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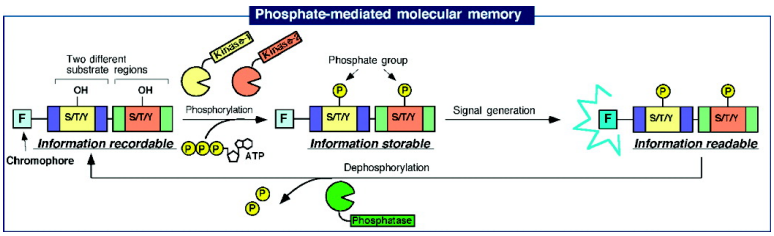

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Phosphate-Mediated Molecular Memory Driven by Two Different Protein Kinases as Information Input Elements

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Abstract: There is increasing interest in studying molecular-based devices that perform Boolean logic operations whose output state (0 or 1) depends on the input conditions (0/0, 1/0, 0/1, or 1/1). So far, great efforts have been devoted to establish molecular-scaled logic gates activated by chemical, physical, and biological inputs. We herein describe the design and synthesis of a tandem protein kinase substrate peptide acting as a phosphate-mediated molecular memory. The molecular-based memory system is comprised of two different phosphorylatable substrate regions joined in series and a spiropyran derivative at the N-terminus. We also demonstrated three basic "AND", "OR", and "NOR" logic operations on the basis of alterations in the spiropyran-to-merocyanine (SP-to-MC) thermocoloration properties of the spiropyran moiety in the peptide upon kinase-catalyzed phosphorylation. The three logic functions were successfully performed by adding ionic polymers as programming elements with preset thresholds of a signal intensity in a microplate format. Throughout this study, information was recorded on the substrate peptide by protein kinase-catalyzed phosphorylation, stored stably as phosphoesters, read according to the extent of the SP-to-MC thermocoloration, and erased by phosphatase-catalyzed dephosphorylation, resulting in the peptide returning to the initial recordable state. Thus, the proof-of-concept experiments described herein could be used to provide clues for developing practical molecular-based processing and computing.

Introduction

There is increasing interest in studying molecular-based devices that perform Boolean logic operations whose output state (0 or 1) depends on the input conditions (0/0, 1/0, 0/1, or 1/1).[1−6] So far, great efforts have been devoted to establish molecular recognition-based logic gates activated by chemical[7−21] and physical[22−27] inputs to miniaturize these devices relative to those commonly used in microprocessors. The potential use of biomolecules as active computing elements also attracts substantial research efforts. This is because gene expression and post-translational modifications of proteins are precisely controlled in a spatiotemporal manner in response to external stimuli in biosystems. Nucleic acid-related molecules[28−37] and proteins/

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enzymes[38−44] have proven to be potential building blocks for molecular logic gates and computational devices. Porphyrinic macrocycles have been also considered for use as charge-storage components in molecular information storage media.[45] One of our major goals is to develop molecular logic gates activated by enzymatic information inputs as an important step toward biosystem-based molecular devices and memories. In this context, we are focusing our efforts on the use of enzymes as active components for driving a molecular-sized memory system.

Protein kinases are enzymes that catalyze phosphorylation of serine, threonine, and tyrosine in protein substrates play quite important roles in the activation of downstream proteins in signaling pathways in response to external stimuli (input) such as drugs, stress, and UV damage. Therefore, phosphate-mediated logic systems with protein kinase substrate peptides would include the following potential features: (i) Information is catalytically recorded on kinase substrates by corresponding protein kinases. (ii) Information to be recorded is diversified by substrate specificities of enzymes. (iii) Information recorded on the kinase substrates is stably stored as phosphoesters under neutral and lower temperature conditions. (iv) Information recorded is read by acquiring signals of a chromophore incorporated into the kinase substrates. (v) Information recorded is catalytically erased by phosphatase-catalyzed dephosphorylation (Figure 1). The features of the phosphate-mediated molecular memory system listed above could cover important aspects of molecular-based processing and computing.

From the viewpoint of improving diversity in input elements, mechanisms of information storage, and signal generation/readout, we examined the potential of using of protein kinases as information input elements to realize such an improved molecular memory system. Our putative design was based on construction of a spiropyran-containing tandem protein kinase substrate peptide composed of two different phosphorylatable substrate regions joined in series and capable of responding to phosphorylation catalyzed by two different representative protein kinases, c-Src protein tyrosine kinase (SrcN1) and cAMP-dependent protein kinase A (PKA) with an enhanced coloration signal from the chromophore.

Spiropyrans are well-known photochromic (and thermochromic) molecules and have been used to study ion transporters[46,47]

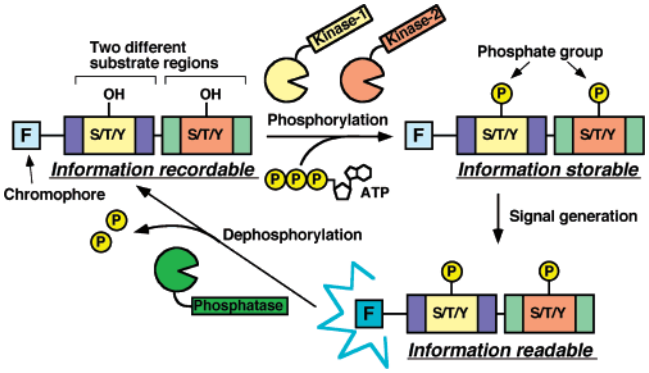

Figure 1. Schematic illustration of a phosphate-mediated molecular memory. Initially, two different kinase substrate regions joined in series in the tandem protein kinase substrate peptide are phosphorylatable (information recordable). Protein kinases catalytically transfer phosphate groups from ATP onto the peptide (information input). Phosphate groups recorded onto the peptide are stably stored as phosphoesters (information storable). Signals are generated in correspondence with the phosphorylated states for the peptide (information readable) and acquired (information output). Protein phosphatase catalytically removes phosphoesters from the phosphorylated peptide (information erasion). Such a phosphorylation/dephosphorylation cycle would be useful for creating molecular-based memories.

and molecular switches/memories[48] and to assay protein kinase activities.[49,50] Spiropyran derivatives are typically in equilibrium ($K_{eq} = \sim 1$) between a colorless "closed" spiropyran (SP) form and a highly colored "open" merocyanine (MC) form in neutral aqueous solution; the latter form has intense absorption and fluorescence bands in the visible region (500−700 nm) (Figure 2A).[51] The SP and MC forms are reversibly produced by visible light irradiation and incubation in the dark, respectively. Conversions between the SP and MC forms depend on the microviscosity surrounding the spiropyran moiety; for example, the isomerization of the spiropyran moiety embedded in polymer matrices is significantly suppressed (Figure 2B).[52,53] These properties imply that alterations in binding modes between tandem kinase substrates and ionic polymers would generate signals corresponding to information recorded on the kinase substrates upon phosphorylation.[49,50,54,55] For instance, a cationic spiropyran-containing substrate peptide with a net charge of $i = +4$ associates strongly with anionic polymers [e.g., poly(L-aspartate)] through electrostatic interactions, whereas diphosphorylation (equally −2/phosphate adduct) of the peptide alters its net charge to neutral ($i = 0$), causing reduction in binding affinity between diphosphorylated peptides and anionic polymers by charge repulsion. Consequently, the former would provide a faint coloration and the latter an intense coloration; that is, the tandem kinase substrate peptide can act as an "AND" logic gate upon diphosphorylation.

Meanwhile, it is also expected that the addition of cationic polymers [e.g., poly(L-lysine)] does not interfere with the SP-to-MC thermocoloration of the spiropyran-containing peptide

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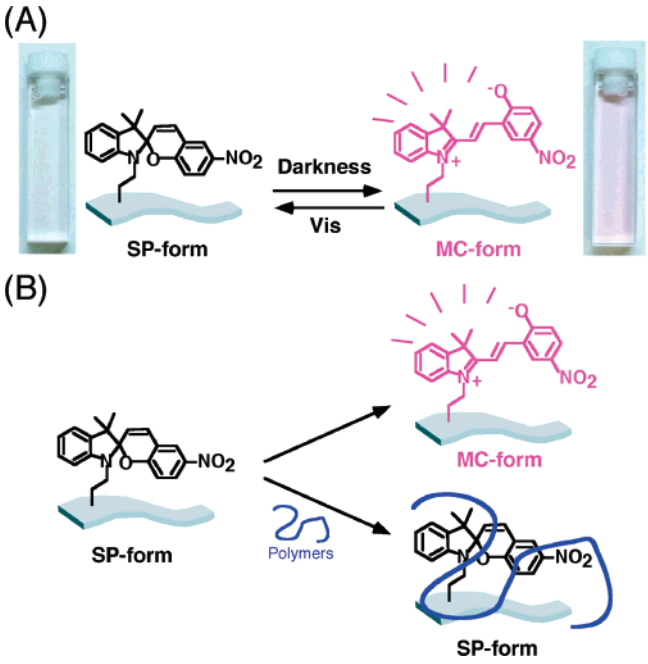

Figure 2. Photochromic and thermochromic properties of spiropyran-containing peptides in neutral aqueous solution. (A) Isomerization from SP to MC forms (thermocoloration) and from MC to SP forms (photobleaching) is controlled by incubation in the dark and irradiation with a visible light, respectively. The MC form is colored in pink and strongly fluorescent, whereas the SP form is colorless and nonfluorescent. (B) The SP-to-MC thermocoloration is significantly suppressed when a spiropyran moiety is embedded in polymer matrices due to elevated microviscosity. The spiropyrans allow both fluorometric and colorimetric signal readouts by regarding generation of the MC form in the peptides.

in a substrate state ($i = +4$) but suppresses such thermocoloration for the diphosphorylated peptides ($i = 0$) by virtue of electrostatic interactions between phosphate groups in the peptides and ammonium groups in the cationic polymers, affording intense and faint colorations, respectively. In this case, a reversed thermocoloration acting as a "NOR (Not OR)" logic gate would be observed. Therefore, it seems that a spiropyran moiety is suitable as a chromophore generating programmable "AND/NOR" logic outputs activated by kinase-catalyzed phosphorylation. Here, we describe the first example of a phosphate-mediated molecular memory system performing logic operations programmable by adding external ionic polymers to generate readout signals that correspond to the "AND" (possibly "OR") and "NOR" logic functions in a microplate format.

Results and Discussion

Molecular Design and Synthesis. We designed and synthesized the following four spiropyran-containing peptides (Figure 3): The SP-YS (net charge, $i = +4$) is a tandem protein kinase substrate peptide that comprises SrcN1 and PKA substrate regions connected in series and a spiropyran derivative at the N-terminus. SP-YS possesses a "chromophore−spacer1−substrate region1−spacer2−substrate region2" format. The substrate regions incorporated in the design are a c-Src tyrosine protein kinase (SrcN1) substrate region (−Ile−*Tyr*−Glu−Phe−Lys−Lys−Lys−) as the substrate regionl[56] and a PKA substrate region (−Leu−Arg−Arg−Ala−*Ser*−Leu−) as the substrate region2,[57] in which phosphorylatable amino acids are in italics. The spacers incorporated in the design are 6-aminohexanoic acids (Aha) to enhance flexibility, thereby facilitating enzymatic reactions. Three phosphorylated peptides, the monophosphorylated peptides SP-pYS ($i = +2$) and SP-YpS ($i = +2$) and the diphosphorylated peptide SP-pYpS ($i = 0$) were also designed as reference peptides to precisely characterize thermocoloration properties in each phosphorylated state. Spiropyran-containing peptides were prepared according to the literature [Figure S1 (Supporting Information)].[22,50,51] Shortly, the compound 1-(2-hydroxyethyl)-3,3-dimethylindolino-6′-nitrobenzopyrylospiran,[22] a spiropyran derivative used in this study, was prepared and treated with *p*-nitrophenyl chloroformate, affording an activated *p*-nitrophenyl carbonate form.[50,51] Peptide-bound resin was prepared by solid-phase peptide synthesis methodology with Fmoc chemistry,[58] attached with the spiropyran unit at the N-terminus of the peptide sequence on the resin, deprotected/deresinated with TFA, and purified by HPLC, followed by characterization by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), affording a fluffy yellow powder. Procedure for the peptide synthesis is detailed in the Supporting Information.

Properties of the SP-to-MC Thermocoloration. First, to understand the photochromic and thermochromic properties of our synthesized spiropyran-containing peptides, we investigated the SP-to-MC thermocoloration processes of SP-YS ($i = +4$) and SP-pYpS ($i = 0$) in the absence and presence of the ionic polymers poly(L-aspartate) and poly(L-lysine). When the yellow powder of the peptide colored by a coexisting protonated MC form was dissolved in neutral aqueous solution, the yellow-colored solution turned to pink immediately, corresponding to deprotonation of the phenolic proton of the MC moiety, resulting in the fluorescent zwitterionic MC form. The resulting solution (a mixture of SP and MC forms) was irradiated with indoor lightning to afford a solution containing only SP form, and subsequent incubation in the dark started the relaxation. The relaxation process was monitored by the increase in fluorescence intensity (in the vicinity of 580 nm, $\lambda_{ex} = 485$ nm) of the MC form on a fluorescence microplate reader.

Changes in fluorescence intensity of SP-YS ($i = +4$) in the absence and presence of poly(L-aspartate) and poly(L-lysine) in neutral aqueous solution as a function of time (0−225 min) are shown in Figure 4A. The SP-YS exhibited a saturation curve in fluorescence intensity with a 4-fold increase at 200 min after incubation started in the absence of ionic polymers. The addition of poly(L-lysine) did not affect the SP-to-MC thermocoloration, but there was a slightly increased fluorescence intensity over that seen in the absence of cationic polymers, as observed in the previous study.[50] By contrast, the addition of poly(L-aspartate) to the peptide solution significantly suppressed the SP-to-MC thermocoloration by tightly binding to the cationic peptides ($i = +4$) via electrostatic interactions. Relative changes in fluorescence intensity at 60 min in the presence vs absence of ionic polymers were found to be 0.053 ± 0.013 and 1.15 ± 0.03 for poly(L-aspartate) and poly(L-lysine), respectively. The SP-to-MC thermocoloration of SP-YS was also monitored by changes in absorbance at 490 nm corresponding to the absorption bands for the MC form of the spiropyran moiety (Figure

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SP-YS (+4):
SP-Aha-Ile-Tyr-Gly-Glu-Phe-Lys-Lys-Lys-Aha-Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH_2

SP-pYS (+2):
SP-Aha-Ile-pTyr-Gly-Glu-Phe-Lys-Lys-Lys-Aha-Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH_2

SP-YpS (+2):
SP-Aha-Ile-Tyr-Gly-Glu-Phe-Lys-Lys-Lys-Aha-Leu-Arg-Arg-Ala-pSer-Leu-Gly-NH_2

SP-pYpS (0):
SP-Aha-Ile-pTyr-Gly-Glu-Phe-Lys-Lys-Lys-Aha-Leu-Arg-Arg-Ala-pSer-Leu-Gly-NH_2

c-Src substrate region PKA substrate region

Figure 3. Amino acid sequences of spiropyran-containing peptides in this study. Numbers in parentheses denote a possible net charge in the peptides in neutral aqueous solution. Abbreviations: SP = spiropyran derivative; Aha = 6-aminohexanoic acid; pSer = phosphoserine; pTyr = phosphotyrosine.

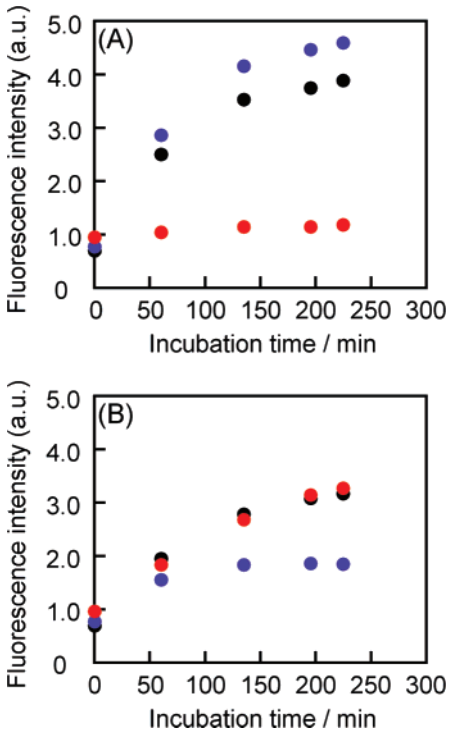

Figure 4. Time courses of the SP-to-MC thermocoloration for (A) SP-YS and (B) SP-pYpS in the absence (black) and presence of poly(L-aspartate) (red) and poly(L-lysine) (blue). Thermocoloration was monitored by changes in fluorescence intensity (excitation filter F485, emission filter = F580 ± 10). [Peptide] = 5.0 μM and [ionic polymer] = 10 μM in 20 mM Tris HCl (pH 7.4) at 25 °C (n = 4, errors within ± 7%).

S2). It was found that time course profiles monitored by absorbance were in good agreement with those by fluorescence intensity, indicating that changes in fluorescence and absorbance during incubation strongly correlated with the amount of the MC form produced by thermocoloration.

Meanwhile, the SP-to-MC coloration profiles of the neutral SP-pYpS in the absence and presence of poly(L-aspartate) were quite similar to each other during the period examined (0.93 ± 0.04, Figure 4B). However, the addition of poly(L-lysine) to the SP-pYpS peptide solution clearly lowered the MC-form content during the relaxation process (0.68 ± 0.06). These alternative thermocoloration properties imply that a thermocoloration with poly(L-aspartate) is switched "on" only when both kinases exceed preset concentration thresholds as the "AND" logic gate, and the "NOR" logic gate is also "on" by the poly(L-lysine)-coupled thermocoloration without kinase treatments.

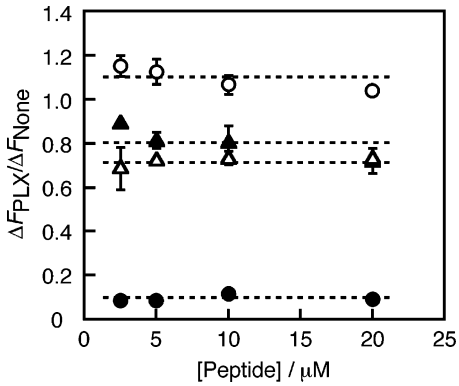

Figure 5. Peptide concentration dependency in the SP-to-MC thermocoloration for SP-YS (i = +4) with poly(L-aspartate) (closed circles) and poly(L-lysine) (open circles) and for SP-pYpS (i = 0) with poly(L-aspartate) (closed triangles) and poly(L-lysine) (open triangles) detected by a fluorescence plate reader (excitation filter F485, emission filter = F580 ± 10). [Peptide] = 2.5−20 μM, [ionic polymer] = 10 μM in 20 mM Tris HCl buffer, pH 7.4 at 25 °C (n = 4, the means ± SD). Abbreviation of the y-axis: PLX = PLD for poly(L-aspartate) or PLK for poly(L-lysine).

Peptide Concentration Dependency in the SP-to-MC Thermocoloration. Repetitive information storage and signal generation steps in a molecular-based memory system sometimes change conditions of the gate molecule-containing solutions. Alterations in concentrations of the gate molecule and viscosity of the solutions are critical for a fluorescence signal readout system. The ratiometric signal readout technique is commonly used in the field of chemical biology to afford reliable and reproducible results independent upon the concentrations of probes applied.[59] Thus, we examined the peptide concentration dependency in the SP-to-MC thermocoloration of SP-YS (i = +4) and SP-pYpS (i = 0) in the absence and presence of ionic polymers in a microplate format. As shown in Figure 5, the relative changes in fluorescence intensity during 60 min of the SP-to-MC thermocoloration in the presence vs absence of ionic polymers were constant at peptide concentrations ranging from 2.5 to 20 μM. The independence of the relative changes in fluorescence intensity upon varying probe concentrations is quite crucial for information storage and signal generation cycles in the molecular memory system. These results (Figures 4 and 5) encouraged us to use the relative changes in fluorescence intensity in the presence vs absence of ionic polymers [$\Delta F_{PLX}/\Delta F_{None}$, where PLX = PLD for poly(L-aspartate) and PLK for poly(L-lysine)] during 60 min of the SP-to-MC thermocoloration

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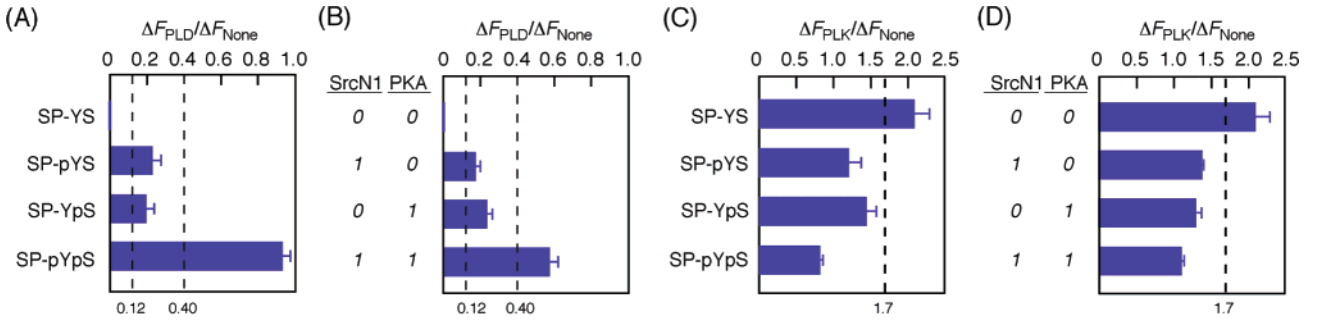

(E)

Input		Output		
		Programming element		
		PLD		PLK
I_1 SrcN1	I_2 PKA	O_1 AND (0.40)	O_2 OR (0.12)	O_3 NOR (1.7)
0	*0*	*0* (< 0)	*0* (< 0)	*1* (2.09 ± 0.21)
1	*0*	*0* (0.17 ± 0.03)	*1* (0.17 ± 0.03)	*0* (1.37 ± 0.03)
0	*1*	*0* (0.23 ± 0.03)	*1* (0.23 ± 0.03)	*0* (1.28 ± 0.08)
1	*1*	*1* (0.57 ± 0.05)	*1* (0.57 ± 0.05)	*0* (1.10 ± 0.04)

(F)

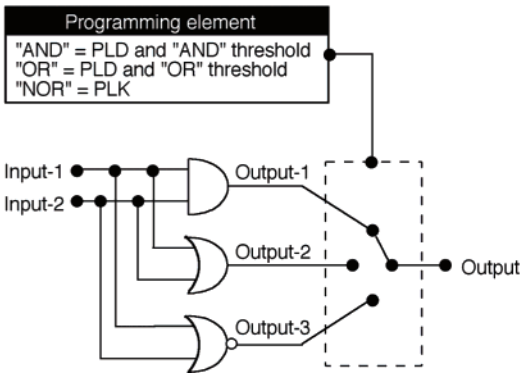

Figure 6. Programmable logic operations using the spiropyran-containing tandem kinase substrate peptide SP-YS. (A) Authentic signals of the SP-to-MC thermocoloration for spiropyran-containing peptides with poly(L-aspartate). (B) "AND" and "OR" logic functions operated with SP-YS, SrcN1, PKA, and poly(L-aspartate). Preset thresholds in the $\Delta F_{PLD}/\Delta F_{None}$ value were 0.40 and 0.12 for the "AND" and "OR" logic operations, respectively. (C) Authentic signals of the SP-to-MC thermocoloration for spiropyran-containing peptides with poly(L-lysine). (D) "NOR" logic function operated by SP-YS, SrcN1, PKA, and poly(L-lysine). A preset threshold in the $\Delta F_{PLD}/\Delta F_{None}$ value was 1.7 for the "NOR" logic operation. (E) Truth table of the phosphate-mediated logic gate programmable with external ionic polymers. (F) Symbolic representation of the programmable logic gate in this study. Output-1 = "AND", output-2 = "OR", and output-3 = "NOR". Input conditions: [SrcN1] = 0/1 = 0 nM/110 nM and [PKA] = 0/1 = 0 nM/60 nM, respectively (n = 4−12, the means ± SD). Abbreviations: PLD = poly(L-aspartate); PLK = poly(L-lysine). Conditions are detailed in the Experimental Section.

to examine functions of the tandem protein kinase substrate peptide as a phosphate-mediated molecular memory.

Logic Operations. The spiropyran-containing peptides, SP-YS (i = +4), SP-pYS (i = +2), SP-YpS (i = +2), and SP-pYpS (i = 0) were treated according to the procedure for the logic operations (detailed in the Experimentaal Section) but lacking kinases before the SP-to-MC thermocoloration starts to precisely characterize their coloration properties (Figure 6A). The diphosphorylated peptide SP-pYpS showed the greatest $\Delta F_{PLD}/\Delta F_{None}$ value (0.93) among the peptides, suggesting that SP-pYpS scarcely bound to poly(L-aspartate) with charge repulsion. The $\Delta F_{PLD}/\Delta F_{None}$ values for monophosphorylated peptides SP-pYS and SP-YpS were 0.23 and 0.20, respectively, and that for nonphosphorylated peptide SP-YS was obtained as ∼0. These discontinuous $\Delta F_{PLD}/\Delta F_{None}$ values obtained for three different phosphorylated states, especially the much greater gaps in the $\Delta F_{PLD}/\Delta F_{None}$ values for diphosphorylated and both monophosphorylated states, allow us to perform the "AND" logic operation using SP-YS as a gate molecule. Expectedly, inputs of both SrcN1 and PKA ([SrcN1]/[PKA] = 110 nM/60 nM = 1/1) at concentrations high enough for complete phosphorylation of each substrate[50] showed the greatest signal (1/1 = 0.57) (Figure 6B). Addition of SrcN1 (110 nM) or PKA (60 nM) as an input element afforded the $\Delta F_{PLD}/\Delta F_{None}$ values for both (1/0) and (0/1) as 0.17 and 0.23, respectively, and that for no kinase inputs (0/0) as ∼0. Therefore, the preset thresholds of the $\Delta F_{PLD}/\Delta F_{None}$ value would be 0.40 for "AND" and 0.12 possibly for "OR" logic operations.

Meanwhile, the SP-to-MC thermocoloration of SP-YS with poly(L-lysine) showed the greatest $\Delta F_{PLK}/\Delta F_{None}$ value (2.09) compared with those for the other three peptides (1.20, 1.44, and 0.83 for SP-pYS, SP-YpS, and SP-pYpS, respectively), suggesting the possibility of the "NOR" logic operation with 1.7 for the preset threshold of the $\Delta F_{PLK}/\Delta F_{None}$ value (Figure 6C). The "NOR" logic function was successfully operated with the preset threshold (1.7) using SP-YS as a gate molecule (Figure 6D). These results are summarized in the truth table (Figure 6E) and as a symbol of the programmable logic gate (Figure 6F), in which two different information inputs (SrcN1 and PKA) can provide three different "AND", "OR", and "NOR" outputs controlled by combinations of external ionic polymers and the preset thresholds of the $\Delta F_{PLX}/\Delta F_{None}$ value. However, the operation using poly(L-lysine) was not applicable for a "NAND (Not AND)" logic function, because the gaps in the $\Delta F_{PLK}/\Delta F_{None}$ values for (1/0 or 0/1) and (0/0) were too close to each other, so that a robust preset threshold for "NAND" operation cannot be defined. Furthermore, differences were observed between the profiles of the $\Delta F_{PLX}/\Delta F_{None}$ values for samples including peptides phosphorylated by kinases and those including authentic phosphorylated peptides. In particular, the $\Delta F_{PLX}/\Delta F_{None}$ values for (1/0) and (1/1) inputs were different from those for the corresponding authentic samples, probably due to the phosphorylatable region for SrcN1 sandwiched between the relatively bulky spiropyran moiety and the PKA substrate region, which likely lowered enzyme accessibility.

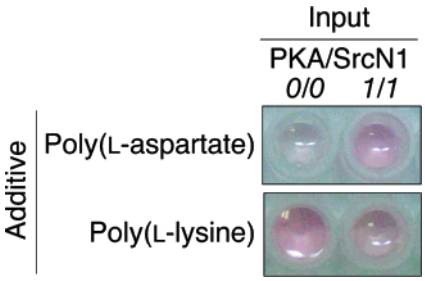

Figure 7. "Naked-eye" signal readouts for SP-YS in a 96-well plate format. Photographs of the reaction mixtures containing peptide, kinases (if required), ATP, cAMP, and external ionic polymers were taken immediately after incubation at 60 °C for 15 min in the dark. Conditions are detailed in the Experimental Section.

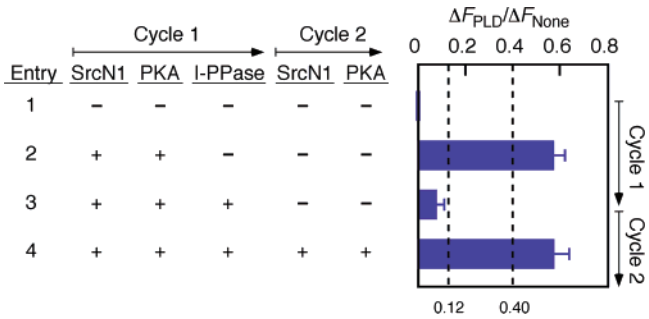

Figure 8. Memory function of the tandem kinase substrate peptide SP-YS with protein kinases SrcN1 and PKA and protein phosphatase lambda (I-PPase). Data for entries 1 and 2 were represented from Figure 6B for comparison. Entry 3 shows the SP-to-MC thermocoloration after information erasion by phosphatase-catalyzed dephosphorylation. Entry 4 shows the second cycle for a memory function where information is recorded by kinase-catalyzed phosphorylations again. The $\Delta F_{PLD}/\Delta F_{None}$ values (0.40 and 0.12) at the bottom denote the preset thresholds for the "AND" and "OR" logic functions, respectively ($n = 3-4$, the means ± SD).

"Naked-Eye" Signal Readout. As one of the potential features of spiropyran derivatives, colorimetric detection of phosphorylated states is quite attractive.[50] Such detection should be possible with spiropyran derivatives because the MC form has intense absorption bands in the visible region, whereas the SP form does not absorb in the visible range. Thus, we performed so-called "naked-eye" signal readouts using the spiropyran-containing tandem kinase substrate SP-YS, which does not require fluorescence spectrophotometers and plate readers to acquire signals (Figure 7). The photographs show the SP-to-MC thermocoloration of SP-YS ($i = +4$) with each of the ionic polymers. The coloration with poly(L-aspartate) was quite intense when both kinases were added into the reaction mixture, whereas the peptide solution lacking both kinases showed little color. By contrast, the coloration with poly(L-lysine) was much more intense for the mixture without both kinases than that with kinases. Thus, the present phosphate-mediated molecular logic system using the spiropyran-containing tandem kinase substrate peptide could be used to perform logic operations in both fluorometric and colorimetric microplate formats.

Memory Function. Generally, a memory system is equipped with a reset function to make itself recordable again. To produce an information record/erasion cycle in the present molecular memory system, we attempted to erase information recorded on the tandem kinase substrate peptide using phosphatase-catalyzed dephosphorylation (Figure 8). Data of entries 1 and 2 were reproduced from Figure 6B for comparison. After treatment of SP-YS with both SrcN1 and PKA under the phosphorylation condition, the reaction mixture was heated to terminate phosphorylation, followed by dephosphorylation with protein phosphatase lambda (I-PPase) to remove the phosphate groups from the diphosphorylated peptide (entry 3). The $\Delta F_{PLD}/\Delta F_{None}$ value observed for entry 3 showed a significant reduction in the $\Delta F_{PLD}/\Delta F_{None}$ value (0.075) due to the increase in the binding affinity of the peptide to poly(L-aspartate) upon dephosphorylation. The $\Delta F_{PLD}/\Delta F_{None}$ value for entry 3 (0.075) was below the "OR" threshold (0.12), indicating that I-PPase effectively removed phosphoesters from the peptide and reset it to the initial recordable state.

Furthermore, to move to the second memory cycle (cycle 2), we separately attempted to phosphorylate the reproduced SP-YS using both SrcN1 and PKA after termination of the dephosphorylation by heating the peptide-containing solution. The $\Delta F_{PLD}/\Delta F_{None}$ value for the diphosphorylated state at the cycle 2 was found to be 0.58, which was coincident with that for the cycle 1, indicating that phosphate groups were effectively incorporated onto the peptide reproduced to be recordable. Although it seemed that the present memory system could be repeatedly operated by a combination of protein kinases and a phosphatase, the fluorescence intensity of the MC form obtained in raw data sets for the cycle 2 decreased to be about 60% of that for the cycle 1 by decomposition of the spiropyran moiety in the peptide resulting from repetitive heating of sample solutions to terminate enzymatic reactions (Figure S3). However, the present concept might be promising to develop a molecular memory system in near future.

Conclusions

We have designed and synthesized a tandem protein kinase substrate peptide composed of two different kinase substrate regions joined in series and a spiropyran derivative at the N-terminus. We successfully used this peptide (gate molecule) to perform three basic "AND", "OR", and "NOR" logic operations on the basis of alterations in the SP-to-MC thermocoloration properties of the spiropyran moiety in the peptide upon kinase-catalyzed phosphorylation in a microplate format. Throughout this study, information was recorded onto the gate molecule by protein kinases, stored stably as phosphoesters, read based on the extent of the SP-to-MC thermocoloration, and erased by phosphatase-catalyzed dephosphorylation, resulting in the gate molecule being reset to the initial recordable state. The use of other combinations of enzymes would diversify logic functions for a molecular-based processing and computing. However, the present phosphate-mediated molecular memory system still has some disadvantages, including a prolonged signal generation process and decomposition of the spiropyran moiety during manipulations. The use of two different types of ionic polymers (positively charged and negatively charged polymers) as additives for the signal readout of the different gates also has disadvantage that gates cannot be combined to work in series different from the sequential operations reported in the literature.[44] However, our memory system is potentially useful for addressing concerns regarding the diversity of input elements and the mechanisms of information storage, signal generation, and signal readout. The ways to sophisticate the enzyme-based memory system would be screening a novel chromophore covering broadly such criteria and/or immobilization of gate molecules onto solid supports (e.g., polystylene

beads) to easily separate the gates from clumsy reaction mixtures, which are now underway. Thus, the proof-of-concept experiments described herein could provide clues for developing molecular-based processing and computing.

Experimental Section

General Methods. The protein kinases, cAMP-dependent protein kinase A (PKA, bovine heart, 170 kDa), c-Src protein tyrosine kinase (SrcN1, recombinant, human, 56 kDa), ATP, cAMP, and poly(L-aspartic acid) sodium salt (M_r 15 000−50 000) were purchased from Sigma. Protein phosphatase lambda (I-PPase, recombinant, 25 kDa) was purchased from Calbiochem. Poly(L-lysine) hydrobromide (M_r 70 000−150 000) was purchased from Nacalai Tesque (Kyoto, Japan). All solvents and reagents (except amino acid derivatives) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used as received. Fmoc-amino acid derivatives and reagents for peptide syntheses were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Acetonitrile (HPLC grade) was used for HPLC analysis and purification. Water was purified with the MilliQ production system. Absorption spectra were acquired on a Shimadzu UV-2550 spectrophotometer equipped with a thermoregulator using a quartz cell (10 mm path length). Fluorescence signals were recorded on a microplate reader ARVO MX1420 multilabel counter (Perkin-Elmer) equipped with an excitation filter F485 and an emission filter F580 ± 10. Absorbance signals were also acquired on the same microplate reader equipped with a 490-nm filter.

Chromatography. Analytical HPLC and purification of peptides were performed on a Hitachi L7000 or a Shimadzu LC10AD system equipped with a Wakosil 5C18 or a YMC ODS-A (4.6 × 150 mm) with a linear gradient of acetonitrile/0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 mL/min for analysis and a YMC ODS A323 (10 × 250 mm) at a flow rate of 3.0 mL/min for preparative purification.

Mass Spectrometry. Mass spectra were obtained on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS, Shimadzu KOMPACT MALDI III, Kyoto, Japan) with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix.

Noncommercial Compounds. Compounds 1-(2-hydroxyethyl)-3,3-dimethylindolino-6′-nitrobenzopyrylospiran (**1**)[22] and *p*-nitrophenyl-carbonate active ester of compound **1** (**2**)[51] were prepared according to the literature.

Peptide Synthesis. Procedures for synthesis of all new spiropyran-containing peptides were detailed in the Supporting Information (Figure S1).

Preparation of the Stock Solutions of Spiropyran-Containing Peptides. Spiropyran-containing peptide lyophilized after purification by HPLC was dissolved in H_2O, and the concentration of the stock solution was adjusted to be 1.0 mM using the absorption coefficient at a peak of 355−360 nm (ϵ = 11 200 M^{-1} cm^{-1} in 1,1,1,3,3,3-hexafluoroisopropyl alcohol containing 0.5% H_2O).[60] The stock solution was safely stored for several months at −20 °C without a light exposure.

Measurement of the SP-to-MC Thermocoloration. A peptide solution (5.0 μM, 500 μL) in 20 mM Tris HCl buffer (pH 7.4) containing poly(L-aspartate) (10 μM) or poly(L-lysine) (10 μM) was irradiated with indoor lightning for 5 min at room temperature to prepare a colorless SP-dominant solution [we assumed concentrations of poly(L-aspartate) (10 mg/448 μL) and poly(L-lysine) (25 mg/453 μL) to be 0.5 mM in 20 mM Tris HCl buffer (pH 7.4)].[50] Four aliquots (100 μL each) were transferred from each solution to a 96-well plate (Costar 3915, black polystylene, Corning Inc.), and the initial fluorescence intensity (F_0) was recorded on the fluorescence microplate reader. The 96-well plate was sealed with a plate seal to avoid contamination and evaporation, wrapped with an aluminum sheet, and incubated at 25 °C in the dark. At the time points requested, the 96-well plate was taken out from the incubator and the aluminum sheet and plate seal were peeled off; subsequently, fluorescence intensity (F) was recorded.

General Procedure for the Signal Generation and Signal Readout. Peptide-containing solutions (several sets of control and sample solutions, 100 μL each) were transferred to a 96-well plate (Costar 3915, black polystylene, Corning Inc.), and the initial fluorescence intensity (F_0) was recorded on the fluorescence microplate reader. The 96-well plate was sealed with a plate seal to avoid contamination and evaporation, wrapped with an aluminum sheet, and incubated at 25 °C in the dark. After 60 min, the 96-well plate was taken out from the incubator and the aluminum sheet and plate seal were peeled off; subsequently, fluorescence intensity (F_{60}) was recorded. Relative changes in fluorescence intensity of the spiropyran moiety in the presence vs absence of ionic polymers [$\Delta F_{PLX}/\Delta F_{None}$, where PLX = PLD for poly(L-aspartate) and PLK for poly(L-lysine)] was calculated with the equation $\Delta F_{PLX}/\Delta F_{None} = (F_{60} - F_0)_{PLX}/(F_{60} - F_0)_{None}$. Data from the wells mishandled were excluded.

"AND" and "NOR" Logic Operations. A mixture (100 μL) of peptide (50 μM), cAMP (1.0 mM), and ATP (1.0 mM) was reacted with kinase(s) (amount requested) in 100 mM HEPES buffer (pH 7.0) containing 5.0 mM $MgCl_2$ at 30 °C for 60 min in the dark. The reaction mixture was irradiated with indoor lightning for 5 min at room temperature to prepare a colorless SP-dominant solution. Two aliquots (45 μL each) were transferred from the reaction mixture to separated eppendorf tubes and mixed with 20 mM Tris HCl buffer (pH 7.4) (405 μL) for a control solution and 20 mM Tris HCl buffer (pH 7.4) (396 μL) and 0.5 mM ionic polymers (9.0 μL) [poly(L-aspartate) for "AND" or poly(L-lysine) for "NOR" logic operations] for a sample solution. Fluorescence data were acquired according to the General Procedure for the Signal Generation and Signal Readout section described above.

"Naked-Eye" Signal Readout. A mixture (100 μL) of SP-YS (100 μM), ATP (1.0 mM), and cAMP (1.0 mM) was reacted with SrcN1 (183 nM) and PKA (60 nM) if required in 100 mM HEPES buffer (pH 7.0) containing 5.0 mM $MgCl_2$ at 30 °C for 2 h in the dark. The reaction mixture was irradiated with indoor lightning for 5 min at room temperature to prepare a colorless SP-dominant solution. The solution was mixed with 50 μL of 20 mM Tris HCl buffer (pH 7.4), and two aliquots (70 μL each) were taken from the solution and transferred separately to a 96-well plate (Costar 3363, polypropylene, Corning Inc.). The resulting solutions were mixed with 0.5 mM poly(L-aspartae) or poly(L-lysine) in 20 mM Tris HCl buffer (pH 7.4) (30 μL), and the 96-well plate was sealed with a plate seal to avoid contamination and evaporation. The plate was wrapped with an aluminum sheet and incubated at 60 °C in the dark. After 15 min, the 96-well plate was taken out from the incubator and the aluminum sheet and plate seal were peeled off; subsequently, photographs were taken within 1 min.

Information Erasion. According to the procedure for "AND" and "NOR" Logic Operations section described above, SP-YS (50 μM) were phosphorylated by SrcN1 (110 nM) and PKA (60 nM) (reaction volume 100 μL). The reaction mixture was incubated at 75 °C for 5 min to terminate phosphorylations and stored on ice. The resulting mixture was diluted with 20 mM Tris HCl (pH 7.4) containing 2.0 mM $MnCl_2$ (100 μL), treated with I-PPase (200 units) at 30 °C for 2 h in the dark, and irradiated with indoor lightning for 5 min at room temperature to prepare a colorless SP-dominant solution. Two aliquots (90 μL each) were transferred from the reaction mixture to separated eppendorf tubes and mixed with 20 mM Tris HCl buffer (pH 7.4) (360 μL) for a control solution and 20 mM Tris HCl buffer (pH 7.4) (351 μL) and 0.5 mM poly(L-aspartate) (9.0 μL) for a sample solution. Fluorescence data were acquired according to the General Procedure for the Signal Generation and Signal Readout section described above.

"AND" Logic Operation (the Second Cycle). According to the procedure in the Information Erasion section described above, SP-YS (50 μM) was phosphorylated by SrcN1 (110 nM) and PKA (60 nM) (reaction volume 100 μL). The reaction mixture was incubated at 75 °C for 5 min to terminate phosphorylations and stored on ice. The

(60) Angelini, N.; Corrias, B.; Fissi, A.; Pieroni, O.; Lenci, F. *Biophys. J.* **1998**, *74*, 2601−2610.

resulting mixture was diluted with 20 mM Tris HCl (pH 7.4) containing 2.0 mM $MnCl_2$ (100 μL), treated with I-PPase (200 units) at 30 °C for 2 h in the dark (total reaction volume 200 μL). The reaction mixture was incubated at 75 °C for 5 min to terminate dephosphorylation and stored on ice. To the solution were added active SrcN1 (final concentration 110 nM), active PKA (final concentration 60 nM), ATP [20 μL, 5.0 mM in 100 mM HEPES buffer (pH 7.0) containing 5.0 mM $MgCl_2$], cAMP [20 μL, 5.0 mM in 100 mM HEPES buffer (pH 7.0) containing 5.0 mM $MgCl_2$], and 100 mM HEPES buffer (pH 7.0) containing 5.0 mM $MgCl_2$ (to adjust the volume of the reaction mixture to be 250 μL), and the resulting mixture was incubated at 30 °C for 1 h in the dark. Two aliquots (120 μL each) were transferred from the reaction mixture to separated eppendorf tubes and mixed with 20 mM Tris HCl buffer (pH 7.4) (330 μL) for a control solution and 20 mM Tris HCl buffer (pH 7.4) (321 μL) and 0.5 mM poly(L-aspartate) (9.0 μL) for a sample solution. Fluorescence data were acquired according to General Procedure for the Signal Generation and Signal Readout section described above.

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Supporting Information Available: Synthesis of spiropyran-containing peptides, SP-to-MC thermocoloration in the absence and presence of ionic polymers, and comparison of the changes in fluorescence intensity for the memory cycles 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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