DOI: 10.1002/chem.201001488

Light-Induced Peptide Replication Controls Logic Operations in Small Networks

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The research of complex systems has received a lot of attention in mathematics, physics, and biology, but until not too long ago was significantly underdeveloped in chemistry. Recently, it has been realized that rationally designed self-organized synthetic networks might provide useful models for the understanding and exploitation of the behavior of complex systems.[1–4] Thus, several relatively complex networks were studied; it was found that it is possible to predict and analyze their connectivity and global topology. Moreover, the networks could be manipulated in various ways to show, just like cellular networks, substantial rewiring following environmental changes, and that they can carry out chemical transformations via various programmed pathways, such as the Boolean logic operations.[5–11] An important family of non-enzymatic systems uses template-directed autocatalysis and cross-catalysis as a means of wiring the network components and controlling their dynamics and replication.[12–19] As such, these networks have received considerable attention with respect to possible scenarios in the origins of life and early molecular evolution.[20–23] Several approaches have been taken to manipulate the systems studied so far based on chemical changes that can affect the replication efficiency. Thus, a common intuitive method was used by changing the concentrations of network constituents, either reactants or templates, in order to enhance specific pathways.[16,18] Changing the characteristics of the principle template-assisted reaction by introducing structural changes to the templates' backbone has been utilized in several cases to control small networks,[15,17,24–26] while changes in the environmental conditions by varying, for example, salt concentrations and/or pH, were used to rewire the networks.[14,27] The ability to test and control the response of non-enzymatic networks to external signals might increase their utility and applicability significantly. Such triggering can be used to shift the self-organization states away from the equilibrium and thus may provide temporary control over the progress of the chemical (replication) reactions and the entire network topology. To the best of our knowledge, this challenge has not yet been met. We describe herein the use of light as an external trigger for quantitative control of peptide tertiary structures and consequently as a tool for controlling peptide-based self-replication, thereby affecting replication-dependent processes in small molecular networks and facilitating selective product formation via the "AND" Boolean function.

Light is an ideal external trigger signal for inducing molecular functionality,[28–30] since it can be manipulated very precisely in terms of time and space, and because the reaction mixtures usually do not react to light. The irreversible photoactivation of proteins has been described in several studies, which showed that it can be used to investigate protein folding and to modulate protein functions.[31–34] The currently replication process takes place by enhanced coupling of two fragments driven by association onto the replicating molecule as a template. While different peptide-based replication systems have been developed and analyzed recently,[26,35] this well-characterized system[36,37] was chosen since it is the only system that performs sequence-specific replication, based on a "residue-to-residue" intermolecular interactions.[16] We thus employ dimeric coiled coil proteins as templates for the association of nucleophilic and electrophilic fragments, which due to the close proximity and appropriate directionality while at the intermediate complex, can be attached together via the native chemical ligation reaction. The light-induced uncaging of the coiled coils is used as a mechanism to initiate the template-assisted replication processes (Figure 1 a), and later on to induce selectivity within replication networks.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201001488.

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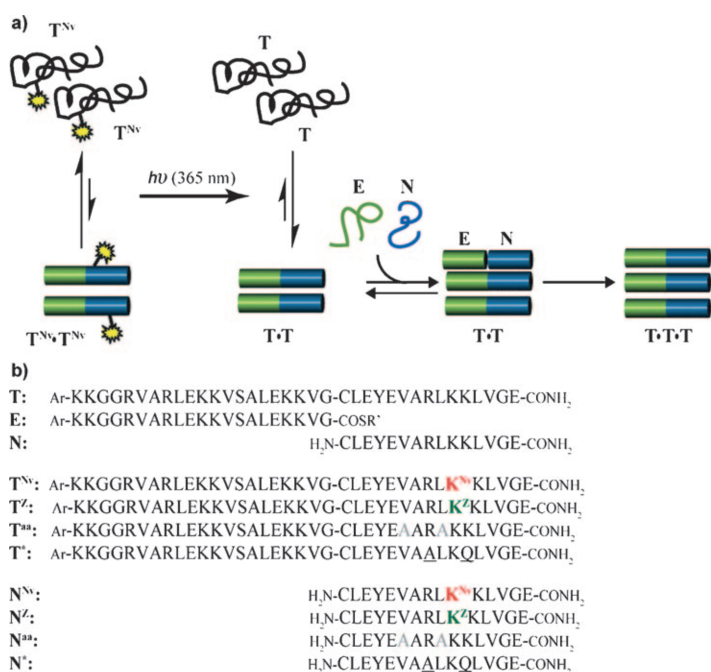

Figure 1. a) Light-induced template assisted ligation scheme. In the dark (left), the caged template $\mathbf{T^{Nv}}$ is catalytically inactive since it cannot dimerize as a coiled coil nor associate with the shorter fragments **E** and **N**. After illumination with monochromatic UV light, it becomes active; the cage-free peptide **T** forms stable dimeric coiled coils complex that serves as a template for replication. b) Peptide sequences employed in this study. $\mathbf{K^{Nv}}$ = Nvoc protected Lys, $\mathbf{K^{Z}}$ = 2-Chlorobenzyloxycarbonyl (2-Cl-Z) protected Lys. $\mathbf{T^{aa}}$ and $\mathbf{N^{aa}}$ posses Leu → Ala mutations (gray) in the hydrophobic core compared to **T** and **N**, respectively. **T*** and **N*** are close analogues of **T** and **N**; respectively, having substituted at solvent exposed non-informational amino acids (underlined). Ar = 4-acetamidobenzoic acid (ABA), and R′ = ethanesulfonic acid.

It has been established that the ligation rate and replication efficiency can be correlated with the unfolding stability of both the dimer template (**T·T**) and the ternary template–product (**T·T·T**) complexes shown in Figure 1 a.[16] Since the stability of these species is dictated by the characteristics of amino acids at the inter-helix recognition interface—occupying positions *a*, *d*, *e* and *g* at the helix heptad repeat (see helical wheel presentation in Figure S1, Supporting Information)—the replication and consequently network behavior in arrays of replicating mutants can be predicted quite accurately.[16] In order to achieve external control over the replication using light, we have synthesized a caged peptide ($\mathbf{T^{Nv}}$, sequences in Figure 1 b) with the photo-cleavable moiety 6-nitroveratryloxycarbonyl (Nvoc) attached at a Lys residue (Figure S2) on a *g* heptad position. As illustrated in Figure 1 a and emphasized using the helical wheel presentation (Figure S1), we envisioned that caging the peptide will interfere with the formation of tertiary structures, thus reducing significantly the concentrations of dimer template and quaternary intermediate complexes, and as a result decrease the availability of $\mathbf{T^{Nv}}$ as a template for replication. Upon short exposure to light and removal of the Nvoc group, a new peptide **T** will be formed, which is expected to fold and aggregate correctly and thus to replicate.

All peptides were synthesized on solid-phase, purified by preparative HPLC, and characterized by HPLC and mass spectra (Figures S3 and S4). The caging Nvoc groups were introduced by first synthesizing the fully-protected nucleophile peptides with the specific Lys side chains orthogonally protected with an Mtt (4-methyltrityl) group, and then selectively removing the Mtt (with 1 % TFA) and reacting the Lys side chain with Nvoc-Cl. $\mathbf{T^{Nv}}$ was prepared by ligating the electrophile **E** with $\mathbf{N^{Nv}}$ (see also in Figure S4).

To compare the unfolding stability of caged and cage-free peptides using circular dichroism (CD), we have synthesized additional caged peptide $\mathbf{T^{Z}}$, analog of $\mathbf{T^{Nv}}$ that is stable during spectroscopic measurements, in which the same Lys residue was equipped with the light-insensitive 2-Cl-Z group (Figure S2). The formation of secondary structures in solutions of **T** and $\mathbf{T^{Z}}$ was deduced from the CD measurements (Figure S5a). Deeper minima at the 208 and 222 nm were found in the spectrum of **T** relative to that of $\mathbf{T^{Z}}$, reflecting 90 and 75 % helix formations at room temperature, respectively. Following the thermal denaturation of both peptides by CD at 222 nm (Figure S5b) revealed melting temperatures (T_m) of 74 and 62 °C for **T** and $\mathbf{T^{Z}}$, respectively, correlating with a free energy difference ($\Delta\Delta G_{caged-uncaged}$) of 2.4 kcal mol^{-1}[38] (~1.2 kcal mol^{-1} per caged residue). Since it was found earlier that 1.0 kcal mol^{-1}, or larger, differences in template stability can be translated to significant differences in replication rates,[5] these results supported our notion that it would be possible to affect the replication process by removal of the caging group. In order to probe if the uncaging triggering can be finely tuned such that the removal of Nvoc from $\mathbf{T^{Nv}}$ takes place rapidly enough to produce large amounts of **T** available for replication, we have analyzed the rate of uncaging by HPLC, and found that the uncaging process proceeds with first-order kinetics characterized by $t_{1/2}$ = 2 min and $t_{90\%}$ = 8 min (Figure S6). Accordingly, we have chosen the 8 min irradiation time as the optimal conditions for the following replication experiments.

Removal of the Nvoc caging group by light produces the active template **T** from $\mathbf{T^{Nv}}$. The self-replication of **T** itself was studied (Figure 2 a), and showed the expected sigmoid plot in product growth versus time (Figure 2 a, insert), reflecting kinetic behavior similar to other previously studied peptides.[37] Although still suffering from slow turnover like almost all other non-enzymatic replication systems—as observed in experiments done with higher amounts of seeded template (24–90 μM, corresponding to 12–45 mol % of **T·T**; Figure S7a)—it exhibited somewhat improved replication order of $p = 0.75$ (Figure S7b). This result was attributed to lower product inhibition, which was obtained due to the addition of a "solubility tail" at the N-terminus made of KKGG residues; this causes destabilization of the template-product complex **T·T·T** (Figure 1). In order to study the effect of cage removal on replication, we have compared the rate of ligation of equimolar amounts of **E** and **N** in the presence of $\mathbf{T^{Nv}}$, in the dark and right after shining light (365 nm; 8 W cm^{-2}) for 8 min. As shown in Figure 2 b, slow formation of **T** was observed in the dark, with a rate similar

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to that of the template-free reaction. A much higher replication rate was observed after releasing **T** by shining light, regaining a replication rate similar to that observed in systems seeded with **T** itself. These data showed, to the best of our knowledge for the first time, the possibility of controlling non-enzymatic molecular replication by light. Since the amounts of released template can be controlled by varying the time of exposure to light (Figure S6), we have further characterized the rate of product formation as a function of exposure time. Figure 2c provides evidence that it is possible to quantitatively control the rate of replication by incremental changes in the amount of light energy provided. In order to show that the new system is useful for temporal control over network behavior, we have studied the possibility of initiating replication at different stages of the reaction. The reaction in the presence of $\mathbf{T}^{\mathbf{Nv}}$ was followed in the dark for different periods of time, before the reaction mixture was exposed to light for 2 min. Higher replication rates were observed during and after irradiation (Figure 2d). The fast formation of **T** during bright light prevented us from accurately determining the amount of newly formed **T** during replication. Thus, this experiment was performed in "quasi-self-replication" mode, in which product **T*** differs slightly from the template by mutations at the non-informative solvent exposed residues (Figure 1b).

The light-induced replication process was further studied as a means to affect competition experiments within ternary networks (Figure 3a). We have synthesized two new nucleophilic fragments, $\mathbf{N}^{\mathbf{aa}}$ and $\mathbf{N}^{\mathbf{z}}$, that compete together with **N** for ligation to **E** as common reactant, but their formation was not expected to be enhanced by any of the templates in the reaction mixture due to Leu→Ala mutations at the hydrophobic core (in $\mathbf{N}^{\mathbf{aa}}$ and $\mathbf{T}^{\mathbf{aa}}$) and caging ($\mathbf{N}^{\mathbf{z}}$ and $\mathbf{T}^{\mathbf{z}}$). The formation of each of the three products within mixtures that included **E**, **N**, $\mathbf{N}^{\mathbf{aa}}$, $\mathbf{N}^{\mathbf{z}}$ and $\mathbf{T}^{\mathbf{Nv}}$ was analyzed in the dark and after light exposure by HPLC (Figure S8). While in the dark, all three compounds were formed with similar rates when $\mathbf{T}^{\mathbf{Nv}}$ was present (Figure S9); after light exposure and uncaging of $\mathbf{T}^{\mathbf{Nv}}$, only **T** was replicating and thus formed much faster (Figure 3b), unlike $\mathbf{T}^{\mathbf{aa}}$ and $\mathbf{T}^{\mathbf{z}}$.

We have noticed that introduction of $\mathbf{T}^{\mathbf{Nv}}$ and light can be considered as two separate reaction components for the study of logic operations within networks. Thus, the competition experiment was studied four times, in which the rates of product formation in the absence or presence of $\mathbf{T}^{\mathbf{Nv}}$, and in the dark or after shining light, were characterized. The Boolean logic presentation of the data in Figure 3c clearly shows selective product formation of **T** obeying the AND gate operation. These data provide the first example of manipulating logic operations in replication systems by shining light, and furthermore, facilitates the formation of the AND gate via an algorithm much simpler than the previously postulated operation using hetero-dimeric protein templates.[11,39]

We have shown herein that the basic template-assisted replication reaction can be controlled by light, provided that the cage moiety is efficiently removed to result in fast switching from inactivated to active conformation. Since the concentration of cage-free template can be quantitatively dictated by the amount of photons provided, either before or after initiating the replication process, we claim that the new system offers a versatile tool for controlling network behavior at different stages. Accordingly, it can be used to reveal latent catalytic pathways that would otherwise be very slow, or to program product formation via protocols that are more complex then the studied AND gate.

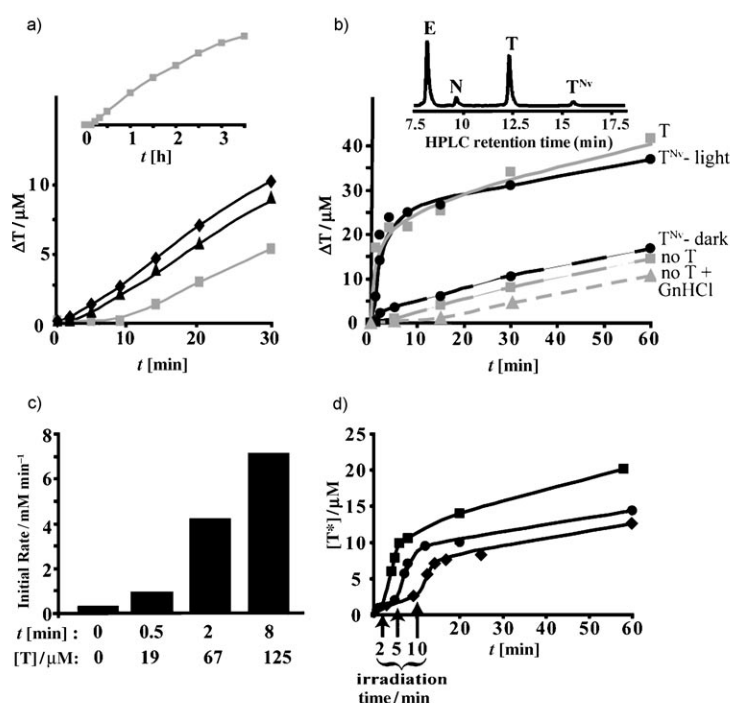

Figure 2. Self-replication in the dark and after shining light. a) Kinetic profile of product formation in reactions between **E** and **N** (100 μм each) in the absence of any template (gray in the main graph and insert) and in the presence of 10 μм (triangles) or 20 μм (diamonds) of **T**. This set of experiments together with the experiments shown in Figure S7 (supporting information) were used to analyze the replication capability of the new system. b) Kinetic profiles of product formation in reactions between **E** and **N** (100 μм each) in the presence of the caged peptide $\mathbf{T}^{\mathbf{Nv}}$ (140 μм) in the dark (black dashed line) and after shining light for 8 min and releasing 125 μм of **T** (solid black line). The kinetics of ligation reactions between **E** and **N** without any template seeded (gray dashed lines) or seeded with premade **T** molecules (100 μм; 50 mol percent of **T·T** with respect to **E** and **N**; gray solid line) are shown for comparison. The product concentrations were calculated from HPLC chromatograms, such as that shown above panel b. c) Initial rates observed for reaction mixtures containing **E**, **N** and $\mathbf{T}^{\mathbf{Nv}}$, which were illuminated before the reaction for the indicated times (*t*). The measured concentrations of **T** released by irradiation are also given. d) Kinetic profiles for reactions between **E** and **N***, to form **T***, in the presence of $\mathbf{T}^{\mathbf{Nv}}$ (80 μм), which were irradiated for 2 min (releasing ~45 μм of **T**) at the indicated times after initiating the reactions in the dark. All experiments were repeated at least two times and showed small variations ($\leq 5\%$) in product formation between consecutive runs. Kinetic profiles shown in panel a were obtained in reactions done at pH 7.0, while those in panel b–d and in Figure S7 at slightly higher pH (7.4).

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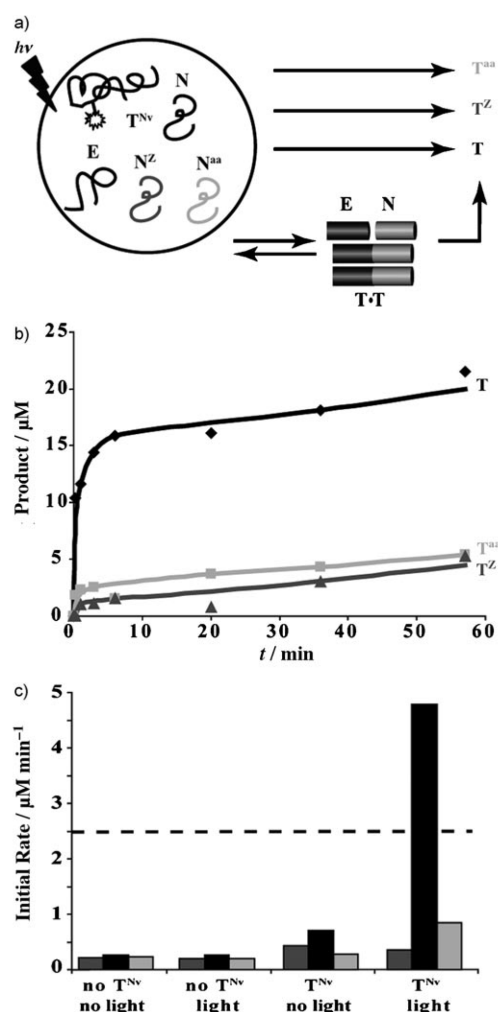

Figure 3. Light-induced selection in small networks. a) Schematic description of the network experiment under illumination. The cage-free template **T** can only form stable complexes with **E** and **N**, facilitating fast template-assisted reaction at the expense of production of the other two templates $\mathbf{T^{aa}}$ and $\mathbf{T^{z}}$. b) Kinetic profiles of reactions between **E** and the three nucleophiles, in the presence of $\mathbf{T^{Nv}}$, to form **T** (black), $\mathbf{T^{aa}}$ (light gray) and $\mathbf{T^{z}}$ (gray) after shining light. c) AND logic gate observed for production of **T** within the network. The initial rates of products formation (output), **T** (black), $\mathbf{T^{aa}}$ (light gray) and $\mathbf{T^{z}}$ (gray), are shown for the various combinations of $\mathbf{T^{Nv}}$ and 365 nm light as inputs.

Acknowledgements

This research is supported by a grant from the Israeli Science Foundation (ISF 1291/08). G.A. acknowledges a Career Development Award from HFSP, and the COST Systems Chemistry action (CM0703). We thank Dr. Rivka Cohen-Luria for her help.

Keywords: complexes · networks · peptides · protein folding · self-replication

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Received: May 9, 2010
Published online: September 14, 2010

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