ChemPhysChem

Research Article
doi.org/10.1002/cphc.202200103

Chemistry Europe
European Chemical Societies Publishing

www.chemphyschem.org

Peptide-Modulated pH Rhythms

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Many drugs adjust and/or control the spatiotemporal dynamics of periodic processes such as heartbeat, neuronal signaling and metabolism, often by interacting with proteins or oligopeptides. Here we use a quasi-biocompatible, non-equilibrium pH oscillatory system as a biomimetic biological clock to study the effect of pH-responsive peptides on rhythm dynamics. The added peptides generate feedback that can lengthen or shorten the oscillatory period during which the peptides alternate between random coil and coiled-coil conformations. This modulation of a chemical clock supports the notion that short peptide reagents may have utility as drugs to regulate human body clocks.

Introduction

Endogenous biological clocks play an essential role in maintaining life functions, notably by enabling organisms to maintain normal physiological ranges of such parameters as body temperature,[1] blood pressure, pH and sugar levels.[2] Rhythm is crucial for homeostasis, and all living systems have multiple fixed rhythms over a variety of time scales. In addition to daily and monthly rhythms, the normal range for the human heartbeat is 60–100/min,[3] the respiratory rate is 12–20/min, gastric peristalsis occurs 3 times per min,[4,5] and neural oscillations have frequencies of 0.5–35 Hz.[6] Abnormal frequencies of circadian rhythms are associated with many mental and metabolic diseases,[7] including bipolar disorder,[8] anxiety,[9] depression,[10] schizophrenia,[11] sleep disorders,[12] diabetes, obesity and atherosclerosis.[13,14] Drugs to treat such dynamical diseases include domperidone, which stimulates gastric muscle contraction by antagonizing the inhibitory effect of dopamine on intermuscular neurons, increasing the amplitude and frequency of gastric antrum and duodenal peristalsis, thereby accelerating the rate of gastric solid-liquid emptying.[15] Bisoprolol treats myocardial infarction and angina pectoris by selectively blocking the production of adrenaline and β-1 receptors, which inhibits cardiac excitation and slows the heart rate.[16] Both are small molecules that work via complex biochemical pathways. Even though such drugs may have negative effects on other biochemical processes, appropriate dosage choices can often avoid disruption of other physiological rhythms.

For mimicking the effects of drugs on biological rhythms in order to seek insight into their interaction, peptides and pH oscillators provide attractive analogues for drugs and bio-oscillatory networks, respectively. pH oscillators[17] typically consist of simple inorganic reaction networks that can exhibit large amplitude pH oscillations in unbuffered media. Their frequencies may be modified by adding reagents that affect their positive and negative feedbacks. Both inorganic pH and biochemical oscillators operate in open far-from-equilibrium systems that provide the high free energy reactants needed to maintain an oscillatory state. When the timescales of their positive and negative feedbacks differ significantly, the waveform becomes decidedly non-sinusoidal – relaxation oscillations – as seen in the insets to Figure 1a.[18,19]

The α-helical coiled-coil is an important structural motif in proteins, such as fibrous muscle protein.[20] It can also bind to DNA and act as a transcriptional regulator.[21] The coiled-coil motif is characterized by a seven-unit repeat sequence, denoted as abcdefg, where positions e and g are typically occupied by hydrophobic residues. These residues are usually charged, which may cause electrostatic attraction or repulsion between the helices, thereby making the coiled-coil stable or unstable.[22] Chmielewski reported peptides that can change their secondary structure and undergo self-assembly and/or self-replication in response to changes in pH or calcium ion concentration.[23,24] These peptides are subject to pH regulation in the range of 4–7, presenting a coiled-coil structure at low pH and random coil-fragments at high pH, likely resulting from hydrogen-bond formation and disruption at low pH and high pH, respectively. It should therefore be possible to drive such a peptide between random coil and coiled-coil structures with a pH-regulated oscillatory system. Polypeptides such as the octapeptide OP (Ac-EALEKELA-$COSC_2H_5$) and the octadecapeptide ODP (H_2N-CLEK-ELGALEK-ELYALEK-$CONH_2$) contain an ethyl thioester end group and/or active carboxyl groups (e/g-position amino-acids) that can participate in pH oscillator feedback reactions to modulate the pH rhythm dynamics. In contrast, the amino acids in the glutathione dimer, GSSG, form a compact structure without any active groups and show no obvious feedback activity to pH oscillations. Molecular structures of these three peptides are shown in Part 1 of the Supporting Information.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cphc.202200103

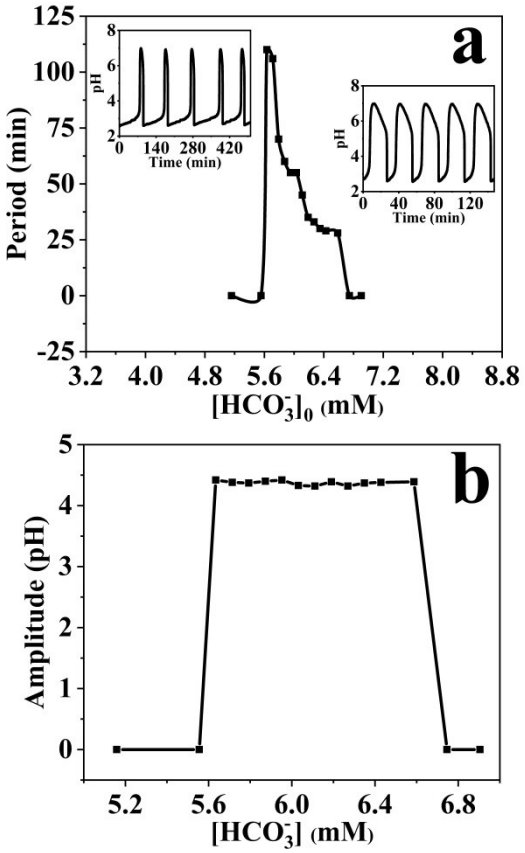

Figure 1. Dependence of the oscillatory period (a) and amplitude (b) on $[NaHCO_3]_0$. Input concentrations: $[BrO_3^-]_0 = 75.18$ mM, $[SO_3^{2-}]_0 = 66.67$ mM, $[H^+]_0 = 11.39$ mM; $k_0 = 3.70 \times 10^{-4}$ s^{-1}.

We seek an appropriate pH oscillator as an analogue to biological rhythms in order to study the effect of peptides on rhythm dynamics. The system should be as biocompatible as possible, which excludes many candidates, such as those involving hydrogen peroxide or iodate, even though these oscillators have been used in conjunction with RNA and DNA, respectively.[25,26] Ideally, our system should oscillate over a pH range of several units, near pH 7, with a period of several minutes to an hour (in order to drive the conformational change of the peptide) and should require only modest concentrations of reactants. The bromate-sulfite (BS) system displays large amplitude pH oscillations, and the oxidizing power of bromate is relatively weak in this pH range.[27] However, under typical flow conditions, i.e., in a continuous stirred tank reactor (CSTR) with relatively low bromate and sulfite concentrations, the period of the BS oscillating system exceeds 2 h. One can shorten the period by heating, but the required increase in temperature brings the system into a range in which biomolecules are easily degraded. Instead, we consider reducing the oscillatory period by introducing an additional feedback agent. Based on the above considerations, we select sodium bicarbonate as the most suitable negative feedback agent, yielding the bromate-sulfite-bicarbonate (BSB) oscillator.[28]

Results and Discussion

Hanazaki has reported BSB oscillations with a period of about 20 min and a pH range of about 3–6.7.[28] However, at the concentrations he employed, carbon dioxide bubbles are generated, which interfere with monitoring the reaction, and it is necessary to pass nitrogen gas above the reaction solution to remove the CO_2. We therefore seek to optimize the BSB system by reducing reactant concentrations so as to eliminate bubbles, while maintaining sufficiently large amplitude and a period of less than 40 min.

After optimization, the concentration of each reactant in our BSB system is much lower than previously reported.[28] Because of the low bicarbonate concentration (< 8 mM) we employ, no bubbles arise during the oscillations. Therefore, it is only necessary to bubble nitrogen through the prepared solution before the experiment in order to remove carbon dioxide. Purging the dissolved CO_2 reduces the oscillatory period from 50 min to 33 min (see Figure S1). The dissolved carbon dioxide, which generates protons, inhibits the negative feedback on the oscillatory reaction, leading to an increase in the oscillation period.

In addition to bisulfite oxidation to dithionate,[28] proton consumption by $NaHCO_3$ constitutes a second negative feedback, and its concentration inevitably affects the oscillatory kinetics. As $[NaHCO_3]_0$ is increased within the oscillatory range, proton negative feedback accelerates, and the reaction period gradually decreases, as shown in Figure 1. At the lower limit of $[NaHCO_3]_0$, the oscillatory period is about 105 min, while at the upper limit, the period is about 30 min. The amplitude remains nearly constant. At higher $[NaHCO_3]_0$, the proton negative feedback is strengthened, which shortens the rise time from the low to the high pH state, so the waveform changes from spikes to broad peaks.

Because of the lower reactant concentrations we employ, the range of $[NaHCO_3]_0$ that supports oscillations is narrower than in earlier studies (Figure S2 in the Supporting Information).[28] When the input concentration of $NaHCO_3$ is low, the system exhibits a low pH steady state. As $[NaHCO_3]_0$ increases, the system begins to oscillate, but when $[NaHCO_3]_0$ exceeds a critical value (~6.7 mM), the system enters a high pH steady state.

All three peptides, GSSG, OP and ODP, show a significant response to pH, as shown in Figure S3 of the Supporting Information. The experimental results in Figure 2 demonstrate that the three peptides produce different effects on the oscillating system under the same experimental conditions. When ODP is added, the oscillation period increases from 33 min to 47 min, while the amplitude remains basically unchanged. After addition of OP, stable pH oscillations appear after a transitory period, the oscillatory period is shortened from 33 min to 12 min, and the oscillatory pH range narrows from 2.56–6.95 to 3.58–6.15. Aqueous 20 mM solutions of OP and ODP have pHs of 3.71 and 3.93, respectively. Buffer indexes of these solutions obtained by titration are 0.090 mM/pH and 0.082 mM/pH (Figure S4 of the Supporting Information), respectively, which are too small to affect the pH amplitude of the

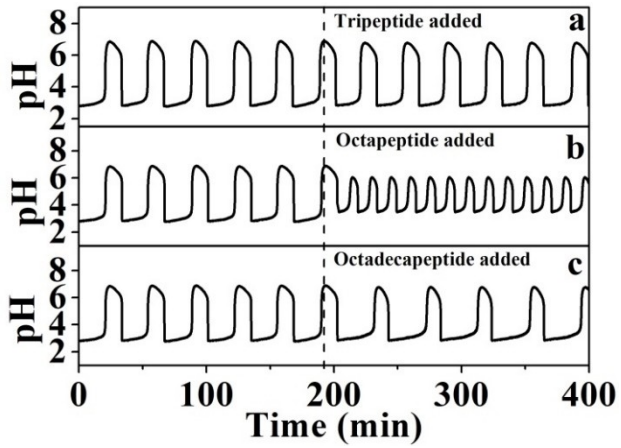

Figure 2. Effect of different peptides on experimental BSB pH oscillations. (a) GSSG; (b) OP; (c) ODP. Input concentrations: $[BrO_3^-]_0 = 75.18$ mM, $[SO_3^{2-}]_0 = 66.67$ mM, $[H^+]_0 = 11.39$ mM, $[HCO_3^-]_0 = 6.27$ mM, [Peptide]$_0 = 20.00$ mM, $k_0 = 3.70 \times 10^{-4}$ s^{-1}.

oscillatory systems. Addition of GSSG produces no significant effect on the oscillations.

The end group of OP is an ethyl thioester, which can hydrolyze to ethyl mercaptan (see structure in Part 1 of the Supporting Information). The generated ethyl mercaptan is a reductant that can react with bromate, producing a new negative feedback process (R1-R2). This influence of the end group reaction on the oscillating system is far greater than that of the e- and g-position amino acids. We describe the effects of adding OP by the following reactions:

$$P_8 \rightarrow RSH + P_8' \quad \text{(R1)}$$

$$BrO_3^- + H^+ + 2RSH \rightarrow HBrO_2 + RSSR + 3H_2O \quad \text{(R2)}$$

$$P_{8\alpha} \leftrightarrow P_8 + 2H^+ \quad \text{(R3)}$$

$$P_8 \leftrightarrow P_{8f} + 2H^+ \quad \text{(R4)}$$

where P_8 = OP, P_8' = OP with hydrolyzed end group, $P_{8\alpha}$ = helical OP in acidic medium, and P_{8f} = helical OP in basic medium.

The ethyl thioester end group in OP hydrolyzes to ethyl thiol in the oscillatory pH range (3–7).[29] Oxidation of the thiol consumes protons, raising the pH minimum of the oscillations. This reaction also generates an increase in the bisulfite concentration via dissociation of sulfurous acid through reaction (M2) of Table S1 in the Supporting Information, resulting in acceleration of the main positive feedback (reaction (M3) of Table S1) at high pH (>5), which lowers the pH maximum of the oscillations. Thus, OP-induced acceleration of both the negative and positive feedbacks reduces the amplitude of the pH oscillations.

The carboxyl groups at the e and g-positions of ODP (5 in total) can release protons (see structure in Part 1 of the Supporting Information), increasing the concentration of hydrogen ions in the system (reaction R5). This reaction delays the negative feedback reaction and causes the pH to rise more slowly. At low pH, protonation of the residues at the e- and g-positions causes the peptide to form a relatively stable helical structure (reaction R6). At high pH, deprotonation of these residues results in electrostatic repulsion, producing random coil fragments. To describe these processes, we introduce the following reactions:

$$P_{18\alpha} \leftrightarrow P_{18} + 5H^+ \quad \text{(R5)}$$

$$P_{18} \leftrightarrow P_{18f} + 5H^+ \quad \text{(R6)}$$

where P_{18} = ODP, $P_{18\alpha}$ = helical ODP in acidic medium, and P_{18f} = ODP deprotonated at the e and g positions in basic medium.

To monitor the peptide conformation, the effluent from the reactor was collected, quickly quenched and subjected to circular dichroism spectroscopy. This analysis showed that the α-helix content of OP and ODP changed periodically, as seen in Figure 3. The α-helix content of the oligopeptides reaches its highest value at the minimum of the pH oscillations.

We also used Berkeley Madonna software[30] to simulate the system with the model and parameters proposed by Rábai,[27] augmented by reactions R1–R6 to account for the effects of the added peptides. The full set of equations and parameters appears in Tables S1 and S2 of the Supporting Information. Adding OP reduced the simulated oscillation period from 36 min to 12 min, as shown in Figure S5 of the Supporting Information, which is in good agreement with the experimental results in Figure 2b. With added ODP, the oscillation period increased from 36 min to 43 min (Figure S6 in the Supporting Information), again in agreement with the experimental results in Figure 2c.

Figure 4 shows the effect of peptide concentration on the oscillatory period and amplitude. For GSSG, no obvious influence is seen. The oscillation period gradually decreases with [OP], an effect that is more pronounced at lower concentrations. ODP has the opposite effect, with the period increasing with [ODP], especially at higher concentrations. The simulated results (dashed lines) agree well with our experiments (solid lines)

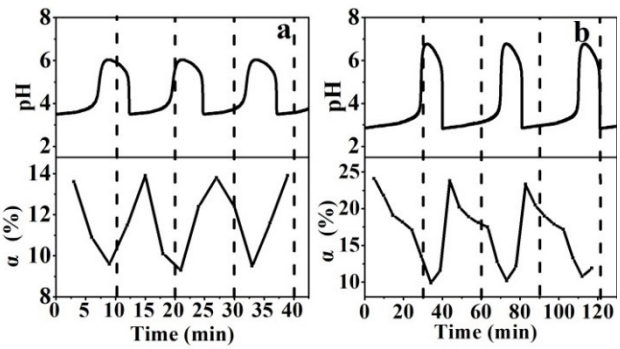

Figure 3. Oscillations in pH and peptide conformation for (a) OP and (b) ODP. Input concentrations: $[BrO_3^-]_0 = 75.18$ mM, $[SO_3^{2-}]_0 = 66.67$ mM, $[H^+]_0 = 11.39$ mM, $[HCO_3^-]_0 = 6.27$ mM, [Peptide]$_0 = 20.00$ mM, $k_0 = 3.70 \times 10^{-4}$ s^{-1}.

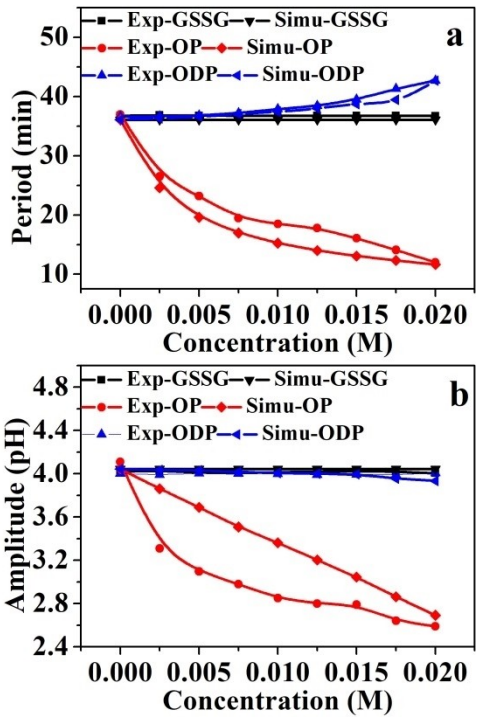

Figure 4. Effects of peptide concentration on the oscillatory period (a) and amplitude (b). Squares, circles and triangles denote tripeptide, octapeptide and octadecapeptide, respectively. Dashed and solid lines represent experiments and simulations, respectively. Input concentrations: $[BrO_3^-]_0 = 75.18$ mM, $[SO_3^{2-}]_0 = 66.67$ mM, $[H^+]_0 = 11.39$ mM, $[HCO_3^-]_0 = 6.27$ mM, $k_0 = 3.70 \times 10^{-4}$ s^{-1}.

The oscillatory amplitude decreases gradually with [OP], while the concentrations of GSSG and ODP have little effect on the amplitude. Although the carboxyl group of ODP is involved in the reaction, it only acts as a buffer, delaying the negative feedback, so it does not affect the amplitude.

Conclusions

In summary, we have optimized the experimental conditions for the bromate-sulfite-bicarbonate pH oscillatory system with respect to quasi-biocompatibility, pH amplitude and period suitable for studying the conformational change and self-assembly of the peptide without interference from carbon dioxide bubbles. We find that OP and ODP affect the negative feedback of BSB pH oscillations through different reaction mechanisms, resulting in shorter and longer oscillatory periods, respectively. Despite important differences between artificial pH oscillators and bio-oscillators, the successful modulation of pH oscillations by small peptides suggests that inorganic oscillators may be useful tools for studying drug-induced feedback mechanisms to regulate biological rhythms.

On a more speculative note, studies of the origin of early life on earth have shown that alpha-helical peptides can bind to DNA and synergistically regulate replication and transcription processes.[31] This observation is consistent with the idea that primordial pH oscillations or fluctuations may have influenced the transmission of genetic information. If oscillators of this type can be built from biocompatible inorganic components, then such molecular oscillations may have played a role in the formation of biological rhythms in living systems. Considering the origin of life from a systems chemistry viewpoint, this work may suggest new directions for prebiotic chemistry research.[32]

Experimental Section

The following analytical reagent-grade chemicals were used without further purification: sodium bromate (Sinopharm Chemical Reagents), sulfuric acid (Sinopharm Chemical Reagents), sodium sulfite (Sinopharm Chemical Reagents) and sodium bicarbonate (Sinopharm Chemical Reagents). Deionized water was supplied by a water purification system (Millipore, Milli-Q Jr.). All peptides were synthesized by Baiger Pharmaceutical Technology Co., Ltd (Hangzhou, China). All input solutions were prepared daily and bubbled with nitrogen gas to avoid air oxidation.

Our experiments were carried out in a flow reactor (Figures S7–S8 in Supporting Information) with a liquid volume of 27.0 mL and three inlet tubes with inner and outer diameters of 3 mm and 5 mm, respectively. The four stock solutions were pumped into the reactor from three channels by a four-channel peristaltic pump (ISMATEC, Switzerland). Before each experiment, the flow rate of each channel of the peristaltic pump was calibrated with water to establish the actual flow rate. Appropriate amounts were weighed and dissolved in distilled water to prepare $NaBrO_3$, Na_2SO_3, $NaHCO_3$ and H_2SO_4 solutions. For the BSB system, three stock solutions were pumped into the reactor through three channels of the peristaltic pump: pure water or peptide solution; sodium bromate solution; mixed solution of sodium sulfite, sulfuric acid and sodium bicarbonate. The reacted solution spontaneously flows out through an outflow channel above the reactor, maintaining constant liquid volume in the reactor. In order to avoid excessive local concentrations of sulfuric acid, sulfuric acid was first added in the mixing process, and then solutions of anhydrous sodium sulfite and sodium bicarbonate were added. To avoid temperature fluctuations, all solutions were preheated in a thermostatic tank to keep the entering solution temperature consistent with the reaction temperature. A circulating water pump (Lauda Instrument, Germany) was used to maintain a constant reaction temperature (44.5 °C). The reaction mixture was stirred at a constant rate of 950 rpm by a Teflon-coated (dimensions, 15 mm × 5 mm) magnetic stirrer (IKA, Germany).

Once stable oscillations were obtained, we replaced the inflow of pure water with a polypeptide solution. When the peptide enters the oscillating system, the original oscillatory balance is broken, and a new state emerges after a transitory period. After the new oscillating condition stabilized, we took the effluent from the reactor and quickly diluted by a factor of 50 to quench the reaction and temporarily freeze the structure of the peptide. The secondary structure of peptides in the diluted effluent was analyzed by circular dichroism spectrometry (Applied Photophysics, the United Kingdom).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 21773304 and 22120102001), the Natural Science Foundation of Jiangsu Province (Grant No.

BK20211242), and the U.S. National Science Foundation (Grant No. CHE-1856484).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: rhythm modulation · pH-responsive peptides · e/g-position amino-acids · ethyl thioester · pH oscillations

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Manuscript received: February 10, 2022
Revised manuscript received: May 31, 2022
Accepted manuscript online: June 1, 2022
Version of record online: June 21, 2022