

# Reversible Photoswitchable Inhibitors Generate Ultrasensitivity in Out-of-Equilibrium Enzymatic Reactions

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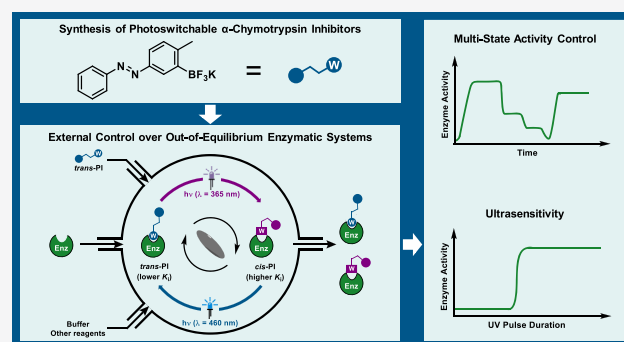


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**ABSTRACT:** Ultrasensitivity is a ubiquitous emergent property of biochemical reaction networks. The design and construction of synthetic reaction networks exhibiting ultrasensitivity has been challenging, but would greatly expand the potential properties of life-like materials. Herein, we exploit a general and modular strategy to reversibly regulate the activity of enzymes using light and show how ultrasensitivity arises in simple out-of-equilibrium enzymatic systems upon incorporation of reversible photoswitchable inhibitors (PIs). Utilizing a chromophore/warhead strategy, PIs of the protease  $\alpha$ -chymotrypsin were synthesized, which led to the discovery of inhibitors with large differences in inhibition constants ( $K_i$ ) for the different photoisomers. A microfluidic flow setup was used to study enzymatic reactions under out-of-equilibrium conditions by continuous addition and removal of reagents. Upon irradiation of the continuously stirred tank reactor with different light pulse sequences, i.e., varying the pulse duration or frequency of UV and blue light irradiation, reversible switching between photoisomers resulted in ultrasensitive responses in enzymatic activity as well as frequency filtering of input signals. This general and modular strategy enables reversible and tunable control over the kinetic rates of individual enzyme-catalyzed reactions and makes a programmable linkage of enzymes to a wide range of network topologies feasible.



## INTRODUCTION

Living systems display unique capabilities, e.g., adaptation to the environment, self-healing, homeostasis, or converting chemical energy into directed motion, growth, and division. These processes are governed by complex chemical reaction networks that operate far from equilibrium and allow a precise regulation of a wide range of cellular mechanisms, e.g., signaling or metabolism.<sup>1–3</sup> A characteristic feature found in many biochemical networks is ultrasensitivity, which means that (in contrast to a standard hyperbolic Michaelis–Menten response) the response to a stimulus yields a sharp, switch-like sigmoidal function (see Figure 1A for a general schematic of the phenomenon).<sup>4–9</sup> This property enables signaling systems to filter out noise and be readily activated once a certain required threshold stimuli is present. Different mechanisms have been identified that can generate this nonlinear input–output relationship, e.g., multisite phosphorylations,<sup>6</sup> molecular titrations (buffering),<sup>8</sup> substrate competition,<sup>9,10</sup> or zero-order kinetics.<sup>4</sup>

A central goal of systems chemistry is to investigate and translate the common design principles of nature into a practical and modular approach, ultimately enabling a programmable and rational design of life-inspired systems exhibiting tunable properties.<sup>11–18</sup> While (light-induced) sigmoidal responses in enzymatic logic gate systems have been reported,<sup>19–22</sup> the bottom-up construction of ultrasensitive, life-inspired enzymatic

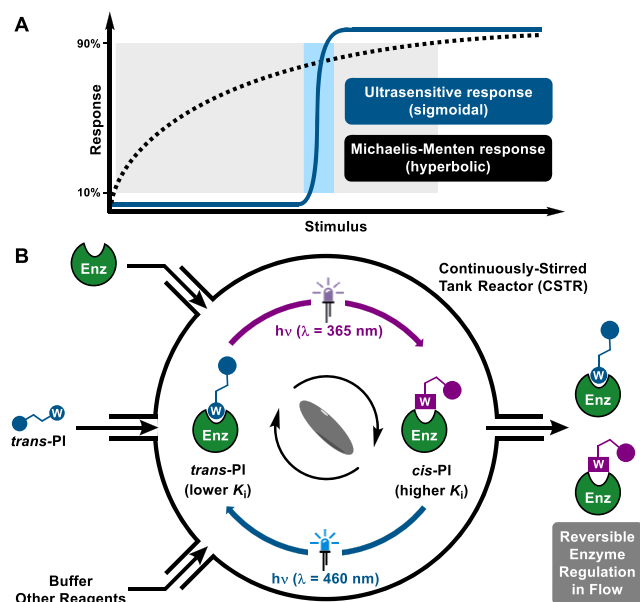
systems remains challenging due to the lack of a general strategy enabling the reversible and tunable regulation of enzymes under out-of-equilibrium conditions.

Although a plethora of different external stimuli to reversibly and spatiotemporally control the activity of enzymes have been applied in the last decades,<sup>23</sup> light is an ideal external control element: it is bioorthogonal ( $\lambda > 360$  nm),<sup>24–27</sup> offers high spatiotemporal resolution, can be precisely tuned in terms of photon flux, and introduces the opportunity to control chemical reaction networks using optoelectronic devices.<sup>28–30</sup> A prominent approach to gain photocontrol over diverse biological processes is the (mostly) covalent installation of photoswitchable chromophores into the biomolecule of interest.<sup>31–35</sup> These so-called “molecular photoswitches” undergo a reversible change in their three-dimensional structure between two or more isomeric forms upon irradiation with light of suitable wavelengths. A number of biological processes, such as protein folding,<sup>36</sup> membrane transport,<sup>37</sup> or transcription and trans-

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**Figure 1.** (A) Schematic of Michaelis–Menten (black) and ultrasensitive response (blue). (B) Incorporation of competitive photoswitchable inhibitors into out-of-equilibrium enzymatic systems enables reversible and tunable activity control using light. Enz = enzyme, W = warhead.

lation,<sup>38</sup> have successfully been photoregulated by the incorporation of photosensitizable units. Thus, an attractive option for obtaining optical control over enzyme function is by installation of molecular photoswitches into the scaffold of enzyme inhibitors.<sup>23,34,39</sup>

Herein, we exploit a general and modular strategy to reversibly regulate the activity of enzymes using light and show how ultrasensitivity arises in simple out-of-equilibrium enzymatic systems upon incorporation of reversible photoswitchable inhibitors (PIs). We studied enzymatic activity of a  $\alpha$ -chymotrypsin/PI-based system under out-of-equilibrium conditions (continuous feeding of substrates and waste removal) using a continuously stirred tank reactor (CSTR) and investigated the response to a series of light pulses (UV (365 nm) and blue (460 nm) light) as input stimuli (Figure 1B). Surprisingly, we discovered a very strong ultrasensitive response to combinations of UV and blue light pulses under out-of-equilibrium conditions. This nonlinear response was also observed in a bottom-up constructed enzymatic cascade based on the proteolytic activity of  $\alpha$ -chymotrypsin (Cr) and aminopeptidase M (Ap). Furthermore, we observed that a combination of flow and reversible activity regulation yields a simple enzymatic system with frequency filtering properties upon gradually increasing the UV pulse irradiation frequency and switching back the PI using blue light.

## RESULTS AND DISCUSSION

### Synthesis and Characterization of Competitive, Photoswitchable Inhibitors of $\alpha$ -Chymotrypsin (PIs).

Due to our long-standing expertise in the bottom-up construction of enzymatic reaction networks using proteases,<sup>29,40–42</sup> we started by synthesizing reversible photoswitchable inhibitors of  $\alpha$ -chymotrypsin (PIs) in order to externally control its enzymatic activity using light. To sufficiently regulate the output of out-of-equilibrium enzymatic

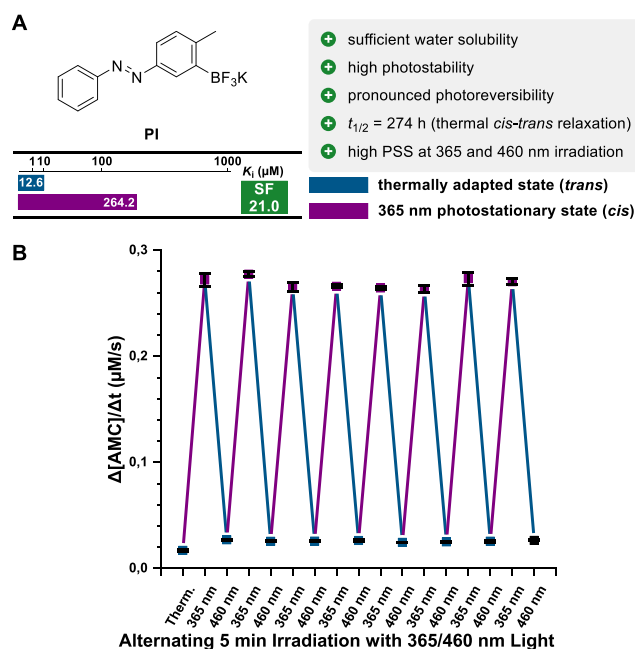
systems, the photoswitchable inhibitors need to fulfill several criteria:

- (1) The switching factor (SF), i.e., the ratio between the inhibition constants of the *cis* and *trans* isomer ( $SF = K_{i,cis}/K_{i,trans}$ ), should be bigger than 10. This ensures that the activity of the respective enzyme can be sufficiently externally modulated using light.
- (2) The PIs should exhibit high photostationary states (PSS, defined as the percentage of *cis* isomer present at equilibrium under irradiation,  $PSS = ([cis-PI]/([cis-PI] + [trans-PI]) \times 100)$  under UV (365 nm) irradiation. A high PSS (>90%) implies superior concentrations of the *cis* isomer relative to its *trans* isomer, thereby enabling decent levels of photocontrol over the activity of the respective enzyme.
- (3) In order to reversibly regulate the activity of enzymes using light, the PI needs to exhibit robust photo-reversibility. This implies that no observable decomposition of the PI should take place upon several consecutive UV/vis switching cycles (>10).
- (4) Ideally, the inhibition constants ( $K_i$ ) of both the *trans* and *cis* isomer of the PI should be in the  $\mu M$  range or lower. Thereby, only small quantities of PIs, enzymes, and substrates have to be used throughout the performance of flow experiments.
- (5) Additionally, the PIs have to be sufficiently water-soluble, should exhibit high photostability under irradiation, and should show slow thermal *cis* to *trans* isomerization, as complete photocontrol over the state of the PIs using light is preferred.

Diverse photoswitchable inhibitors for the photoregulation of the activity of proteases have been described in the literature.<sup>43–47</sup> These effectors were mostly rationally designed by incorporating a molecular photoswitch (usually an azobenzene) into a previously identified pharmacophore (a reversible inhibitor functionality; “warhead”), thus applying a so-called chromophore/warhead strategy.<sup>34</sup> However, the reported PIs exhibit suboptimal properties for successful application in out-of-equilibrium enzymatic systems due to several reasons, e.g., the best Cr PI, bearing a trifluoromethyl ketone warhead, had a SF of only 4.7.<sup>47</sup>

Therefore, we set out to design and synthesize improved photoswitchable Cr inhibitors to fulfill these criteria and render them suitable for application in out-of-equilibrium enzymatic systems. The synthesis of PIs was performed by applying a chromophore/warhead strategy,<sup>34</sup> aiming to maximize the difference in  $K_i$  between both isomeric states (switching factor), while keeping the other (photo-)physical requirements for later flow applications in mind. Due to their excellent photophysical properties and straightforward synthetic construction, azobenzenes were chosen for the chromophore.<sup>48</sup> Based on initial experiments, phenylalanine-, tyrosine-, valine-, tryptophan-, boronate-, or boronic-acid-based warheads were identified as unsuitable when installed into the azobenzene scaffold. Weak inhibitory properties, negligible differences in  $K_i$  between both isomeric states, and insufficient water solubility or (photo-)chemical degradation of the respective PIs over time were observed. Inspired by a report from Smoum et al. on the competitive inhibition of  $\alpha$ -chymotrypsin by trifluoro(organo)-borates,<sup>49</sup> we aimed to synthesize azobenzene-based photoswitchable inhibitors bearing this warhead. A systematic structure–activity–relationship (SAR) analysis was performed,

in which the position of the  $\text{BF}_3\text{K}$  warhead on the azobenzene chromophore was altered and diverse substituents with different steric and electronic properties were introduced (see 2.1–2.5 of the Supporting Information (SI) for details). In total, 21 photoswitchable  $\alpha$ -chymotrypsin inhibitors have been synthesized throughout the performed SAR study. The synthesized PIs have been carefully characterized with respect to (photo)physical and inhibitory properties (see 3.1–4.6 of the SI for detailed results). Among them, we identified  $\alpha$ -chymotrypsin inhibitor **PI**, bearing a *meta*-trifluoroborate functionality, as an ideal candidate for our envisaged applications (Figure 2A, see 2.1–2.5 of the SI for details).



**Figure 2.** (A) Structure and enzymological data of the photoswitchable  $\alpha$ -chymotrypsin inhibitor **PI** identified throughout structure–activity–relationship analysis. Inhibition constants were determined using a fluorogenic assay. For details on other synthesized photoswitchable  $\alpha$ -chymotrypsin inhibitors, experimental procedures, and results, see 2.1–4.6 of the SI. SF = switching factor; PSS = photostationary state. (B) Batch photoswitching using **PI** illustrating reversible photoswitching and competitive mode of inhibition.  $[\text{Cr}] = 10$  nM,  $[\text{PI}] = 400$   $\mu\text{M}$ ,  $[\text{AAPF-AMC}] = 80$   $\mu\text{M}$  (see 4.5 of the SI for details).

**PI** exhibits a good solubility in commonly used aqueous protease buffers (TRIS (200 mM),  $\text{Ca}^{2+}$  (20 mM), pH = 7.81), and no substantial hydrolysis of the trifluoroborate was observed within 24 h using  $^{19}\text{F}$  NMR analysis. In addition, the photophysical characterization revealed both a high photostability and photoreversibility of **PI** upon 365/460 nm excitation and a very slow thermal *cis* to *trans* relaxation ( $t_{1/2} = 274$  h), thereby enabling full photocontrol over the isomeric state of **PI** using 365/460 nm light. Importantly, the content of *cis* isomer obtained upon 365 nm irradiation of **PI** in the protease buffer was sufficiently high (PSS = 93%, see 3.1–3.4 of the SI for details regarding photophysical characterization of **PI**).

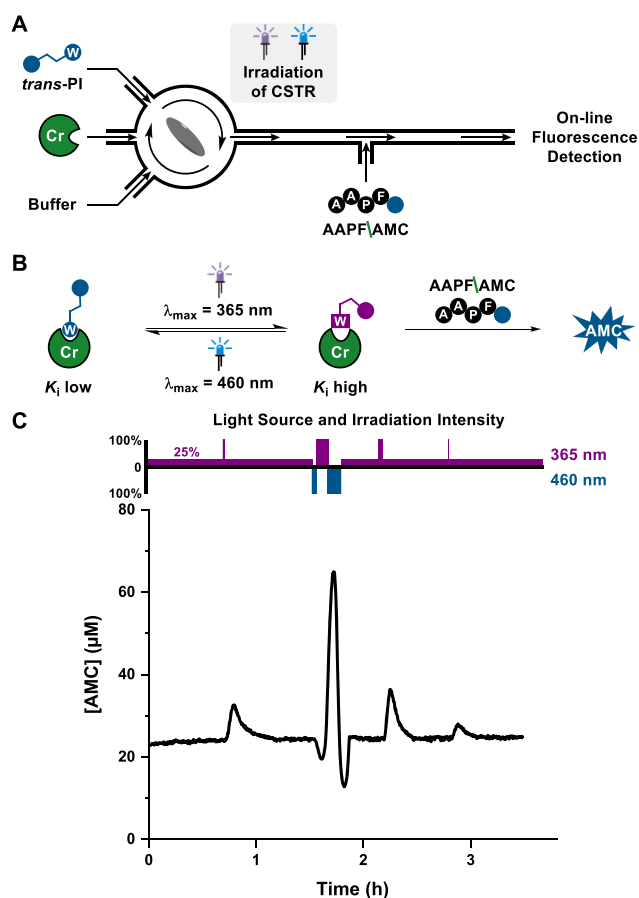
Using **PI**, the protease  $\alpha$ -chymotrypsin, N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (AAPF-AMC) as substrate and TRIS/ $\text{Ca}^{2+}$ /BSA as buffer (200 mM, 20 mM, 0.1 wt %, pH = 7.81), we determined the inhibition constants ( $K_i$ ) for the enzyme/inhibitor interaction of *trans/cis-PI* and Cr

using a fluorogenic assay.<sup>42</sup> To our delight, *trans-PI* was identified to be a (moderately) strong competitive inhibitor of  $\alpha$ -chymotrypsin ( $K_{i,trans} = 12.6 \pm 0.6$   $\mu\text{M}$ ), whereas *cis-PI* exhibited significantly weaker inhibitory properties ( $K_{i,cis} = 264.2 \pm 50.6$   $\mu\text{M}$ ). The three-dimensional structural change caused by photoinduced *trans* to *cis* isomerization thus leads to weaker interactions of *cis-PI* and Cr compared to *trans-PI*. Combined, this leads to a good switching factor of 21.0 and enables excellent reversible photoregulation of Cr activity.

Having identified **PI** as a suitable Cr inhibitor, we experimentally probed and verified its reversible photoswitching properties upon consecutive 365/460 nm irradiation cycles as well as its competitive mode of inhibition. We performed a batch photoswitching experiment, in which a mixture of **PI** and  $\alpha$ -chymotrypsin in a TRIS/ $\text{Ca}^{2+}$ /BSA (200 mM, 20 mM, 0.1 wt %, pH = 7.81) buffer was successively irradiated several times for 5 min with light of a 365 or 460 nm LED, followed by the addition of AAPF-AMC as substrate to an aliquot of the mixture (see 4.5 of the SI for detailed procedure and results). As depicted in Figure 2B, the degree of AMC production over time was reproducible throughout multiple 365/460 nm photoswitching cycles, thereby demonstrating the reversible switching properties of **PI** as well as its competitive mode of  $\alpha$ -chymotrypsin inhibition. In addition, these results point toward high photostability of Cr with respect to irradiation, as its performance is still consistent and reproducible after a total of 40 min of each 365 and 460 nm irradiation.

**Multi-State Activity Control over Cr in Enzymatic Systems.** With suitable PIs in hand, we aimed to regulate the activity of the protease Cr in a reversible and tunable manner under out-of-equilibrium conditions (continuous addition and removal of **PI**, enzyme, and buffer to/from CSTR) using 365/460 nm irradiation pulses. To this end, a microfluidic flow setup with a CSTR was constructed. The latter could be selectively irradiated with light from surrounding 365 and 460 nm LEDs, which in turn were externally controlled by connecting both to an Arduino device. The construction of this setup enabled a programmable timing of the irradiation source and its intensity (photon flux), the precise control over the flow rates of the respective stock solutions, and on-line fluorescence monitoring (Figure 3A; see 5.1 and 6 of the SI for experimental details of the flow setup and computer code to control irradiation patterns).

To demonstrate multi-state activity control over Cr in this setup, we used **PI** ( $K_{i,trans} = 12.6 \pm 0.6$   $\mu\text{M}$ ;  $K_{i,cis} = 264.2 \pm 50.6$   $\mu\text{M}$ ) and AAPF-AMC as fluorogenic substrate (Figure 3B). Irradiation with 365 nm light converts the thermally adapted *trans-PI* into the weaker inhibitory *cis* isomer, which induces an increase in Cr activity. By consecutive irradiation with 460 nm light, the *cis* isomer of **PI** is converted back to the stronger inhibitory *trans* isomer, leading to a decrease in Cr activity. The amount of *cis-PI* present at the 365 nm photostationary state depends strongly on the applied photon flux,<sup>48</sup> and variation in light intensity therefore introduces an easy route to modulate the effective inhibitor strength. Thus, similar to the recently described light engineering of the PSS for wavelength-gated adaptation of hydrogel properties,<sup>50</sup> a wide range of enzymatic activity can be accessed by utilizing PIs, which enables fine-tuning the kinetics of enzyme-catalyzed reactions using light. Figure 3C shows how irradiation with 365 and 460 nm light is translated into a well-controlled temporal pattern of Cr activity. By applying the depicted light pulse sequence, which is characterized by changes in the photon flux of the 365 nm light source and differently long



**Figure 3.** (A) Schematic of the experimental setup developed for the performance of flow experiments. (B) Network motif of the Cr-catalyzed cleavage of AAFP-AMC to give AMC. (C) Precise reversible photocontrol over the activity of  $\alpha$ -chymotrypsin under out-of-equilibrium conditions: generation of an electrocardiogram heartbeat AMC-output pattern. See 5.2.1 of the SI for details regarding timing, duration, and intensity of 365 and 460 nm irradiation. Concentrations in the flow cuvette:  $[\text{Cr}] = 40 \text{ nM}$ ,  $[\text{PI}] = 200 \text{ } \mu\text{M}$ ,  $[\text{AAFP-AMC}] = 80 \text{ } \mu\text{M}$ . Flow rates: Cr, PI =  $400 \text{ } \mu\text{L/h}$ ; AAFP-AMC, buffer =  $200 \text{ } \mu\text{L/h}$ . Reactor volume =  $196 \text{ } \mu\text{L}$ .

pulses of 365 and 460 nm irradiation with 100% power, a characteristic pattern could be introduced (see 5.2.1 of the SI for details regarding timing, duration, and intensity of 365 and 460 nm irradiation). Interestingly, it appears that irradiation with blue light (460 nm) can sharpen the peak generated by irradiation with 365 nm (see large peak in the center). The photoinduced *trans* to *cis* isomerization of PI is rapidly reversed upon 460 nm irradiation, leading to the formation of a stronger inhibitor and thus to a sharper decrease in Cr activity than observed when the inhibitor concentration decreases only due to efflux from the reactor (see smaller peaks).

**Ultrasensitivity in Out-of-Equilibrium Enzymatic Systems by Incorporation of PIs.** Encouraged by the initial results shown above, we decided to study the impact of varying the dynamics of the UV input. In cellular signaling pathways, information is encoded not only in the amplitude of a certain signal, but also in its dynamics.<sup>51,52</sup> Hence, cellular output decisions can be altered by differences in signal duration and frequency of the input.<sup>53</sup> It has been reported that changes to dynamic signal transduction properties, e.g., by cancer mutations or drugs targeting the respective pathway, can lead to improper cell decisions and dysfunction.<sup>54–56</sup>

Inspired by these biological processes, we first systematically altered the duration of irradiation with 365 nm light as input stimuli, both with and without irradiation by blue light immediately afterwards, in order to optionally switch the chromophore back (Figure 4A). Due to the very slow thermal *cis* to *trans* isomerization of PI ( $t_{1/2} = 274 \text{ h}$ ), the isomeric state of the photoswitchable inhibitor is controlled exclusively by 365 and 460 nm irradiation. Thus, PI remains in its *cis* state after 365 nm irradiation and can only be converted back to the corresponding *trans* state by 460 nm irradiation. In both pulse sequences, the duration of UV irradiation (365 nm) was gradually increased from 1 s to 30 min. The CSTR was irradiated with the next UV pulse only after the AMC concentration had stabilized to the initial concentration prior to UV irradiation. In the absence of blue light irradiation, PI cannot undergo photoinduced *cis* to *trans* isomerization. Hence, the AMC output of the system to values prior to irradiation can only be recovered “passively” by relying on flow to flush the *cis* isomer out of the CSTR over time. In the pulse sequence with blue light irradiation, however, a 15 s long pulse of blue light directly after UV irradiation induces complete *cis* to *trans* photoisomerization, thereby directly resetting the system to the Cr activity state prior to UV irradiation. Figure 4A shows the enzymatic systems response to the two pulse sequences, where the UV pulse duration is logarithmically plotted against the corresponding normalized AMC output recorded on-line using our flow setup.

In the absence of a blue light pulse, a sigmoidal response curve was observed in which higher concentrations of AMC are formed with increasing UV irradiation times. Just 5 s of UV irradiation led to an increase in AMC output. The maximum Cr activity of the enzymatic system was reached at UV irradiation times of 5 min and longer. By irradiating the CSTR with a 15 s long pulse of blue light immediately after each UV irradiation step, a significantly different AMC response curve was obtained. Only UV pulses of 3 min or longer led to an increase of AMC production. Shorter UV pulses are filtered out, as the combination of flow and the 460 nm induced *cis* to *trans* isomerization results in a quick recovery of the system to the Cr activity prior to UV irradiation. In both pulse sequences, we observed an ultrasensitive input–output relationship, but especially pulsing with blue light resulted in sharp, switch-like behavior.<sup>5</sup> This ultrasensitive response is caused by the interplay of residence time of the Cr/PI mixture within the CSTR and the duration and intensity of UV/blue light pulses. We wish to remind the reader that an ultrasensitive response formally refers to an increase from 10% to 90% of the maximum response upon a *less than 81-fold* change in input stimulus. We can quantify the degree of ultrasensitivity by fitting our experimental data to the Hill equation (eq 1):

$$\text{Response} = \frac{\text{Stimulus}^n}{(\text{EC}_{50}^n + \text{Stimulus}^n)} \quad (1)$$

where  $\text{EC}_{50}^n$  is the stimulus value needed to produce 50% of the maximum response. This plotting allowed us to determine the Hill coefficient ( $n$ ), which is a quantitative measure of an ultrasensitive response as defined according to eq 2:<sup>57</sup>

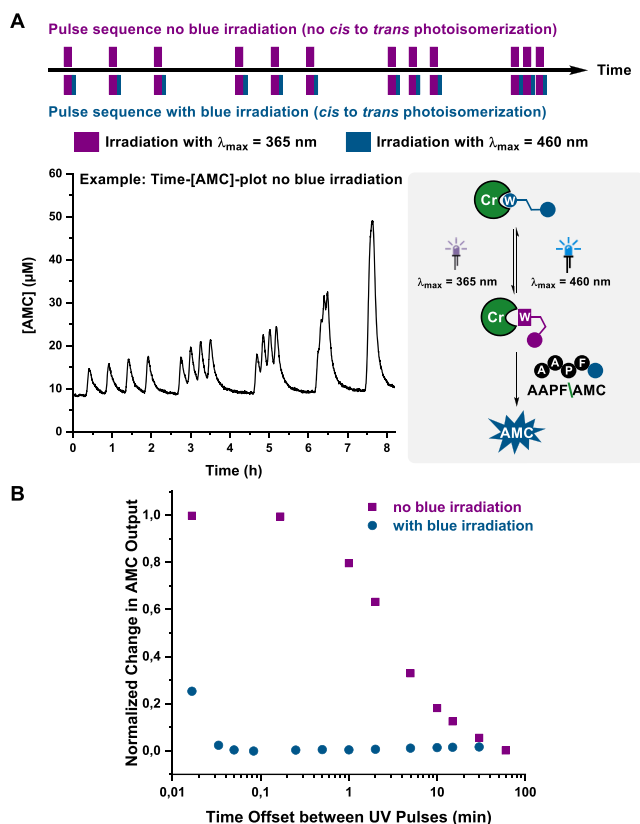
$$n = \frac{\log[81]}{\log\left[\frac{\text{EC}_{90}}{\text{EC}_{10}}\right]} \quad (2)$$

Herein,  $\text{EC}_{90}$  and  $\text{EC}_{10}$  are the stimulus values needed to produce 90% and 10% of the maximum response, respectively.



selectively modulate the activity of enzymes within multi-enzymatic systems.

**Stimulus Frequency Filtering in Out-of-Equilibrium Enzymatic Systems by Incorporation of PIs.** Finally, we sought to characterize the temporal resolution of our strategy to regulate the activity of enzymes under out-of-equilibrium conditions (continuous addition and removal of PI, enzyme, and buffer to/from CSTR) by applying different stimuli frequencies to our system (Figure 5A). In two applied pulse



**Figure 5.** (A) Impact of different UV pulse periodicity (pulse duration = 30 s) and pulse sequences (with and without 5 s of 460 nm CSTR irradiation following UV pulse) on the enzymatic systems AMC output over time.  $[Cr] = 40 \text{ nM}$ ,  $[PI] = 200 \mu\text{M}$ ,  $[AAPPF-AMC] = 80 \mu\text{M}$ . Flow rates: Cr, PI = 400  $\mu\text{L/h}$ ; AAPPF-AMC, buffer = 200  $\mu\text{L/h}$ . Reactor volume = 196  $\mu\text{L}$ . (B) Plot of the normalized change in AMC output of the system as a function of the time offset between multiple UV pulses (see 5.2.4 of the SI for details).

sequences, the offset between four UV pulses of 30 s duration was gradually increased, ranging from as short as 1 s to 60 min. If no blue light pulses are installed after UV irradiation, the PI within the CSTR remains in its weaker inhibitory *cis* state due to the very slow thermal *cis* to *trans* isomerization ( $t_{1/2} = 274 \text{ h}$ ). Consecutively, the depicted exemplary  $[AMC]-t$  plot of the pulse sequence without blue irradiation shows that the temporal resolution of the system is insufficient to separate the four UV pulses at higher pulse frequency, and they fuse into a single peak. As a result, the maximum AMC concentration observed after four UV pulses increases. Figure 5B shows the normalized change in AMC output of the system as a function of the time offset between multiple UV pulses (see 5.2.4 of the SI). In the absence of blue light irradiation, a pulse offset of 30 min or shorter is not sufficient to completely flush out all formed *cis*-PI of the reactor, and the residual *cis*-PI leads to a higher AMC

output upon the next consecutive UV pulse. Thus, in the absence of a blue light pulse, the temporal resolution of our regulatory strategy is strictly limited by the underlying flow properties. In contrast, a similar sequence with 5 s long blue pulses after UV irradiation shows strong “filtering” of the UV input pulses. The blue light immediately switches PI back to its strongly inhibiting *trans* state, and infrequent pulses will thus not lead to an appreciable buildup of active enzyme. Only when the pulse offset is decreased to 2 s or shorter do we observe an increase in AMC response. This is a first promising demonstration that a combination of flow and reversible enzyme activation yields a system with frequency filtering properties.

## CONCLUSIONS

Ultrasensitivity is an emergent property of many biochemical reaction networks. It enables these networks to generate switch-like responses upon small changes in input parameters. Introducing ultrasensitivity in synthetic systems is a desirable goal in systems chemistry, as it would open up new routes to control life-inspired, complex systems. However, ultrasensitivity in biological systems is often the result of rather complex motifs, which are challenging to recapitulate in synthetic systems. Here, we have shown that enzymatic reactions operating under out-of-equilibrium conditions generate an ultrasensitive response in a surprisingly simple topology upon incorporation of photoswitchable inhibitors. Introduction of a photoswitchable inhibitor with sufficiently large differences in inhibitory properties between the two photoisomers yielded switch-like responses in enzymatic activity. Furthermore, we discovered that by reversibly switching the photoisomers, even single enzymatic reactions can filter out short pulses under flow conditions. In the absence of photoinduced *cis* to *trans* irradiation (blue light pulse), a period of over 30 min between two UV light pulses was required to ensure an “on–off” switching of the system, whereas short (5 s) blue light irradiation immediately after UV pulses allowed us to detect pulses that were only 5 s apart.

The simple building blocks introduced here set the scene for more complex motifs that are now becoming feasible. We can introduce photoswitchable inhibitors with different chromophores, couple multiple enzymes into reaction networks, and explore more complex motifs. The ability to modulate the activity of individual enzymes in such networks using light will provide an exceedingly versatile tool for creating ever more advanced complex molecular systems.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c12956>.

Experimental details, photophysical and enzymological characterization data, copies of NMR spectra of new compounds, simulation details, and assay procedures (PDF)

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## Notes

The authors declare no competing financial interest.

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