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

Karuthapandi Selvakumar, Leila Motiei, and David Margulies*

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel 76100

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.

T
he 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 biosensors2 and stimulus-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 processes, the surface of the target protein.6 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 GST isozymes.8 Activity-based protein profiling and related methods, in which enzyme groups are discriminated by their

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′).

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′).

Received: September 4, 2014
Published: March 28, 2015

DOI: 10.1021/jacs.5b02496
J. Am. Chem. Soc. 2015, 137, 4892−4895
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,” 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, isozyme 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’s enhancement due to 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 bicyclic Zn(II) complex was selected as the catalytic unit (A) for this biomimetic because complexes of this type can generally act as esterase mimics\textsuperscript{13,14} that accelerate the hydrolysis of fluorogenic substrates, e.g., cleavage of fluorescent 1-hydroxypropene-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.\textsuperscript{14} For a specific protein binder (B), we used a bisethacrynic amide (bis-EA) inhibitor, which has been shown\textsuperscript{15} to simultaneously bind the two active sites of these enzymes with nanomolar affinities. The role of the acidine 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\textsubscript{3})\textsubscript{2}, 6H\textsubscript{2}O 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 S1) was applied to determine the catalytic parameters (Table S2), e.g., substrate binding constant \(K_{\text{M}} = 18.8 \pm 1.4 \mu\text{M}\), pseudo-first-order rate constant \(k_{\text{cat}} = 0.90 \text{ h}^{-1}\), and rate enhancement \(k_{\text{cat}}/k_{\text{uncat}} = 300\). As expected from the cooperativity between the metal centers, the apparent second-order rate constant, or the specificity constant, \(k_i = k_{\text{cat}}/K_{\text{M}}\) for 1 (0.0479 \(\mu\text{M}^{-1} \text{ h}^{-1}\)) was 25 times higher than that of the monocyclen Zn(II) complex (0.0019 \(\mu\text{M}^{-1} \text{ h}^{-1}\)). 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.\textsuperscript{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 isoforms in the presence and absence of a large excess (1 \(\mu\text{M}\)) 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
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 I, 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 I 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 I and using linear discriminant analysis (LDA), a pattern recognition algorithm that can effectively classify unknown samples (SI),16 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 I. In the next step, the same sample was added to I, 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 I from related systems used to differentiate GSTs by arrays of various protein surface receptors or multiple inhibitors and substrates12 is that enzyme recognition...
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 separate 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 LDA 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.

## AUTHOR INFORMATION

### Corresponding Author

*david.margulies@weizmann.ac.il*

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This research was supported by the Minerva Foundation, the International HFSP Organization, and a European Research Council Starting Grant. Postdoctoral fellowship of Dr. Selvakumar was partly funded by the Council for Higher Education of Israeli Government under VATAT program.

## REFERENCES


(4) Oshovsky, G. V.; Reinhoudt, D. N.; Verbloom, W. Angew. Chem., Int. Ed. 2007, 46, 2366.


