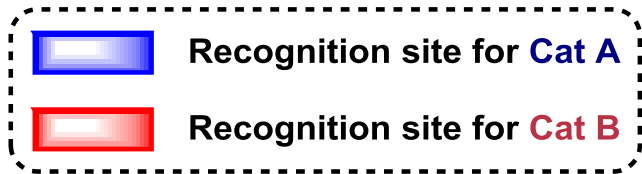
but

slow demodification reaction
(low k_5)

slow modification reaction
(low k_4)

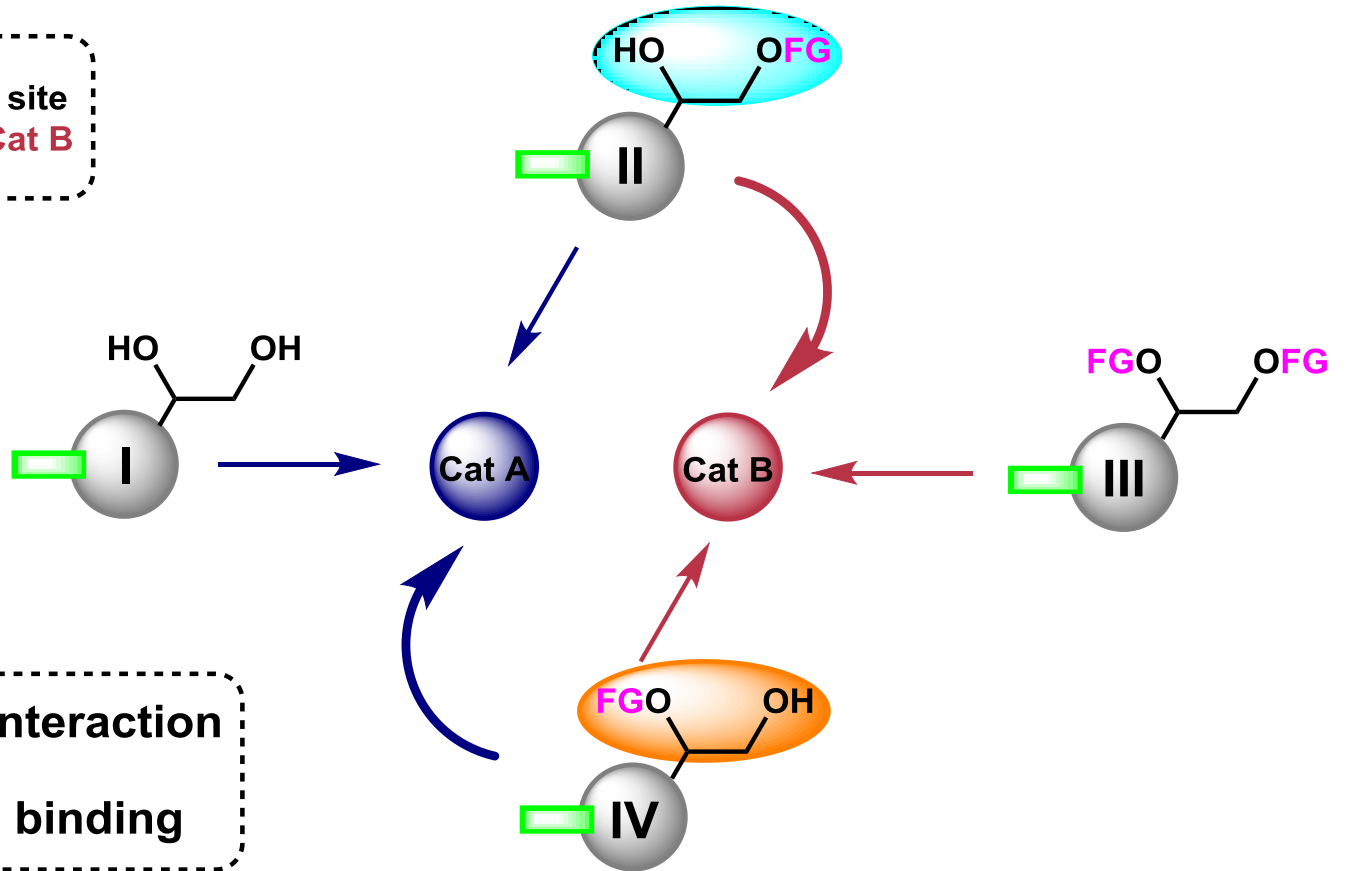
Plus, I and III do not strongly bind to any catalysts.

What Needed To Be Designed?



What Needed To Be Designed?

 Recognition site for **Cat A** & **Cat B**



Q & A

Q1. 何故生物はself-oscillationする必要があるか
太陽光への引き込みではいけないのか

A1. self-oscillationは2つの安定状態(KaiCであれば比リン酸化/リン酸化状態)を行き来するdynamic stabilityを持ちます。そうすると外部刺激によるゆらぎ(温度変化や他分子濃度の増減)をキャンセルして(動的に)安定化することができるので、体内調節の機構として適しているのだと思います。

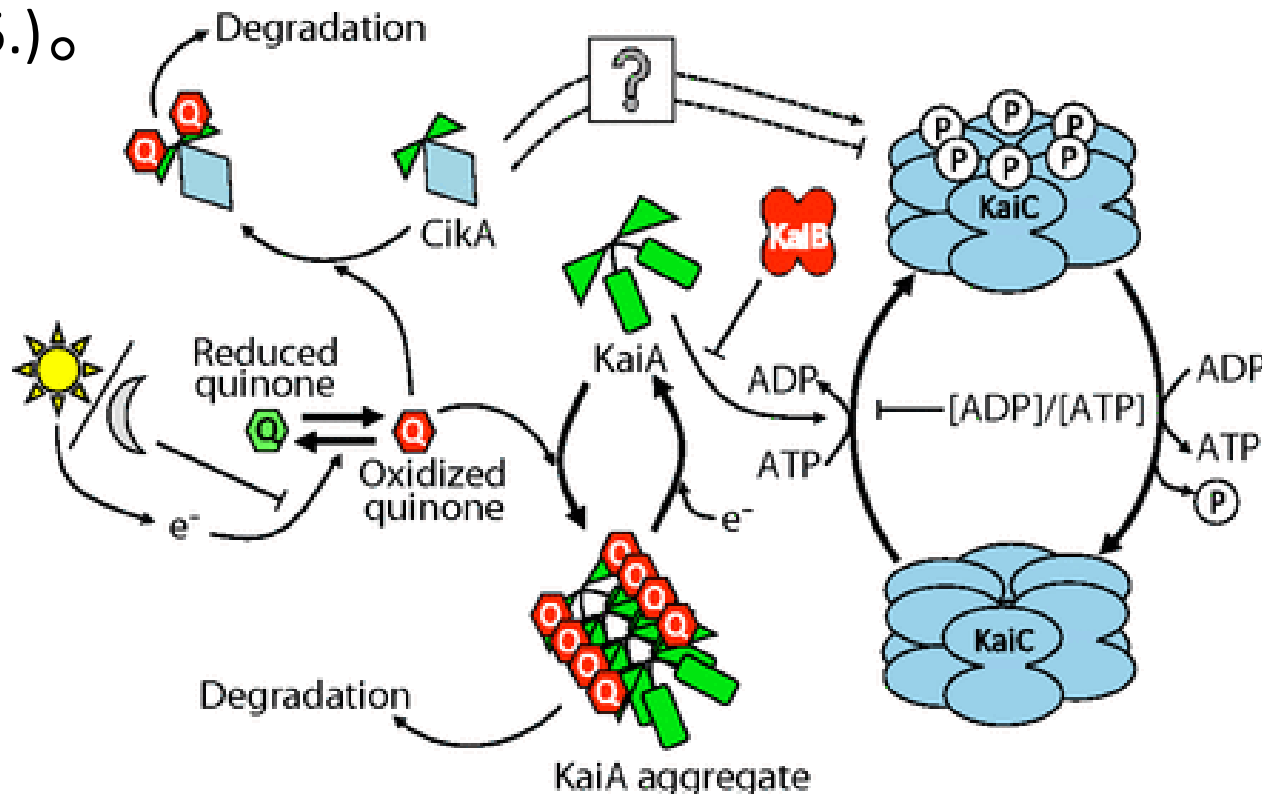
Q2. 集団としてのoscillationはどう説明するの？

A2. KaiC hexamer間でのモノマー交換が重要であることがわかっています(app.1-3参照)。

Q & A

Q3. KaiCは時計タンパク質の一種ですが、光調節もここに関与しているのか

A3. Vitroでは関係しません。Vivoでは下図のような機構で光が関与することが報告されています (PNAS, 2012, 109, 17765.)。



Q4. 各モノマーのC2部位はお互いに独立している？

A4. 同期についての論文によると、隣接モノマー間での位相引き込みを仮定していますので、おそらくC2部位の修飾状態はお互いに何らかの相互作用をしていると思われる。勉強不足でわかりません。

Q5. 化学反応で振動させたい場合にBZ反応のような既存の系を活かすことはできるか。

A5. オリジナルの振動系(BZ反応)をmodifyした振動系はたくさんの変種があります。

例えばメタルをRuに変え、redox activeリガンドを用いて光応答性にすることで振動のタイミングなどを自由に操ることができます。

ただ、新しい振動系を立ち上げることが次の大きなステップだと思います。

Q6. 振動を医療に適用するとして、再生医療のほう
が先に実現してしまいそう？化学分野での技術進展
を加速するmethodologyはどのようにあればよいと思
う？

A6. 振動を用いる場合には外科的な操作が不要なの
である程度の住み分けは可能かと思います。そして
振動現象を研究する意義として医療応用というのは
もう少し発展してから考えるべき内容で今現在のモ
チベーションは生命現象の理解、等かと思います。
化学分野を加速するためには、XXさんがまずベン
チャーを立ち上げるところからだと思います。

Q & A

Q7. 有機化学においてこのような修飾・脱修飾システムは今あるのか？またどのような結合生成が重要になると思うか？

A7. 僕が知る限りありません。惜しいもの、転用できそうな(気がする)反応はたまに見かけるので、あとは分子デザインをしっかりと行えるかどうかかなあと思います。

使用する結合(または反応)の種類は分子デザインに適したものを選ぶべきで今のところあまりアイデアはありません。

Q8. simulation studyで得られたシステムは生体応用以外にも何か応用先がありますか？

A8. 僕としては理学的な意義が一番大きいと思っています(生命現象の理解への寄与(Q12)、とか)。生体医療材料への応用を目指している研究を除くと、視覚的なおもちゃになりえますね(BZとかは化学演習によく使われます。)

Q9. 生体内で、ほかのoscillationの仕組みはどうなっている？

A9. 今のところ発見されているほぼ全ての振動系は feedback mechanism です (app. 5 参照)。
具体的なメカニズムを考えていくとそれぞれの系にかなり個性があって一概には言えません。

Q10. 12時間周期を早めたりすることはできるか？

A10. 例えばこのKaiCの系では温度保障性という性質もあって、温度をあげただけでは周期は変わりません。タンパク質の構造を改変すればもちろん周期をいじることは可能だと思います。

また、KaiCではありませんが、名古屋大の伊丹先生が小分子を用いて概日周期を変化させる分子群を見出しています。

Q & A

Q11. Feedback typeのほうがchemistにとってaccessし易いということはないでしょうか？

A11. 十分に考えられます。勉強中です。
ただこちらのほうが複雑でデザインに求められるセンスのファクターが大きいような・・・

Q & A

Q12. 有機化学が振動する、ということが達成できたとしてどのようなoutputが得られたら大きなインパクトになるのか？

A12. 振動現象をもう少し大きな枠組で考えると、ノーベル化学賞を受賞したプリゴジンが提唱している「散逸構造」の一分野になります。

通常有機化学が熱力学的な(静的な)安定状態を扱うのに対して、この分野は(動的な)安定性を対象とします(今回のKaiC oscillationは非リン酸化・リン酸化を行き来するdynamic stabilityを持ちます)。

振動する化学反応系を自由にデザインできるようになることはこの分野の発展に寄与し、化学だけでなく生命(動的な安定性を持った個体)の理解につながると思っています。

Q & A

Q13. KaiCはリン酸化ですが、ほかの振動するタンパク質だとどのような化学反応を用いていますか？

A13. 現在発見されている中で最も多いのは、**遺伝子発現によるタンパク質合成(アミノ酸合成)**と、**ユビキチンなどによる分解経路(加水分解)**によってタンパク質濃度が振動する系だと思います。

KaiC以外では、グルタチンの酸化還元酵素である Peroxiredoxinの酸化状態が概日周期に連動して振動することがわかっています(生物の全ドメイン、すなわち真正細菌、古細菌、真核生物を通じて保存! *Nature*, 2012, **485**, 7399.)。

僕が知っている遺伝子が関与しない系はこの2例です。

Q14. アロステリックサイトを用意して別の部分の修飾・脱修飾、というのでもいける？

A14. 2箇所での修飾部位・2つの触媒を考えるよりデザインすべき部位は減らせるので魅力的だと思います(機構的にも面白い)。

メカニズムの的には可能だと思うので考えてみます。