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Protein–Protein Communication and Enzyme Activation Mediated by a Synthetic Chemical Transducer

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Supporting Information

ABSTRACT: The design and function of a synthetic "chemical transducer" that can generate an unnatural communication channel between two proteins is described. Specifically, we show how this transducer enables platelet-derived growth factor to trigger (*in vitro*) the catalytic activity of glutathione-*s*-transferase (GST), which is not its natural enzyme partner. GST activity can be further controlled by adding specific oligonucleotides that switch the enzymatic reaction on and off. We also demonstrate that a molecular machine, which can regulate the function of an enzyme, could be used to change the way a prodrug is activated in a "programmable" manner.

There is a growing interest in developing synthetic protein binders based on oligonucleotide (ODN)-small molecule or ODN-peptide conjugates that, in response to external stimuli, undergo a major conformational change that enables them to modulate the activity of their protein targets, akin to allosteric proteins.[1] The use of ODNs for scaffolding such binders not only facilitates projecting the synthetic conjugates in specific orientations[2] but also enables one to change the conformation of these constructs by adding complementary strands. It has been shown that when this structural change affects the affinity of such systems, they can operate as allosteric switches that reversibly interact with different protein partners.[3]

One of the key roles of allosteric proteins in nature is to mediate signal transduction pathways in which the rise and fall of one protein remotely affects the activity of another protein. Such communication networks become possible owing to the function of various allosteric signaling proteins (e.g., adaptors, mediators, amplifiers, and modulators) that reversibly interact with different protein partners and activate or inactivate them.[4] It occurred to us that by endowing ODN-synthetic molecule hybrids with the ability to bind different proteins, it may be possible to obtain allosteric signaling switches that can mediate unnatural signal transduction steps. Herein, we present an artificial chemical transducer that enables a platelet-derived growth factor (PDGF) to trigger (*in vitro*) the catalytic activity of glutathione-*s*-transferase (GST), which is not its natural enzyme partner. By adding specific ODNs to the system, the chemical transducer–enzyme interaction can be reversibly controlled, which allows one to switch the enzymatic reaction on and off. We also show that the system can be used to reconfigure the conditions needed for prodrug activation, in a way that resembles the activation setting of an electronic logic circuit.

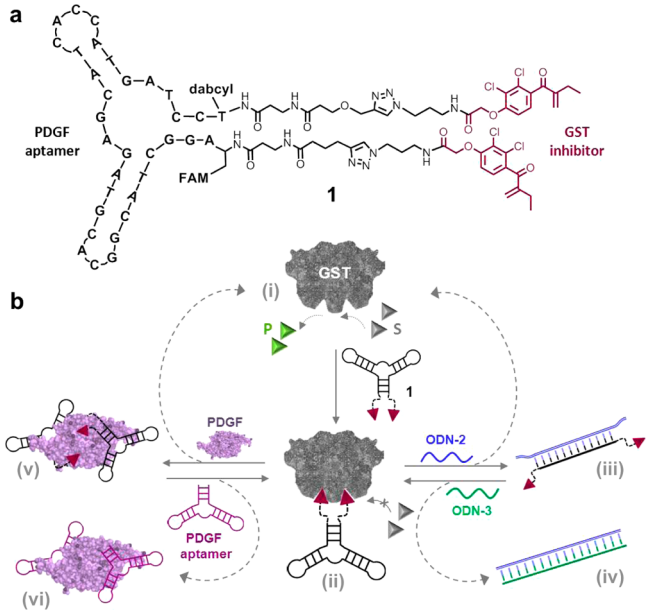

Figure 1. (a) Structure of artificial "chemical transducer" **1** integrating a PDGF aptamer, a bivalent GST inhibitor, a fluorophore (FAM), and a quencher (dabcyl). (b) In the presence of **1** the activity of GST is inhibited, owing to the binding of the two inhibitor units at the enzyme active sites (i → ii). The catalytic activity can then be restored by adding a complementary strand ODN-2 (ii → iii) or PDGF (ii → v) that binds **1** and disrupts its interaction with GST. The subsequent addition of ODN-3 (iii → ii) or a PDGF aptamer (v → ii) liberates **1** from the **1**-ODN-2 or **1**-PDGF complex, allowing it to inhibit the enzyme one more time.

The activation of PDGFR kinase by its PDGF binding partner is an important signal transduction step that is mediated by a receptor that connects the enzyme (i.e., kinase) to PDGF.[5] Based on this principle, we designed an artificial "chemical transducer" **1** (Figure 1a) that can interact with both PDGF and GST and, as a result, can mediate unnatural protein–protein communication, in which a growth factor (PDGF) activates an unrelated enzyme (GST). The structure of **1** integrates a DNA aptamer and a bis-ethacrynic amide (EA) inhibitor, which serve as PDGF and GST binders, respectively. The flexible scaffold, consisting of a DNA backbone and elongated linkers, provides the system with allosteric switching capabilities. A bivalent inhibitor was used for targeting the GST dimer because bis-EA derivatives have been shown to be much better inhibitors than monovalent EA

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compounds.[2a] We also incorporated a fluorophore (FAM) and a quencher (dabcyl) in the vicinity of the 3′ and 5′ termini to allow monitoring the binding of **1** to its targets by fluorescence spectroscopy.

The operation of the system is schematically illustrated in Figure 1b. In addition to depicting the principle underlying PDGF- GST communication (state ii → v → i), this scheme also shows how the function of **1** could be SET and RESET by adding specific DNA strands. In the absence of an inhibitor, the dimeric enzyme catalyzes the conversion of the substrate (S) into a chromophoric product (P) [state (i)]. In the presence of **1**, however, the enzymatic reaction is inhibited owing to the simultaneous binding of the two EA units of **1** to the two active sites of GST [state (ii)]. The activity of the enzyme can then be regenerated by adding ODN-2, which is complementary to the PDGF aptamer, and, hence, can form with **1** a rigid duplex that projects the two EA groups in opposite directions [state (iii)]. This conformational change transforms the bivalent GST inhibitor into a much weaker monovalent one,[3a] which leads to reactivation of the enzyme. ODN-2 was designed to contain two terminal "toehold" sequences that are not complementary to the PDGF aptamer. Therefore, with the addition of ODN-3, which is fully complementary to ODN-2 [state (iv)], **1** can be displaced from the duplex and can inhibit GST one more time.

A more challenging goal than using a synthetic input signal (ODN-2) to induce an enzymatic reaction is to activate the enzyme with another protein (PDGF), which would correspond to an artificial signal transduction step. We have recently shown that bringing a synthetic agent in the vicinity of a protein is likely to promote interactions between the synthetic molecule and the surface of this protein.[2a] We therefore expected that the strong binding of PDGF to the aptamer unit of **1** would make the two EA groups less available for binding [state (v)] as well as create streric hindrance that would prevent **1** from inhibiting GST. Note that PDGF does not necessarily need to interact with the GST-bound **1** [state (ii)], rather, it can bind to the excess of free **1** in the solution, which would shift the equilibrium toward dissociation of the **1**-GST complex. This process could also be reversed by adding an unmodified PDGF aptamer that can displace the PDGF-bound **1** [state (vi)] and enable it to reinhibit the enzymatic reaction.

We first confirmed that **1** individually binds each of its targets (GST, PDGF, and ODN-2) by performing fluorescence measurements[6] and by using an enzymatic assay that follows the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) (Supporting Information(SI), Figures S1a and S3).

Next, we investigated whether in the presence of **1**, the activity of GST would be triggered by the synthetic ODN-2 and, most importantly, by PDGF, which is not its natural binding partner (Figure 2a). To this end, GST (10 nM) was incubated with **1** (500 nM), and the enzymatic activity was followed in the presence of increasing concentrations of ODN-2 (Figure 2a, left). As expected, a dose-dependent response was observed, showing almost full reactivation of the enzyme with 1 μM of ODN-2. We then performed a similar experiment with an incremental addition of PDGF (Figure 2a, right). Remarkably, PDGF (1 μM) successfully restored the activity of GST, confirming the possibility of inducing communication between two unrelated proteins. These measurements were also used to confirm the operating mechanism of **1** by comparing the observed initial velocities (V_0) with the theoretical values calculated using the Michaelis–Menten model according to the IC_{50} of **1** and the K_d of the PDGF-**1** and ODN-2-**1** interactions (SI). As shown in Figure 2a, similar values were obtained for the calculated and observed V_0 values, indicating that the enhanced activity of GST results from the competitive binding of PDGF or ODN-2. The higher V_0 value observed upon the addition of 250 nM PDGF most likely results from the formation of the $\mathbf{1}_2$:PDGF complex (Figure 1b) when there is an excess of **1** in the medium, which leads to a more significant reduction in the concentration of the free inhibitor.

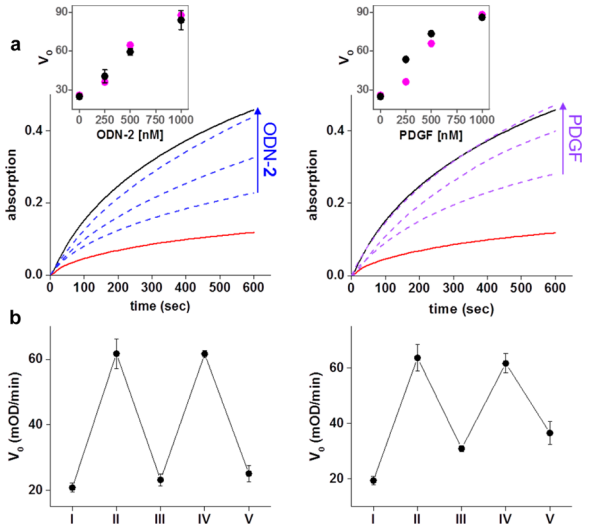

Figure 2. (a) Enzymatic activity of GST (10 nM) before (black, —) and after (red, —) the addition of **1** (500 nM) and after subsequent additions of increasing concentrations (250, 500, or 1000 nM) of ODN-2 (blue, ---) (left) or PDGF (purple, ---) (right). Insets: Calculated (magenta, ●) vs measured (black, ●) V_0 values. (b) Left: Initial velocity (V_0) measured after sequential additions (II → V) of ODN-2 and ODN-3 to the **1**-GST complex: (I) none, (II) ODN-2 (2 μM), (III) ODN-3 (3 μM), (IV) ODN-2 (4.5 μM), and (V) ODN-3 (6 μM). Right: A similar experiment performed with the addition of PDGF and PDGF aptamer: (I) none, (II) PDGF (750 nM), (III) PDGF aptamer (4 μM), (IV) PDGF (5 μM), and (V) PDGF aptamer (10 μM).

The reversibility of our system was also demonstrated by monitoring the response of the **1**-GST complex to the sequential addition of ODN-2 and ODN-3 (Figure 2b, left) or PDGF and its aptamer (Figure 2b, right), which resulted in inhibition/activation cycles. The gradual loss of on/off signals observed in the PDGF/aptamer cycle results from the similar binding affinities of **1** and the unmodified aptamer, which slow down the displacement process. These changes in the reaction rate were also monitored in real time by adding each input while measuring the enzymatic activity (Figure 3). As shown in Figure 3a, an immediate enhancement of the reaction kinetics was observed when ODN-2 (left) or PDGF (right) was added to a solution containing the **1**-GST complex 3.5 min after adding the substrates. Similarly, a rapid decrease in the reaction rate was observed (Figure 3b) upon the addition of ODN-3 (left) or the PDGF aptamer (right), which reversed the previous effect. Taken together, these experiments (Figures 2 and 3) demonstrate that, in addition to inducing PDGF-GST communication, **1** can operate as a molecular machine that can be carefully controlled, namely, it is reversible and can rapidly adapt to changes in the environment by responding to different input signals in real time.

The activation and deactivation of enzymes play an important role in controlling the responses of cells to various environmental signals.[4] In the following proof-of-principle experiments (Figures 4 and 5), we demonstrate how "chemical transducers" such as **1**, which can alter the natural regulation mechanisms of enzymes,

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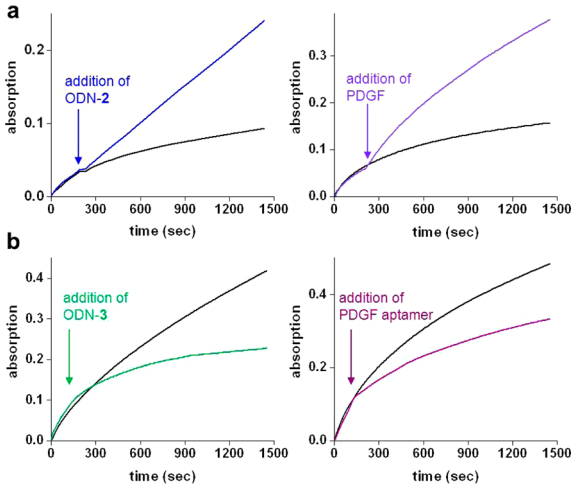

Figure 3. (a) Real-time enhancement of the enzymatic activity observed in a solution containing GST (10 nM) and **1** (500 nM) upon the addition of 1 μM ODN-2 (left) or 750 nM PDGF (right) at t = 3.5 min. (b) Left: Decrease in the enzymatic reaction rate observed upon the addition of ODN-3 (3 μM) to a solution containing GST (10 nM), **1** (500 nM), and ODN-2 (1 μM) at t = 1.5 min. Right: Addition of a PDGF aptamer (5 μM) to GST (10 nM), **1** (500 nM), and PDGF (750 nM) at t = 1.5 min. Black line corresponds to reactions observed without addition of inputs.

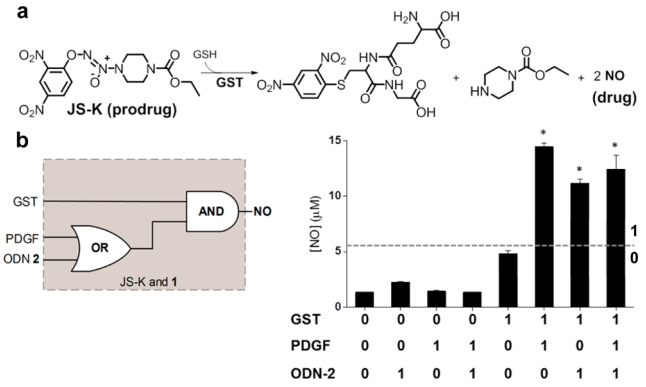

Figure 4. (a) JS-K activation reaction. (b) A logic circuit (left) in which the output signal corresponds to the release of NO (right) in a solution containing JS-K (45 μM), GSH (750 μM), and **1** (750 nM), upon the addition of different combinations of inputs: GST (10 nM), PDGF (2 μM), and ODN-2 (2 μM). *p < 0.001.

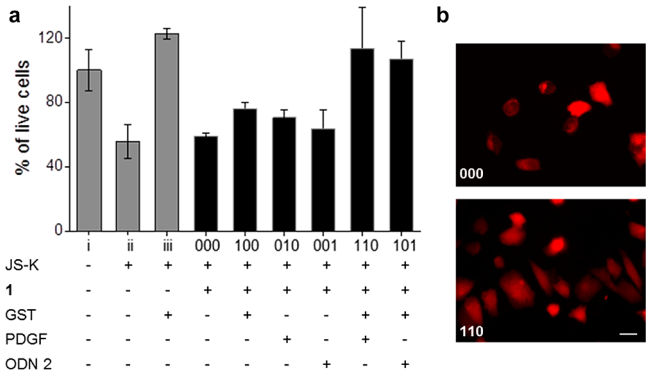

Figure 5. (a) Gray bars: viability of cancer cells incubated with PBS buffer (i), JS-K (ii), or JS-K and GST (iii). Black bars: cells incubated with **1** and JS-K and different combinations of PDGF, GST and ODN-2. (b) Fluorescent images of representative cell samples (000 vs 110). The scale bar represents 20 μm.

could be used to control the way environmental changes affect cells. We tested the effect of **1** on the activation of JS-K, an anticancer prodrug whose intracellular cleavage by GST induces the release of toxic nitric oxide (NO) (Figure 4a).[7] Initially, different combinations of GST (10 nM), PDGF (2 μM), and ODN-2 (2 μM) were added to a PBS buffer solution containing **1** (750 nM), JS-K (45 μM), and GSH (750 μM), and the release of the drug (NO) was measured using two colorimetric assays that either monitor JS-K metabolism or directly follow the production of NO (SI). To elucidate how **1** affected the activation of JS-K, we applied principles of molecular logic,[8] which have been effectively used to describe the function of various multistimuli responsive therapeutics.[9] Accordingly, GST, PDGF, and ODN-2 were denoted as digital inputs (0 or 1) and NO as a digital output (0 or 1), which depends on the concentration of NO with respect to the threshold line[8] (Figure 4b, right). The resulting activity-based truth table corresponds to the logic of a digital circuit (Figure 4b, left). This Boolean logic representation[8] shows that only when GST was combined with PDGF or ODN-2, or with both, a significant amount of drug was released, which indicates that the metabolism of prodrugs could be altered either by "external" stimuli (e.g., ODN-2) or by specific protein biomarkers such as PDGF,[5] which is known to be secreted in high concentrations by several cancer cells. The fact that some JS-K cleavage was also observed in the presence of GST alone and that micromolar concentrations of inputs were used indicates that more potent "transducers" should be generated before considering such systems for therapeutic applications. What distinguishes this system from related logic-based therapeutics that respond to several input signals[9a,10] is that here, the medication can be used as is. Namely, the drug does not have to be chemically modified[10a−c] or be loaded on an auxiliary molecular computational device.[10d−l] Instead, the "chemical transducer" "reprograms" the natural regulation mechanism of the activating enzyme (i.e., GST), which changes the conditions needed for prodrug activation.

The ability to alter the kinetics of JS-K cleavage according to an additional protein present in the solution (i.e., PDGF, Figure 4b) is of particular importance, because activation of prodrugs by specific enzymes is a common and effective tool for achieving selective drug release. The activating enzymes could be recombinant enzymes linked to antibodies that direct them to the outer membrane of specific cells.[11] Alternatively, they may be natural enzymes that are overexpressed in cancer, which leads to high enzyme concentrations within the cell and/or at the extracellular space (ECS). Elevated levels of GST, in particular, have been detected both within cancer cells, as well as in extracellular fluids.[12] To demonstrate how "chemical transducers" could be used to control the effect of prodrugs on cells according to the presence of a specific protein in their immediate environment, breast cancer cells (MDA-MB-231) that stably express a fluorescent Cherry-Red protein were treated with the same concentrations of prodrug (10 μM), GSH (200 μM), and "chemical transducer" (750 nM), but with a different combination of GST (10 nM), PDGF (2 μM) and ODN-2 (2 μM) (Figure 5). This model system was mainly intended to demonstrate how communication between a growth factor (PDGF) and an enzyme (GST) at the ECS can induce JS-K cleavage outside the cell, which would prevent intracellular prodrug activation by cytosolic GST and consequently cell death. After 3 h of incubation, live cells were counted by a hemocytometer (Figure 5a), which showed a decrease in cell viability in the absence of inputs (000) (59 ± 2%) or when only PDGF (010) (70% ± 5%), ODN-2 (001) (74 ± 12%), or GST (100) (76 ± 4%) was present in the medium. In

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contrast, in the presence of GST and PDGF (110) or GST and ODN-2 (101) the viability remained intact (113 ± 25% or 107 ± 11%, respectively). Namely, it is similar to that of control cells, which were not treated with the prodrug (Figure 5a, (i)).

These differences in cell viability can also be visualized using a fluorescent microscope. As shown in Figures 5b and S5, cell death (000 vs 110) leads to changes in the morphology of the cells, transforming them into smaller spherical shapes, as well as to a reduction in the number of imaged cells owing to their detachment from the surface. Thus, under these conditions the activation of GST by PDGF or ODN-2 induces an extracellular degradation of JS-K, which prevents the release of NO inside the cells. Thus, this model system indicates the feasibility of controlling the way prodrugs affect cells through an artificial regulatory system that makes the activating enzyme responsive to the presence of specific proteins or synthetic stimuli in its surroundings. This could be used, for example, to protect or damage specific cells (Figure 5) upon treatment with broad-spectrum medications.

To summarize, the main concept highlighted in this work is the ability to design synthetic agents that mimic the function of signaling proteins and, therefore, can generate *de novo* communication channels between proteins. Whereas in nature PDGF activates its PDGFR enzyme partner, we have shown that in the presence of a synthetic "chemical transducer" the same growth factor can trigger the enzymatic activity of an unrelated enzyme (i.e., GST). Another important property of the system is the ability to regulate it in real time by using specific ODN inputs. The strength of a molecular machine, which can change the way an enzyme is regulated, was further demonstrated by using it to induce differential cell death by "reprograming" the conditions needed for prodrug activation. Although this "transducer" prototype does not fully inhibit the enzyme and is currently limited to controlling prodrug activation outside the cell, it demonstrates a general approach that could potentially be applied to activate other classes of prodrugs as well as to generate more effective, cell-permeable transducers that regulate the function of enzymes by mediating intracellular protein−protein communication. Given that many of the cell's functions are mediated by signaling proteins that continuously activate and deactivate enzymes, we believe that mimicking the function of these proteins may open up new possibilities for controlling biological processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b01123.

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Notes

The authors declare no competing financial interest.

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