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Published on Web 04/21/2010

Exploring Peptide Space for Enzyme Modulators

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Abstract: A method is presented for screening high-density arrays to discover peptides that bind and modulate enzyme activity. A polyvinyl alcohol solution was applied to array surfaces to limit the diffusion of product molecules released from enzymatic reactions, allowing the simultaneous measurement of enzyme activity and binding at each peptide spot. For proof of concept, it was possible to identify peptides that bound to horseradish peroxidase, alkaline phosphatase, and β-galactosidase and substantially altered enzyme activity by comparing the binding level of peptide to enzyme and bound enzyme activity. This basic technique may be generally applicable to find peptides or other small molecules that modify enzyme activity.

Introduction

Enzyme regulation plays an important role in biological metabolism[1] and the ability to control enzyme activity through noncovalent interactions is central to therapeutics.[2] The modulation of enzymes is also important for industrial production of products and in enzyme-based assays.[3,4] Screening libraries of small molecules, peptides, and nucleic acids has been used to identify ligands that bind to proteins and modulate their function.[5,6] Peptides are promising molecules for the modification of enzyme function because of the large chemical diversity available[7] and established methods for library synthesis.[8] In principle, assaying high-density microarrays of molecular libraries provides a high-throughput approach to screening for molecules that alter enzymatic function. Microarrays have been used for this purpose in the past,[9,10] by constructing arrays of small molecules[5,11,12] or peptides,[13,14] printing the enzyme substrate on the surface,[14−16] and activity-based protein profiling.[17] However, in general, the ability to measure enzyme activity on standard slide-based arrays is limited by diffusion of reaction products away from the sites of enzyme action. This problem is normally overcome by physically separating the array elements in such a way that enzymatic reaction products cannot diffuse between them, for example, microwell[18] and microdroplet arrays.[19,20] Hydrogel, which contains large quantities of solvent and behaves as an intermediate between dry and wet systems, can maintain the activity of biomolecules, or even cells immobilized on it,[21] and has applications in many biological processes, such as protein[22] or cell immobilization,[23] bioresponsive sensing,[24,25] and biomedical applications.[26−28] Recently, hydrogels have been applied to protein arrays for assaying enzyme activity[29] and protein−ligand interactions.[30]

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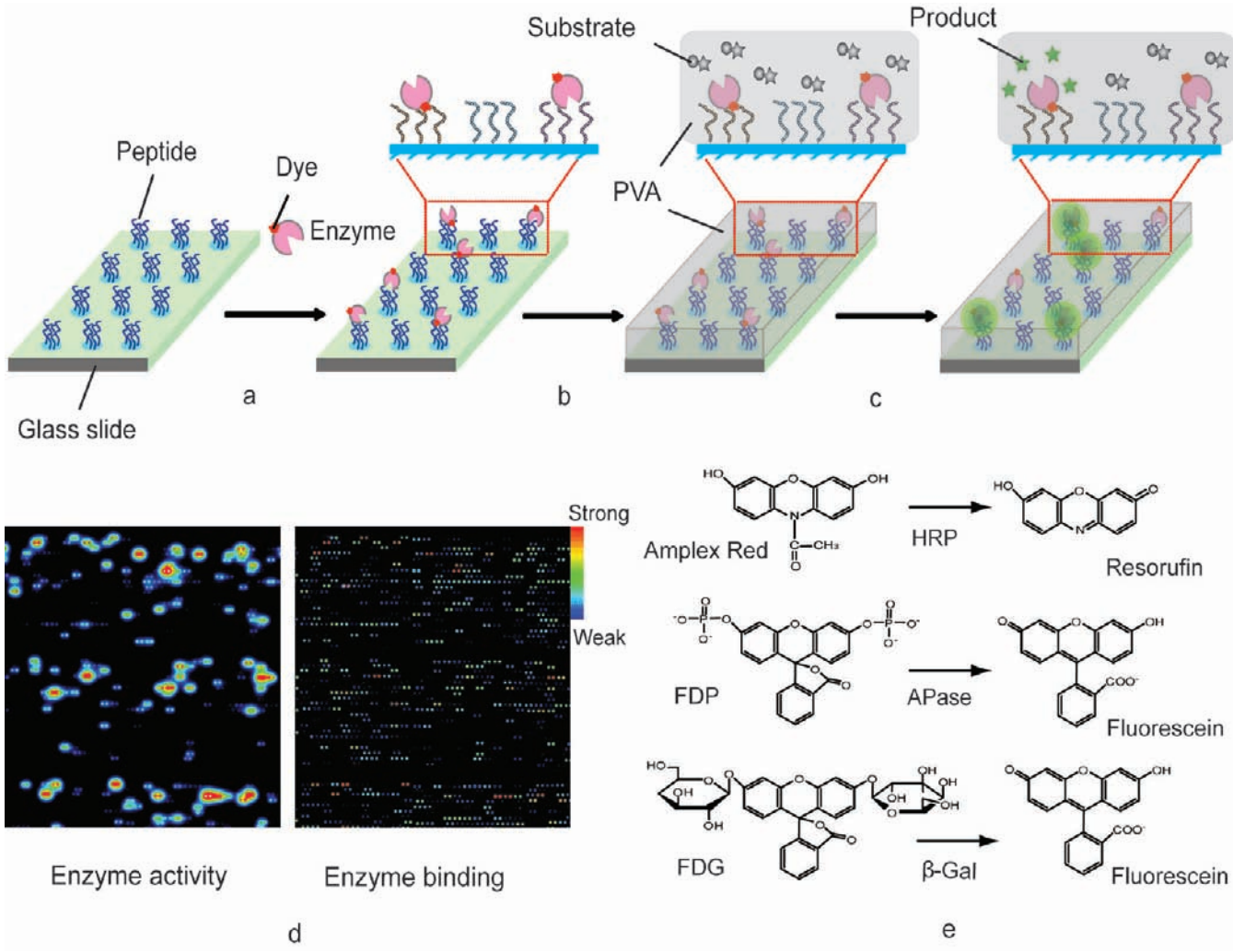

Figure 1. The overall process for screening peptide/enzyme interactions using peptide arrays. (a) Enzyme incubation. (b) PVA coating on the array surface. (c) The enzymatic reaction takes place in the PVA layer. (d) Fluorescent scanning images (a representative region) of binding (Alexa 647) and activity (fluorescein) for β-Gal on the PVA-coated array. Conditions: β-Gal, 5 nM; incubation time, 2 h at room temperature; substrate, 50 μM FDG; PVA concentration, 5%; reaction time, 3 min at room temperature. (e) Substrate analogues Amplex Red, FDP, and FDG used for evaluating activity of HRP, APase, and β-Gal, respectively.

In this work, a method for identifying modulators of enzyme function is described that involves screening an array of 10 000 defined and addressable peptides on a polymer-coated glass slide for the ability to interact with an enzyme and change its activity. This is performed by simultaneously monitoring both the binding and activity of the enzyme at each peptide spot on the microarray surface.

Experimental Section

Chemicals. AmplexRed, fluorescein di-β-D-galactopyranoside (FDG), resorufin β-D-galactopyranoside (RBG), fluorescein diphosphate (FDP), and Alexa Fluor 647 were purchased from Invitrogen (Eugene, OR). Phenylethyl β-D-thiogalactoside (PETG), horseradish peroxidase (HRP), β-galactosidase (β-gal, *Escherichia coli*), alkaline phosphatase (APase), poly vinyl alcohol (PVA, MW: 124 000−186 000), 4-nitrophenyl phosphate (PNPP), phosphate buffered saline (PBS), and Tris buffered saline (TBS) were obtained from Sigma (St. Louis, MO). A 4 mg/mL stock solution of β-Gal was prepared in 10 mM potassium phosphate buffer with 0.1 mM $MgCl_2$ at pH 7.4. A 1.2 mg/mL stock solution of APase was prepared in 0.1 M Tris containing 50 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ at pH 8.2. A 2.5 mg/mL HRP stock solution was prepared in pH 6, 10 mM sodium acetate.

Microarray Fabrication. Peptide microarrays were generated using our established, in-house printing method.[31] Each microarray was prepared by robotically spotting approximately 10 000 distinct polypeptide sequences, in duplicate, on a glass slide possessing an amino-silane surface coating. Synthesized peptides (70% purity) were purchased from Alta Biosciences Ltd. (Birmingham, U.K.). Each polypeptide was 20 residues in length and the 17 amino-terminal positions were randomly chosen from 19 amino acids (excluding cysteine) using a pseudorandom computational process. The last three carboxy-terminal positions of each peptide constituted

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a glycine-serine-cysteine (GSC) linker, used for conjugating the peptides to amino-silane surfaces through the C-terminal cysteine via a maleimide linker, Sulfo-SMCC (Pierce, Rockford, IL). A Telechem Nanoprint60 was used to spot approximately 500 pL of 1 mg/mL peptide per feature on glass slides with 48 Telechem series SMP2 style 946 titanium pins.

Enzyme Assays on PVA-Coated Arrays. As shown in Figure 1, a microarray containing 10 000 20-mer, random-sequence peptides was first incubated with a solution containing dye-labeled enzyme (Alexa 647), allowing the enzyme to bind with peptides on the array surface (Figure 1a). Unbound enzyme was washed off, and a substrate analogue (fluorescent-based) was mixed with a 5% PVA buffer solution and spin-coated onto the array surface to form a ~50 μm layer (Figure 1b). The PVA-coated array was then incubated in a constant humidity chamber to allow the enzymatic reaction to occur. The substrate molecules in the PVA layer were converted to products, by the enzymes bound to specific peptides on the array surface (Figure 1c), and remained localized because of the PVA viscosity. For each of the 10 000 peptides in the array, both the relative binding level of Alexa 647-labeled enzyme and the relative amount of fluorescein produced during the incubation period were determined by dual color scanning (Figure 1d). Each array experiment was repeated at least three times under the same conditions for statistical analysis. Horseradish peroxidase (HRP),[32] Alkaline phosphatase (APase),[33] and β-galactosidase (β-Gal)[34] were chosen as representative enzymes due to the availability of substrate analogues (Amplex Red, FDG and FDP in Figure 1e) and the wealth of structural and mechanistic information available for these enzymes. (For a detailed protocol, please see Supporting Information.)

Microarray Data Analysis. Array images were first processed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA), and then, microarray data was imported into GeneSpring 7.2 (Agilent, Foster, CA) for statistical analysis. To enable statistical comparisons between experiments, each slide was median-normalized: the raw data was normalized to the median signal of each array. Because enzyme activity sometimes appeared artificially low at the edge of the array due to insufficient PVA coating, peptides in these regions were not selected as candidates for further analysis.

Solution-Based Enzyme Assays. Peptides selected from microarrays were synthesized and purified for use in solution-based enzyme assays, which were performed on a SpectraMax M5 96 well plate reader (Molecular Device, Sunnyvale, CA). Peptides were first incubated with enzyme for half an hour, and then the substrate was added to the wells to measure the enzyme activity. At least three replicates were tested in parallel. The IC_{50} of each inhibitor was determined by fitting the concentration versus inhibition curve to the function 'Fit LogIC50' in the GraphPad program using the fitting equation "$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{X-\text{LogIC}_{50}})$". The "Bottom" term was constrained to 1, which represents the maximal inhibition of 100%. The "Top" term was constrained to 0, which represents the minimal inhibition of 0%. Each data point is the average of at least 3 replicates.

Results and Discussion

To limit the diffusion of the product so that it remained in the immediate vicinity of the bound enzyme on the array, we applied the enzyme substrate in a thin coating of PVA. PVA is nonfluorescent, optically transparent, water-soluble, and highly viscous and has applications in many biological assays.[35,36] The diffusion coefficient of fluorescein in a layer of 5% PVA in phosphate buffer was measured via fluorescence recovery after photobleaching (FRAP)[37] and found to be ~50 $\mu m^2/s$, roughly 6-fold slower than in phosphate buffer without PVA (Supporting Information Figure S1). As shown in Figure 1d, it was relatively easy to resolve the enzyme activity in the spots with little cross contamination. This is because of the spacing of the spots, the viscosity chosen for the PVA, and time of reaction. In addition, each peptide is duplicated side-by-side producing a distinctive oval around the active spot. In general, cross contamination is only an issue for peptides that bind to the enzyme very weakly (Supporting Information Figure S2) and those peptides are normally of little interest. Less cross contamination can be obtained by either increasing the space between spots or using more viscous PVA. In Figure 2, the ability of enzymes to function in the PVA medium was demonstrated by real-time imaging of bound β-Gal activity on PVA-coated peptide arrays. One can see that the fluorescent products continued to ac-

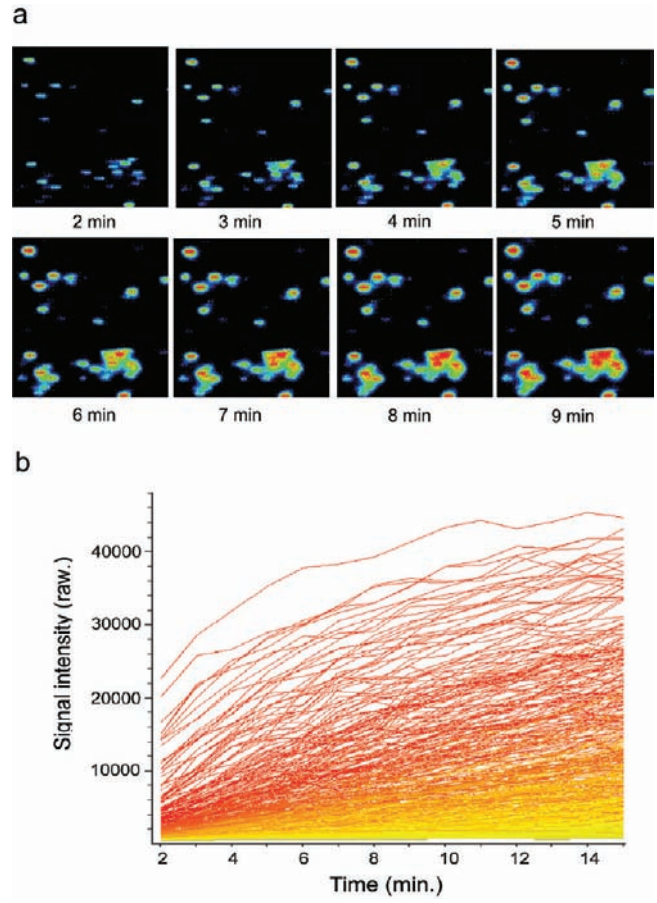

Figure 2. The ability of enzymes to function in PVA polymer. (a) Real-time imaging of β-Gal activity on PVA-coated peptide arrays. Fluorescence is generated due to the production of resorufin released from RBG hydrolysis. A 10 mm ×20 mm region of the slide containing ~1000 spotted peptides was monitored (only a very small part of entire slide area). Conditions: substrate, 10 μM RBG; PVA concentration, 5%; Scanning interval, 1 min. (b) Kinetics measurements of RBG hydrolysis catalyzed by bound β-Gal on peptide arrays. Each trace represents the kinetics at a particular spot (a particular attachment peptide) on the array.

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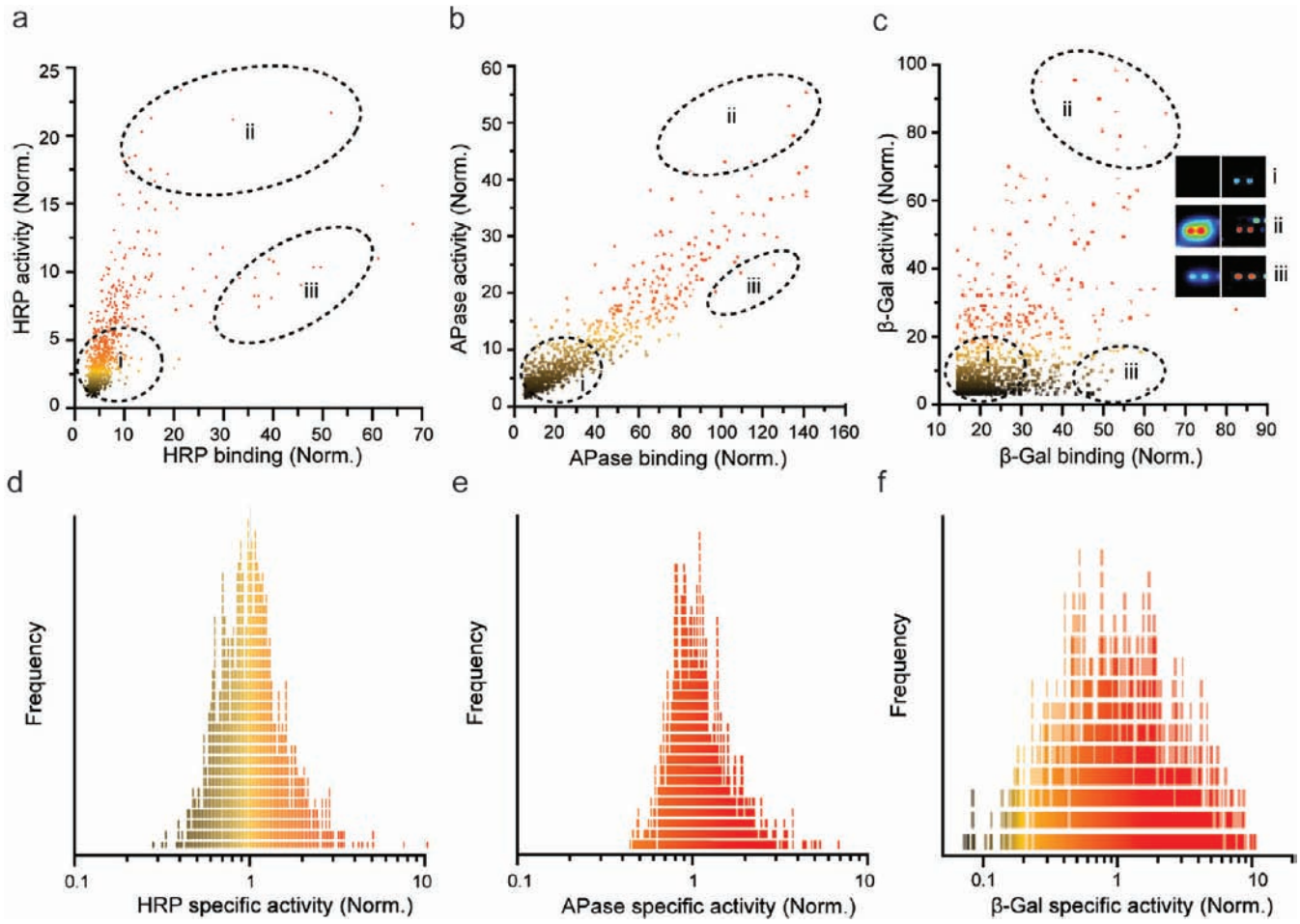

Figure 3. The median-normalized activity of bound (a) HRP, (b) APase, and (c) β-Gal on the microarrays as a function of the amount of enzyme bound to a particular peptide on the array for the 1000 strongest binding peptides. Frequency distribution of surface specific activity of (d) HRP, (e) APase, and (f) β-Gal. Examples of raw fluorescence images associated with specific classes of peptides in the array are shown as an inset of panel c. (i) Weak enzyme activity with weak enzyme binding intensity, (ii) strong enzyme activity with strong enzyme binding intensity, (iii) weak enzyme activity with strong enzyme binding intensity.

cumulate over the entire time of the measurement, indicating that the enzyme remained active during that period. This assay was also used to determine the best period of time to run the enzyme reactions for subsequent analyses. The greatest resolution between spots was observed after the first few minutes of incubation at the substrate concentrations used here.

Three enzymes were tested on the peptide microarray in Figure 3: HRP, APase, and β-Gal which are monomeric, dimeric, and tetrameic proteins, respectively. The binding level and activity of HRP on the peptide array for the 1000 top binders are shown in Figure 3a. As expected, the total activity generally increases with the amount of enzyme bound. Peptides exhibiting weak binding and lower enzyme activities are mainly distributed in Region (i) (lower left). Peptides that show both strong binding and enzyme activities are distributed in Region (ii) (upper right). The peptides that appear to bind and inhibit enzyme activity are distributed in Region (iii), showing relatively weak enzyme activity compared to the level of enzyme binding. The surface-specific activity of HRP was calculated for each of the spots, in Figure 3a, by dividing the total bound enzyme activity by the total binding intensity (Figure 3d). The median-normalized specific activities ranged from 0.33 to 11, suggesting that the nature of the interactions between the enzyme and the peptides on the surface was affecting enzyme activity. Higher than median activities for particular peptides could be due to favorable orientation of the enzyme by the peptide or peptide stabilization of a more active conformation (Supporting Information Figure.S3a).

Figure 3b shows the binding level and activity of APase on the peptide array for the 1000 top binders. As shown, the increase of total activity with the amount of enzyme bound forms a significantly tighter correlation compared to HRP, with the median-normalized specific activity ranging from 0.42 to 6.6 (Figure 3e). APase (from *E. coli*) is a homodimeric enzyme which possesses an unshared active site in each subunit[33] (structure independent). Therefore, it may be that only one of the two subunits interacts with a surface peptide at any given time, and thus, only the activity of that subunit is modulated (Supporting Information Figure.S3b). This idea is consistent with the fact that the lowest activities were about half of the median surface-specific activities of the enzyme. The seven peptides with the lowest surface-specific activities were selected, resynthesized, and tested in solution. Four of them were able to inhibit the enzyme in solution as well as on the surface, with IC_{50} values (concentration of 50% inhibition) between 400 and 900 μM (Supporting Information Figure.S4).

In contrast to APase, β-Gal (*E. coli*) is a tetramer with the active site on the interface of two subunits and has known allosteric inhibition and activation.[34,38] As shown in Figure 3c, there is much more variation in β-Gal surface specific activity

Table 1. Solution Test of Selected Peptides Inhibiting β-Gal Activity[a]

peptide	sequence	enzyme activity (norm.)	enzyme binding (norm.)	surface specific activity (norm.)	IC_{50} (μM) (β-Gal)	IC_{50} (μM) (APase)
1	RVFKRYKRWLHVSRYYFGSC	0.9 ± 0.4	50 ± 10	0.08	1.7 ± 0.2	80 ± 5
2	KFHHFWKWHWRWHHRPFGSC	1.9 ± 1.9	49 ± 10	0.18	1.2 ± 0.2	>250
3	PASMFSYFKKQGYYYKLGSC	2.3 ± 2.5	64 ± 7	0.16	13 ± 2	>200
4	LGRMFAYRWRLKIKHRLGSC	2.6 ± 1.5	47 ± 11	0.25	10 ± 1.2	>175
5	FLMRKYNKQRVFYIAFRGSC	0.8 ± 0.6	48 ± 9	0.07	10 ± 1.4	>150
6	FNAPIWWYIYPRHVRHAGSC	0.8 ± 0.5	42 ± 5	0.09	6 ± 1	>75
7	FRNFPVPVIFRYLNPWPGSC	2.3 ± 1.1	52 ± 8	0.20	7 ± 2	>200
8	GVFPRRFGYVWVHLTEKGSC	0.8 ± 0.3	51 ± 2	0.07	30 ± 3	>100
9	HIPWWWQNYPSWYPYRLGSC	1.5 ± 0.9	43 ± 6	0.16	insoluble	-
10	SYMLYHHFIWFKTHYSQGSC	2.4 ± 1.2	47 ± 8	0.22	>120	>120
11	YHNNPGFRVMQQNKLHHGSC	92 ± 13	38 ± 6	11	>500	>500
12	EFSNPTAQVFPDFWMSDGSC	0.7 ± 0.3	0.4 ± 0.3	-	>1000	>400
13	ESVPTDLPMDTMEGKNWGSC	1.2 ± 0.5	0.5 ± 0.4	-	350 ± 30	>400
14	Phenylethyl β-D-thiogalactoside				35 ± 3	>1000

[a] Peptides 1−10 are selected inhibitors, Peptides 11−13 are the negative control peptides and 14 is a known competitive inhibitor of β-Gal. The IC_{50} of the peptide inhibition is measured at 25 °C with a substrate concentration of 100 μM RBG (resorufin β-D-galactopyranoside) and a β-Gal concentration of 150 μg/L. APase concentration, 200 μg/L; PNPP (4-nitrophenyl phosphate), 200 μM. '-' unavailable data for poor solubility or weak inhibition.

than APase activity as a function of binding to different peptides. Examples of the raw fluorescence images associated with each region of the activity versus binding plot are shown in the right panel of Figure 3c. Strong inhibition was seen in Region (iii) with the median-normalized specific activity lower than 0.2. If the peptides were acting as simple active site inhibitors, one might expect that β-Gal would never have less than about one-half to three-fourths of the median activity of the enzyme due to its tetrameric nature and the likely ability of peptides on the surface to interact with only one or two subunits at a time. However, the strong inhibition in Region (iii) of Figure 3c suggests that some peptides may trigger conformational changes in the entire tetramer and inhibit the whole enzyme (Supporting Information Figure.S3c). Consistent with this senario, activities as low as 0.07 of the median surface specific activity are observed (Figure 3f). These results suggest that the simple ratio assay described here may provide a general approach for directly detecting peptides that allosterically inhibit particular enzymes.

Ten peptides inhibiting β-Gal (in Region (iii) of Figure 3c) and resulting in low surface-specific activities on the array were synthesized and purified for solution-based enzyme inhibition assays. Eight peptides (1−8) were found to inhibit β-Gal activity in solution with a range of IC_{50} values from 1.2 to 30 μM (Table 1). As controls, several peptides from Region (ii) of Figure 3c (strong binding and high activity, e.g., peptide 11) and from Region (i) (weak binding and weak activity, e.g., peptides 12 and 13) were also synthesized and tested for the inhibition of β-Gal in solution. These peptides showed much higher IC_{50} values (>300 μM) than the selected peptide inhibitors. These results imply that modulation of enzyme activity via surface-bound peptides corresponds, in most of cases, to the effects of those peptides in solution. As an indication of the specificity of the selected peptide inhibitors, they were also tested for their effects on APase activity. Most showed much weaker inhibition of APase than β-Gal (>20-fold higher IC_{50}).

In addition to inhibitors, peptides that enhanced the surface-specific activities of β-Gal were also found (e.g., Region (ii) in Figure 3c) which suggests that peptides can stabilize the active form of each enzyme (or alternatively optimize its orientation and function on a surface). In room-temperature solution tests, some peptides in that region enhanced β-Gal activity by about 50% but did not show better activation of β-Gal in solution

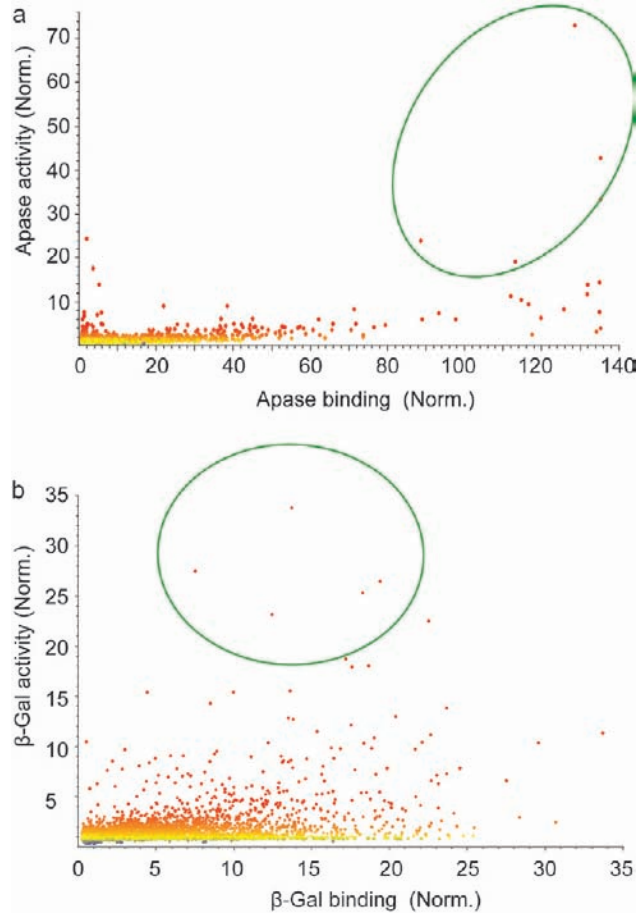

Figure 4. Thermal-stability test of APase and β-Gal on a peptide microarray. (a) APase was first bound to a peptide microarray at room temperature, then incubated in Tris buffer at 61 °C for 1 h. The activity was measured by coating the APase bound slides with 50 μM FDP and incubating for 3 min at room temperature. (b) β-Gal was first bound to microarray at room temperature, then incubated in phosphate buffer at 55 °C for 1 h. The activity was measured by coating the β-Gal bound slides with 50 μM FDG and incubating for 3 min at room temperature. The selection region (circled) contains peptides binding to the enzyme with the highest specific activity after incubating at high temperature.

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than negative control peptides (weak binder peptide 12 and 13), possibly because β-Gal is stable and nearly at its maximum possible activity in solution at room temperature. However, this result suggests that it might be possible to discover peptides that enhance stability of the enzyme on the surface or under other conditions that might favor inactive conformations of the enzyme (e.g., high temperature, pH). To test this, the enzymes were bound to peptides on microarrays at room temperature and then the arrays were incubated at higher temperatures (61 °C for APase and 55 °C for β-Gal) for 1 h and assayed for activity at room temperature (HRP was not tested in this way). As shown in Figure 4, most of the peptide-bound enzymes lose activity after incubation at high temperature (compared to Figure 3). However, there are a few peptide-bound enzymes that remain stable after this treatment. For Apase, up to a 14-fold improvement in remaining activity over the median level is observed after extended exposure to a temperature of 61°, and for β-Gal, up to 31-fold improvement in remaining activity over the median after exposure to 55 °C is observed. These results suggest that both enzymes can be stabilized by binding to particular peptides.

Conclusions

The approach described above represents a surprisingly simple and general means of discovering modulators for enzyme activity using a library of 10 000 peptides on a surface and performing parallel measurements of activity and binding for the entire array. The peptide slides can be printed inexpensively and rapidly analyzed given an appropriate enzyme assay. As a result, many enzymes can be processed in parallel under almost any set of desirable conditions. All HRP, APase, and β-Gal tested in this work demonstrated a wide variation in binding to the 10 000 spotted peptides (median-normalized binding levels from 0.2 to 70 for HRP, from 0.1 to 150 for APase, and from 0.3 to 85 for β-Gal). A >10-fold variation over the array was found in the surface specific activity for HRP and APase and >100-fold for β-Gal. In most cases tested, enzyme inhibition observed on the surface was also demonstrated in solution-based measurements. Not only was it possible to rapidly and easily discover enzyme inhibitors in this fashion, but enzyme stabilizing peptides were also found; some of the peptides were able to promote maintenance of enzyme activity on the surface even after prolonged exposure to high temperatures. Such peptides might be useful in enzyme immobilization applications, resulting in improved enzyme activity and stability. The method as demonstrated used a profluor as substrate of the enzymes assayed. This may be a limitation in applying this technology to other enzymes. To overcome this, other approaches are under development including enzyme-linked assays (assays where the product of the enzyme under study is used by a second enzyme in the PVA film that is easier to monitor). Finally, this approach is not limited to peptides; any small molecule that is arrayable could also be searched in this format for enzyme modifiers.

Acknowledgment. This work was supported by Technology and Research Initiative Funds (TRIF) provided to Drs. Neal Woodbury and Stephen Albert Johnston by ASU, and the Edson Student Entrepreneur Initiative provided to Mr. Jinglin Fu at Arizona State University. We thank John Lainson and Mitch Magee for the peptide array production; Seongmin Hahn, Donnie Shepard, and Loren Howell for the peptide synthesis and purification. We also thank Dr. Phillip Stafford for the helpful discussions. We are grateful to Ms. Carole Flores for critical reading of the manuscript.

Supporting Information Available: Detailed methods and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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