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Enzyme–Artificial Enzyme Interactions as a Means for Discriminating among Structurally Similar Isozymes

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Supporting Information

ABSTRACT: We describe the design and function of an artificial enzyme-linked receptor (ELR) that can bind different members of the glutathione-S-transferase (GST) enzyme family. The artificial enzyme–enzyme interactions distinctly affect the catalytic activity of the natural enzymes, the biomimetic, or both, enabling the system to discriminate among structurally similar GST isozymes.

The remarkable efficiency by which enzymes catalyze chemical reactions has stimulated chemists to develop myriad tools to mimic their function.[1] These advancements have led to the creation of biomimetics with catalytic turnovers, and they also inspired the development of biosensors[2] and stimuli-responsive catalysts that utilize principles of cooperativity and allostery,[3] akin to natural enzymes. Although, to date, molecular-scale artificial enzymes cannot compete with the catalytic efficiency of natural proteins, these model systems significantly contribute to the realization of "supramolecular chemistry in water"[4] by advancing our understanding of the parameters required to obtain synthetic receptors that can catalyze reactions in aqueous medium at neutral pH and at ambient temperature.[5]

Beyond their ability to accelerate important biochemical reactions, several groups of enzymes called enzyme-linked receptors (ELRs)[6] are also involved in signal transduction processes in which binding of an enzyme to a protein partner affects its catalytic activity and the consequent downstream signal cascade. In addition to having a catalytic site, ELRs possess a protein recognition domain[7] that enables them to interact with several proteins and regulate the transfer of chemically encoded information across cells. Here we show that it is also possible to endow artificial enzymes with the ability to interact with multiple enzyme partners and that these interactions can distinctly affect the catalytic activity of the natural enzymes, the biomimetic, or both. We also show that information gained from the artificial ELR-enzyme interactions enables the system to discriminate among structurally similar isozymes.

Recently, we demonstrated that attaching a highly specific protein receptor to a nonspecific protein surface binder results in binding cooperativity, giving the latter enhanced affinity toward the surface of the target protein.[8] We also showed that, when combined in arrays (the so-called "chemical noses/tongues"[9,10]), targeted protein surface sensors of this class can generate unique optical "fingerprints" that enable them to discriminate among structurally similar glutathione-S-transferase (GST) isozymes.[8] Activity-based protein profiling and related methods, in which enzyme groups are discriminated by their activities and specificities toward synthetic inhibitors and/or substrates, have also been used to identify isozymes.[11] GSTs, in particular, have been effectively differentiated using arrays of multiple inhibitors and substrates.[12] It occurred to us that by using an enzyme mimic as a nonspecific protein surface binder (Figure 1a), one should be able to obtain an artificial ELR that, similar to natural ELRs, can change its catalytic activity upon binding to a small set of protein partners (Figure 1a, isozymes i and iii). In addition, we expected that, by endowing the ELR mimic with the ability to target an enzyme family (isozymes i–iv), this monomolecular system could be used to differentiate among multiple isozymes by measuring the changes in the catalytic activity of both the synthetic and the natural enzyme (Figure 1b). Four representative complexes, generated by binding the ELR mimic to different isozymes, are shown in Figure 1a. Differences in the reaction rates for each catalyst can be followed optically by observing the formation of two different products (P,P′) from their colorimetric substrates (S,S′).

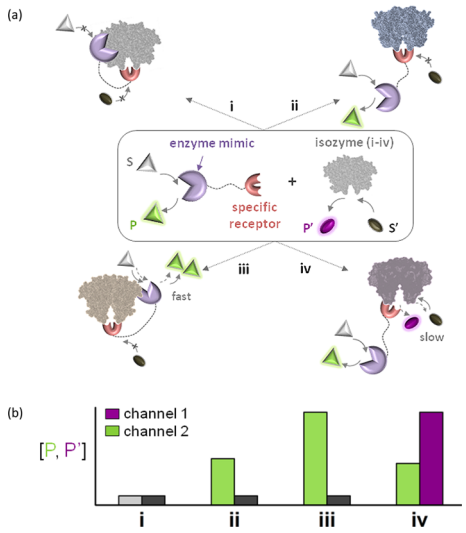

Figure 1. (a) Operating principles of an artificial ELR integrating a catalytic site (i.e., enzyme mimic) with a specific protein binder (i.e., receptor). Binding of the biomimetic to isozymes with different surface characteristics (isozymes i–iv) inhibits their activity and, at the same time, distinctly affects the association of the synthetic catalyst with their surfaces and, consequently, its catalytic efficiency. (b) Unique ID for each isozyme can be obtained by following the formation of the products (P,P′) from their colorimetric substrates (S,S′).

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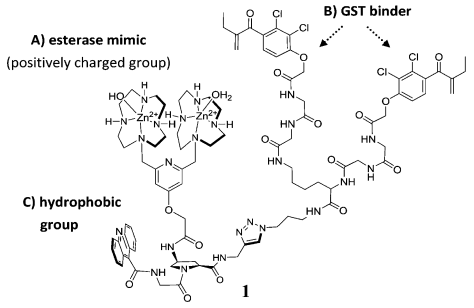

Figure 2. Chemical structure of an artificial ELR consisting of an esterase mimic (A), a broad-spectrum GST binder (B), and a hydrophobic recognition element (C).

Binding of the ELR mimic to the active site of isozyme i, for example, inhibits the catalytic activity of the enzyme and brings the synthetic catalyst in the vicinity of the protein's surface. As a result of the "multivalency effect",[8] electrostatic and hydrophobic interactions and hydrogen bonding between the artificial enzyme and the protein's surface are enhanced, blocking the biomimetic's catalytic site and inhibiting its activity. In contrast, isoform ii, with distinct surface characteristics, does not interact with the catalytic unit of the artificial ELR; thus, only the catalytic activity of the natural enzyme is affected. Distinct catalytic responses are obtained upon binding the ELR mimic to isozymes iii and iv. Binding to isozyme iii inhibits the enzyme's activity and enhances the activity of the biomimetic due to allosteric effects on the protein's surface. The activity of isozyme iv, on the other hand, which binds the biomimetic with the lowest affinity, is only partially inhibited upon forming the biomimetic-enzyme complex, whereas the activity of the synthetic catalyst remains unchanged.

Based on these principles, we generated an artificial ELR (Figure 2, **1**) that can interact with different members of the GST enzyme family. GSTs are detoxifying enzymes that assist in removing a wide range of xenobiotics (e.g., 1-chloro-2,4-dinitrobenzene, CDNB) from the body by catalyzing their conjugation to glutathione (GSH) (Figure 3, channel 1). The structure of **1** consists of a *cis*-amino proline scaffold appended with an artificial esterase (**A**), a broad-spectrum GST inhibitor (**B**), and a hydrophobic aromatic group (**C**). A biscyclen Zn(II) complex was selected as the catalytic unit (**A**) for this biomimetic because complexes of this type can generally act as esterase mimics[13,14] that accelerate the hydrolysis of fluorogenic substrates, e.g., cleavage of fluorescent 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS) from its ester precursor (Ac-HPTS) (Figure 3, channel 2). Biscyclen metal catalysts have been shown to be more efficient catalysts than the monometallic compounds, due to the synergy between the two metal centers.[14] For a specific protein binder (**B**), we used a bisethacrynic amide (bis-EA) inhibitor, which has been shown[8,15] to simultaneously bind the two active sites of these enzymes with nanomolar affinities. The role of the acridine group (**C**) is to promote hydrophobic and π-interactions, which will, together with the positively charged Zn(II) complexes, facilitate the recognition of the aromatic and negatively charged substrate (Ac-HPTS), as well as negatively charged and hydrophobic patches on the protein's surface.

Following its synthesis, apo-**1** was incubated with $Zn(NO_3)_2 \cdot 6H_2O$ to afford the Zn(II) complex catalyst **1** (Supporting Information (SI)). The catalytic activity of **1** was followed by monitoring the hydrolysis of Ac-HPTS into the fluorescent HPTS (Figure 4b), and the Michaelis–Menten model (Figure

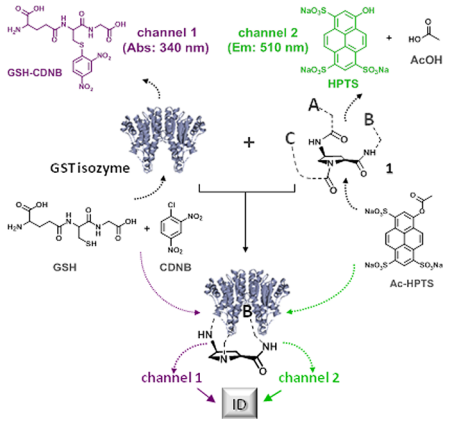

Figure 3. Colorimetric assays used to measure the activity of the natural GST enzymes (channel 1) and artificial ELR (channel 2). Upon formation of the **1**-GST complex, the signal in each channel is distinctly affected, enabling the system to generate a unique ID for each isozyme.

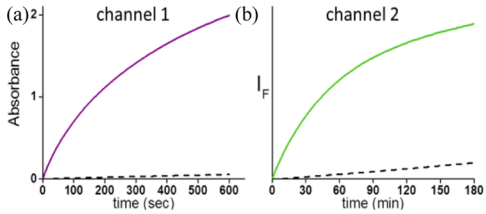

Figure 4. (a) Enzymatic activity of a representative isozyme (GST M1). (b) Catalytic activity of **1**. Uncatalyzed background reactions are denoted by a dashed line.

S1) was applied to determine the catalytic parameters (Table S2), e.g., substrate binding constant $K_M = 18.8 \pm 1.4\ \mu M$, pseudo-first-order rate constant $k_{cat} = 0.90\ h^{-1}$, and rate enhancement $k_{cat}/k_{uncat} = 300$. As expected from the cooperativity between the metal centers, the apparent second-order rate constant, or the specificity constant, k_2 ($= k_{cat}/K_M$) for **1** ($0.0479\ \mu M^{-1}\ h^{-1}$) was 25 times higher than that of the monocyclen Zn(II) complex ($0.0019\ \mu M^{-1}\ h^{-1}$). In parallel, we tested the conditions for following the enzymatic activity of different GST isoforms (Table S1) by following the formation of the GSH-CDNB conjugate (Figure 4a). Eleven GST isoforms (GST A1, GST A2, GST A3, GST M1, GST M2, GST P1, GST K1, GST S1, GST T1, GST Z1, and GST O1) were screened for enzymatic activity. As shown in Table S1, GST A1, GST A2, GST M1, GST M2, and GST P1 exhibited good enzymatic activity at 10 nM concentration, whereas GST A3, GST S1, and GST K1 required a higher enzyme concentration (50 nM). No activity was detected for GST Z1, GST T1, and GST O1 under these concentrations, in agreement with previous reports.[16] Although these findings show that the CDNB-GSH assay can be used to discriminate among isozyme groups (Table S1), i.e., highly active (I), moderately active (II), and inactive (III), they also show that enzymatic activity alone is not a sufficient parameter for multi-isozyme differentiation.

After optimizing the colorimetric assays for measuring the activity of **1** and GSTs, we used these assays to test the interaction between them. By measuring the enzymatic activity of the different isozymes in the presence and absence of a large excess ($1\ \mu M$) of the ELR mimic, we confirmed that **1** can bind to different GSTs from groups I and II (Figure 5, dashed line). The activity of most GSTs was strongly inhibited, indicating that **1** binds the active site of these enzymes, as expected from our

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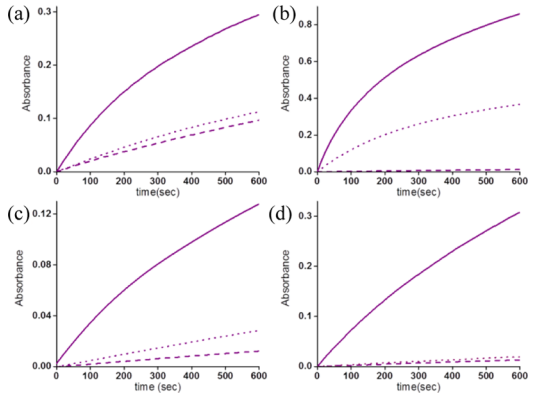

Figure 5. Inhibition of GST isozymes by artificial ELR **1**. Catalytic activities of 20 nM (a) GST A1, (b) GST M1, (c) GST A2, and (d) GST P1 were monitored at 340 nm in the absence (−) and presence of 100 nM (···) or 1 μM (---) **1**.

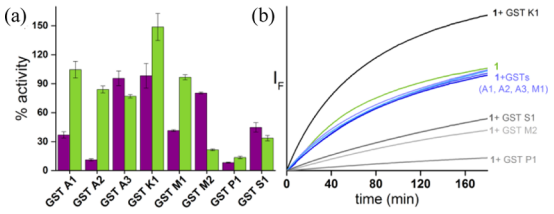

Figure 6. (a) Activity plot summarizing changes in the catalytic activity of various GSTs by **1** (purple) and of **1** by GSTs (green). (b) Changes in the catalytic activity of the artificial ELR **1** by various GST isozymes; λ_{ex} = 450 nm, λ_{em} = 510 nm.

design. Next, we followed the enzymatic activities of the different GSTs with decreasing concentrations of **1** to obtain enzymatic inhibition curves (Figure S2), which were used to identify the appropriate concentrations for inhibiting different GSTs with different magnitudes. As shown in Figures 5 (dotted line) and 6a (purple bar), with 100 nM **1**, the catalytic activities of GST P1 and GST A2 from group I were strongly inhibited, whereas the enzymatic activities of GST M1 and GST A1 were decreased by 60 and 64%, respectively. GST M2 was inhibited by 20%. From group II, GST S1 was inhibited by 53%, whereas GST A3 and GST K1 were not inhibited under these conditions.

We then set out to determine how the different GSTs would affect the catalytic activity of **1**. We expected that, upon the binding of **1** to the active site of GSTs, the interactions between its catalytic unit and some of the isozymes' surfaces would affect the rate of cleavage of the fluorogenic substrate (Figure 1a). Inspecting the catalytic activity of **1** at different concentrations (Figure S3) revealed that 0.5 μM is the minimal amount of catalyst required to obtain catalytic activity and that 2 μM is the minimal concentration needed to attain a stable and reproducible signal. By following the fluorescence signal generated by 2 μM **1** in the absence and presence of 1.5 μM GSTs, we could determine which of the isozymes affects its catalysis. As shown in Figure 6b (and 6a, green bar), GST P1 inhibited the catalytic activity of **1** by 90%, whereas GST S1 and GST M2 reduced the activity by about 65 and 74%, respectively. Interestingly, GST K1 induced 49% enhancement in the catalytic activity, although this isozyme did not seem to be inhibited by **1**. This enhancement might thus result from the use of much higher concentrations of the enzyme and **1**, which can enhance specific or nonspecific interactions between them; both of them can change the configuration of **1** and, thereby, its catalytic response. To assess the contribution of

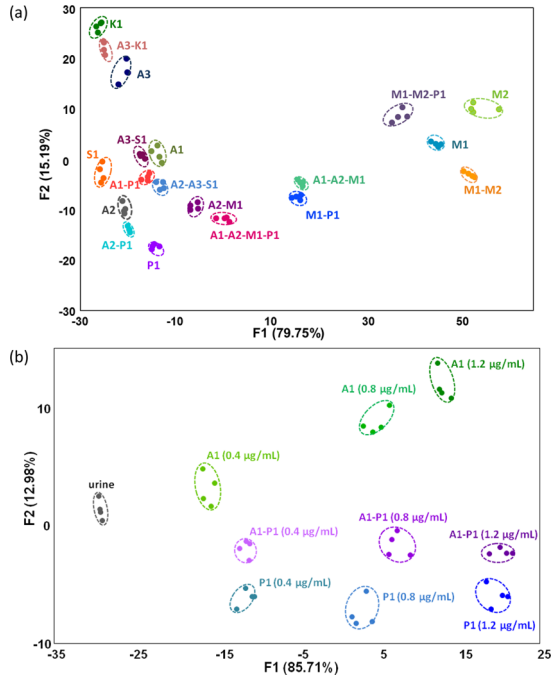

Figure 7. (a) LDA mapping of catalytic activity in channels 1 and 2 for different GSTs and their combinations (Table S4). (b) Analysis of urine samples containing GST A1, GST P1, or both.

the specific binder to the observed catalytic responses, we performed two additional experiments. In the first (Figure S4), we measured the effect of other proteins on the catalytic activity of **1**, and in the second, we tested how GSTs affect the activity of a control compound lacking the bis-EA unit (Figure S5). The facts that five different proteins did not significantly change the activity of **1** and that the activity of the control compound was only weakly affected by the presence of GST M2, S1, and K1 indicate the role of the bis-EA unit in inducing the observed effects. GST P1 did inhibit the activity of the control catalyst; however, this compound was also found to be an inhibitor of GST P1 (Figure S6), most likely due to the high affinity of this isozyme to metal complexes.[17]

These results not only indicate the feasibility of obtaining an ELR mimic that responds to the presence of specific proteins - they also open the way for using it for GST identification. One can couple the inhibition results obtained in channels 1 and 2 to obtain a unique ID for most isozymes (Figure 6a). When these data are combined with the activity of the enzymes in the absence of **1** and using linear discriminant analysis (LDA), a pattern recognition algorithm that can effectively classify unknown samples (SI),[10] isozyme differentiation could be further improved. LDA converted these parameters into two factors (F1,F2) that were plotted graphically (Figure 7a) to obtain well-separated clusters of individual GSTs and combinations of two, three, and even four isozymes (Table S4). To identify unknown GST samples, the catalytic activity of each sample was followed in channel 1, in the absence and presence of **1**. In the next step, the same sample was added to **1**, and the change in its catalytic activity was measured in channel 2. By associating the LDA results with the corresponding clusters (Figure S8), 49 of 54 randomly selected samples could be identified, indicating 91% accuracy. What distinguishes **1** from related systems used to differentiate GSTs by arrays of various protein surface receptors[8] or multiple inhibitors and substrates[12] is that enzyme recognition

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is based on a unimolecular biomimetic device that can interact with both the active site and the protein's surface. A limitation of this prototype is the lower catalytic activity of the artificial esterase when compared with that of natural enzymes, requiring higher concentrations of **1** and GST in channel 2.

The discriminatory ability of the system was further tested by using it to differentiate among isozymes in human urine. GST P1 and GST A1 were selected as the analytes for this study because elevated concentrations of these isozymes in urine have been detected in kidney-related diseases.[18,19] These urinary biomarkers exhibit very similar activities, and hence, conventional enzymatic assays, which can be used to diagnose high levels of these isozymes, cannot determine their identity (Figure S7). In a proof-of-principle experiment, human urine spiked with different concentrations of GSTs, including medicinally relevant concentrations (0.4−0.8 μg/mL),[18] was analyzed by our system. To eliminate background reaction by urine esterases, the GST content of each sample was enriched by a GSH column prior to testing in channel 2 (SI). A LDA plot (Figure 7b) showed a clear differentiation of different combinations and concentrations of these isozymes, which enabled detection of 26 of 27 unknown samples with 96% accuracy.

In conclusion, a novel enzyme mimic that integrates a catalytic site and a protein recognition domain was created and used to discriminate among structurally similar isozymes. In addition to demonstrating a biomimetic approach to differential protein sensing, this study highlights an important principle that could be applied in future artificial enzyme design. The ability of **1** to be engaged in enzyme−artificial enzyme interactions shows that although enzyme mimics cannot yet compete with the catalytic turnovers of natural enzymes, in terms of biomolecular interactions, they can exhibit nanomolar binding affinities comparable to those of natural proteins. Hence, beyond modeling enzyme active sites, artificial enzymes of this class could help to elucidate the parameters needed to couple protein recognition with catalysis, a fundamental principle underlying the function of signaling allosteric enzymes such as ELRs. Considering the simplicity by which synthetic protein binders can be attached to current artificial enzymes, we believe that various other ELR mimics could be designed and contribute to the development of stimuli-responsive biomimetics, biosensors, and allosteric catalysts.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and kinetic experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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