Detection of small-molecule enzyme inhibitors with peptides isolated from phage-displayed combinatorial peptide libraries

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Background: The rapidly expanding list of pharmacologically important targets has highlighted the need for ways to discover new inhibitors that are independent of functional assays. We have utilized peptides to detect inhibitors of protein function. We hypothesized that most peptide ligands identified by phage display would bind to regions of biological interaction in target proteins and that these peptides could be used as sensitive probes for detecting low molecular weight inhibitors that bind to these sites.

Results: We selected a broad range of enzymes as targets for phage display and isolated a series of peptides that bound specifically to each target. Peptide ligands for each target contained similar amino acid sequences and competition analysis indicated that they bound one or two sites per target. Of 17 peptides tested, 13 were found to be specific inhibitors of enzyme function. Finally, we used two peptides specific for *Haemophilus influenzae* tyrosyl-tRNA synthetase to show that a simple binding assay can be used to detect small-molecule inhibitors with potencies in the micromolar to nanomolar range.

Conclusions: Peptidic surrogate ligands identified using phage display are preferentially targeted to a limited number of sites that inhibit enzyme function. These peptides can be utilized in a binding assay as a rapid and sensitive method to detect small-molecule inhibitors of target protein function. The binding assay can be used with a variety of detection systems and is readily adaptable to automation, making this platform ideal for high-throughput screening of compound libraries for drug discovery.

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Introduction

In recent years, the rate of accumulation of genomic sequence information has rapidly accelerated. The DNA sequences of yeast, nematode and 22 microbial genomes have been completely determined and the sequencing of the human, mouse and 71 additional microbial genomes is underway (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/bact.html). This information has aided in the identification of genes whose products could serve as targets for pharmacological intervention. Although the accumulation of sequence information has been rapid, functional information for the corresponding gene products has lagged behind. For example, 40% of the open reading frames in *Escherichia coli* encode proteins of unknown function, even though this microbe has been scrutinized for decades [1]. This lack of functional information makes it difficult to develop assays to exploit these genes as potential drug targets. Even when detailed knowledge of the target protein is available, it is often difficult to develop an assay amendable for use in a high-throughput screen of compound and natural-product libraries. Techniques and tools that facilitate the screening process independent of functional assays are therefore needed to efficiently exploit available targets. One approach to this problem is to develop probes that are specific for functional sites of target proteins and use them in a binding assay to detect inhibitors. Functional sites are regions on the target protein where the binding of another molecule can modulate the activity of the target. Functional sites of enzymes can be the site of enzyme–substrate interaction (the active site) or allosteric regulatory sites. Combinatorial peptide libraries have been used as a source of surrogate ligands that bind to various macromolecules. Libraries have been synthesized on beads [2], pins/crowns, polysomes, in solution [3–5] and displayed on the surface of phage [6–8] or *E. coli* [9,10]. Each of these approaches has been successful in identifying peptides that bind to many different types of proteins including antibodies [8], enzymes [11], oncoproteins [12,13] and receptors [14]. The most exploited method, phage display, presents random peptides on the surface of bacteriophage M13 (reviewed in [15,16]). The large size of the libraries ($> 1 \times 10^9$ members), the renewable nature of the library and the relative ease

Table 1

Enzymes targeted by phage display.

Target	Enzyme class	Reaction(s) catalyzed
Tyrosyl tRNA synthetase (*Haemophilus influenzae*)	Ligase	Tyrosine + ATP → Tyrosyladenylate + PP_i Tyrosyladenylate + tRNA → Tyr-tRNA + AMP
Proline RS (*E. coli*)	Ligase	Proline + ATP → Prolyladenylate + PP_i Prolyladenylate + tRNA → Pro-tRNA + AMP
Alcohol dehydrogenase (*Saccharomyces cerevisiae*)	Oxidoreductase	Ethanol + NAD^+ → Acetaldehyde + NADH + H^+
Carboxypeptidase B (*Sus scrofa* – pig)	Hydrolase	$(\text{Amino acid chain})_n \rightarrow (\text{Amino acid chain})_{n-1}$
β-glucosidase (*Agrobacterium faecaelis*)	Hydrolase	$(\text{Glucose})_n \rightarrow (\text{Glucose})_{n-1}$ + Glucose
Hexokinase (*S. cerevisiae*)	Transferase	Glucose + ATP → ADP + Glucose 6-phosphate
Glycogen phosphorylase a (*Oryctolagus cuniculus* – rabbit)	Transferase	$(\text{Glucose})_n + P_I \rightarrow (\text{Glucose})_{n-1}$ + Glucose 1-phosphate

List of targets used in this study, the enzyme class to which each belongs and a summary of the reaction catalyzed by each.

with which the libraries are screened make it a powerful technique for identifying peptide ligands. Iterative rounds of binding, washing, elution and amplification give rise to populations of phage with an increasing proportion displaying peptides that bind to the target protein. The sequences of peptides isolated using phage display can often be aligned to identify consensus-binding motifs, and peptides with similar sequences often bind to a common site on the surface of the target protein.

We hypothesized that phage display could be used to isolated peptides that bind to regions of biological interaction on target proteins, specifically sites of enzyme–substrate interaction, and that these peptides can be used to detect compounds that bind at these sites. A series of well-characterized enzymes with diverse structures, functions and established biochemical assays were selected as targets for the isolation of peptide ligands by phage display. Peptides affinity-selected for each target were grouped on the basis of primary sequence homology. A synthetic peptide representative of each sequence cluster was then tested for its capacity to inhibit enzyme function and the kinetic mechanism of inhibition was assessed. Finally, we examined the utility of peptide ligands as screening tools by investigating the ability of two peptides to detect known inhibitors of the *Haemophilus influenzae* tyrosyl-tRNA synthetase.

Results

The goal of this study was to test whether peptides isolated by phage display would bind preferentially to functional sites on enzymes allowing the peptides to modulate enzyme function and if these peptides could then be used as probes to detect enzyme inhibitors. A variety of enzymes from a wide range of classes were selected for study (Table 1). Phage were selected for binding from 12 libraries displaying a total of ~1.5×10^{10} peptides. Results for each enzyme are described below.

Identification of peptide ligands and inhibition studies

TyrRS

Tyrosyl-tRNA synthetase (TyrRS) from *H. influenzae* catalyzes the charging of the amino acid tyrosine to $tRNA^{Tyr}$ (Table 1). Phage were affinity selected on TyrRS immobilized by method 1 (see the Materials and methods section) and the selected peptide sequences are presented in Table 2. The sequences fell into two distinct but related sequence homology clusters, plus two additional unrelated peptides. Two peptides from each cluster as well as the two unrelated peptides were synthesized for further analysis (Table 2).

To determine whether the peptides were binding to the same site, we attempted to block the binding of individual phage with synthetic peptides or with an inhibitor that mimics the aminoacyladenylate intermediate. Synthetic peptides Tyr-1 and Tyr-2 (cluster 1) effectively competed for binding with phages 1 and 14 (the respective parent phage), but did not affect the binding of phages 22, 24, 25 and 26. Synthetic peptides Tyr-3 and Tyr-4 (cluster 2) were effective competitors with phages 1, 14, 22 and 24. Synthetic peptides Tyr-5 and Tyr-6 did not compete for binding with any of the tested phage. Preincubation of the enzyme with a small-molecule inhibitor effectively blocked the binding of phage from the first cluster, but had no effect on the binding of the remaining phage (data not shown). It is not clear why peptides from the second cluster block the binding of phage from clusters 1 and 2, whereas peptides from the first cluster block binding of

Table 2

Peptide ligands for the enzymes in this study.

	Phage #	Sequence cluster	A_{405} (10^9 pfu/ml)	Peptide synthesized
(a)	1	S R L Y S W P D E Q Y E R P	0.46	Tyr-1
	2	S R F G F Y G W P D D Q Y	ND	
	3	M Y T W P G S P Y L Q M	ND	
	4	M Y S W P G E H Y T V H	ND	
	5	S S M Y A W P D S S E L E K	ND	
	6	M Y S W P G V	ND	
	7	Y Y G W P S E	ND	
	8	S R D R V Y G W P P F E E	ND	
	9	S S A Y H W P W V E S E W	ND	
	10	S R G Y S W P W P D D N A	ND	
	11	S S I Y S W P W P S N E N	ND	
	12	Q Y T W P W P	ND	
	13	S S Y S W P W P D F N E T	ND	
	14	S S A Y S W P W H D T V D	0.56	Tyr-2
	15	S S W D G F A W P M H Q T	ND	
	16	S S W P W G G F E W P K L	ND	
	17	S S R Y W W P D W G S R E	ND	
	18	S S L W W P E W G V Y T G	ND	
	19	S S Y F W W P D W G S S A	ND	
	20	S S D R G W W W P S W G V S R	ND	
	21	S R G Y W W P D W G S G Q	ND	
	22	S R A E Y W W P D W G F F	0.70	Tyr-3
	23	S S R L Q Y W W P D W G P	ND	
	24	S R M Y W W P N W G S Q E	0.42	Tyr-4
	25	S S W L D G L P L Y H E V	0.14	Tyr-5
	26	S R D T V R K D L L L E R E	0.22	Tyr-6
(b)	1	S R D W G F W D W G V D R	0.83	
	2	S R D W G F W R L P E S M A	0.83	
	3	S R E W H F W R D Y N P T	0.85	Pro-3
	4	S R D W S F W D V R D W A	0.70	
	5	S R E W G F F D P R F L P	0.74	
	6	S R D W H M F F T G P G Q	0.74	
	7	S R D W H Y F H G F P S V	0.78	
	8	S R D W A L W H P P L S L	0.74	
	9	S R D W G Y W H L G E I V	0.57	
	10	S R D W G Y W K V P A P S	0.70	
	11	S R D W G F W R L P N P T E	0.76	
	12	S R E W G F F R M P L H D	0.70	
	13	S R D W G F W N M P P G S	0.58	
	14	S R D W G F W N P I G P L	0.80	
	15	S R D W A F W R G D A S G	0.47	
	16	S R E W A F F R N W D S V	0.70	
	17	S R C G W A A E V R G V C	0.70	
	18	S S C H W G R E V R G L C	0.58	
	19	S S E R G S G D R G E K G	0.55	
(c)	1	S R P M W M N L V N Y V S	1.07	ADH-1a
	2	S S W Q G N V L L G N W I S	0.60	
	3	S S V L C R G F C W T D T	0.89	ADH-4c
	4	S S W Q C R G F C W A G E	0.68	
	5	S S P T C R G F C W S T S	0.20	
	6	S S N N A F C R F G C W Q	0.60	
	7	S S A G T T R H E E L I P	0.20	
	8	M P P P E P R	0.14	
(d)	1	S S Q T D W R K I F Q S L	0.36	
	2	S S S T D W L N V W R Q L	0.15	
	3	S S A T D W G R V Y S I L	0.94	Glu-3
	4	S S A S Y A P W P I Y F A	0.76	Glu-4
	5	S S G A F K P W P V Y S F	0.36	
	6	S R Q V E V F K P W P V Y	0.44	
	7	S S S F K P W P I Y L G S	0.52	
	8	S S E P F S V W P I Y K H	0.44	Glu-8
	9	S S S V P F A P W P V Y A	0.62	
	10	S S T S L P F N R W P I Y	0.22	
(e)	1	S R Y D N W G W T E K L T K	0.64	
	2	S R Y D D W G W V H R L S	0.50	
	3	S S Y D D W D W V V R L N	0.66	Hex-3
	4	S S M Y D E D Q W I L K L N	0.48	
	5	S S M F D D P S W T M L M R	0.61	
	6	S R W E W Q E F L D G P L	0.55	
	7	S S V R W W S D D E W R M	0.83	Hex-7
(f)	1	S S E E G R K F R W G W L	0.62	
	2	S S L P E E R K L R W G W L	0.77	Pa-2
	3	S S E D N R K L R W G W L	0.58	
	4	S S D A R T C R W C W L G	0.44	
	5	S S W F M D C E W Q C F T	0.15	
	6	S R W C G E H D R F C R Q A	0.38	
	7	S R T R F C S H D D A F C	0.92	Pa-7
(g)	1	S R L L E V S P G W W Q M	0.12	CP-1
	2	S S F R E L K P G W W S Y	0.09	
	3	S S W G D Y F N W R D G L	0.09	

Peptide sequences encoded by phage that bound specifically to **(a)** TyrRS, **(b)** prolyl-tRNA synthetase (ProRS), **(c)** alcohol dehydrogenase, **(d)** β-glucosidase, **(e)** hexokinase, **(f)** glycogen phosphorylase a and **(g)** carboxypeptidase B were aligned by maximizing the number of conserved residues in the same primary sequence. The sequences shown are the peptides displayed on the surface of the mature phage. For the peptides that begin with 'SR' or 'SS', the first two residues are encoded by cloning sites and these residues are therefore fixed. Peptides that contain two cysteine residues (underlined) may form disulfide bridges between the cysteines to display a constrained peptide. The ELISA signal corresponding to absorbance at 405 nm for 10^9 pfu/ml were used to determine the relative affinities to the target for each phage clone as described in the Materials and methods section (phage affinity titrations). ND, not determined.

phage only from the first cluster. It might be that the peptides from the first cluster bind in a deep pocket on the enzyme, whereas those in the second cluster bind near the top of this pocket and prevent the access. Collectively, these results imply that peptides Tyr-1–Tyr-4 bind to overlapping sites whereas peptides Tyr-5 and Tyr-6 appear to bind to distinct sites on TyrRS.

Each of the synthetic peptides was tested for its ability to act as an inhibitor of enzyme activity. Peptides Tyr-1–Tyr-4 were found to inhibit enzyme function with K_i values in the nanomolar range (Figure 1 and Table 3). The two unrelated peptides, Tyr-5 and Tyr-6, did not inhibit enzymatic activity. Within a cluster, the relative K_i values obtained for the peptides corresponded to the affinities observed in the phage-titration experiments.

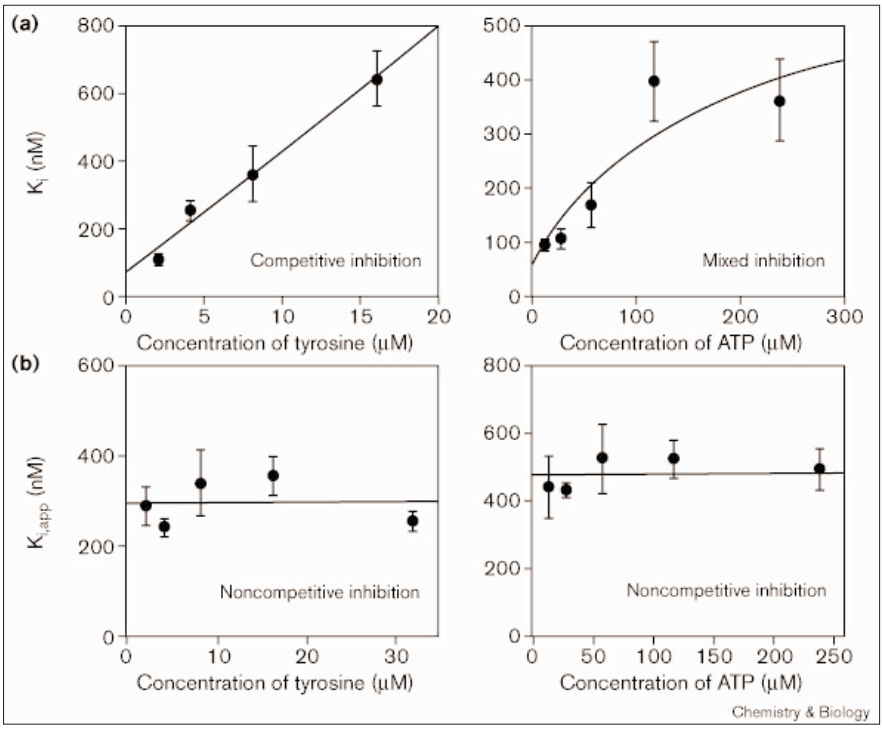
ProRS

Prolyl-tRNA synthetase (ProRS) from *E. coli* catalyzes the charging of the amino acid proline to $tRNA^{Pro}$ (Table 1). Peptides, affinity selected for ProRS immobilized using methods 3 or 4 were grouped into three distinct sequence-homology clusters (Table 2). Phage titrations indicated that several peptides had relatively high affinity for ProRS. Competition assays between phage and an inhibitor of ProRS resulted in a tenfold reduction in the phage ELISA signal, indicating that phage binding was sensitive to the presence of an inhibitor (data not shown). One of the tightest binding peptides, Pro-3, was synthesized and kinetic analysis revealed that this peptide inhibited ProRS activity with a nanomolar K_i value (Table 3).

Alcohol dehydrogenase

Alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* catalyzes the conversion of ethanol to acetaldehyde using NAD^+ as a cofactor (Table 1). Affinity selection of peptides was carried out on ADH immobilized using methods 2–4. Two distinct sequence-homology clusters and several outlying sequences that bound specifically to ADH were identified (Table 3). Phage titrations were conducted to rank the relative affinity of the displayed peptides and two of the tightest binding peptides were chosen for further analysis. Kinetic analysis revealed that both

Figure 1



Inhibition of tyrosyl-tRNA synthetase activity by peptides Tyr-1 and Tyr-4. Enzyme inhibition of TyrRS was monitored using a charging assay as described in the Materials and methods section. **(a)** Secondary plots of the apparent K_i versus substrate concentration with peptide Tyr-1 indicating competitive inhibition with respect to tyrosine and mixed inhibition with respect to ATP. **(b)** Secondary plots of the apparent K_i versus substrate concentration for the peptide Tyr4 indicating noncompetitive inhibition with respect to both tyrosine and ATP. Similar experiments were conducted for each peptide–enzyme pair to determine the mechanism of inhibition in Table 3.

synthetic peptides inhibited ADH activity at nanomolar concentrations (Table 3). The relative K_i values obtained for the peptides correspond to the affinities observed in the phage titration experiments.

β-Glucosidase

β-Glucosidase from *Agrobacterium faecalis* catalyzes the hydrolysis of β-glucosides (Table 1). Affinity selection of phage was conducted on β-glucosidase immobilized using methods 3 or 4 and two distinct sequence clusters of peptides were identified (Table 2). Immobilized β-glucosidase was pre-incubated with 3 mM conduritol, a covalent inactivator of the protein that acts via acylation of an active-site glutamate residue, to prevent binding at the active site. Phage displaying peptides from the second cluster (sequences 4–10) were prevented from binding. In contrast, binding of phage from the first cluster (sequences 1–3) was not affected. Phage titrations were conducted to rank the phage in terms of affinity (Table 2) and three of the highest affinity peptides were synthesized for kinetic evaluation. Peptides Glu-4 and Glu-8 inhibited β-glucosidase activity with K_i values of 400 nM and 4 μM, respectively, but Glu-3 did not inhibit enzyme activity (Table 3). For the peptides that inhibited β-glucosidase activity, the relative K_i values correspond to the affinities observed in the phage titration experiments. It is interesting to note that peptides derived from phage that were sensitive to conduritol inactivation also inhibit enzyme activity.

Hexokinase

Hexokinase (HK) from *S. cerevisiae* catalyzes the phosphorylation of D-glucose by ATP to yield D-glucose 6-phosphate (Table 1). Peptides affinity selected for HK were isolated using method 4. Seven peptide sequences were identified, six of which showed similarity and could be grouped into a sequence homology cluster (Table 2). Phage titrations were conducted to rank the affinity of the phage (Table 2) and two of the highest affinity peptides, Hex-3 and Hex-7, were synthesized for more detailed analysis. Both peptides blocked the binding to HK of the all phage tested, whereas addition of an unrelated peptide had no effect (data not shown). Therefore, even though the peptides are unrelated by primary sequence, it is likely that they bind to overlapping sites.

Kinetic analysis indicated that peptides Hex-3 and Hex-7 inhibited HK with K_i values ranging from low micromolar to low nanomolar, respectively (Table 3). The relative K_i values obtained for the peptides correspond to the affinities observed in the phage titration experiments.

Glycogen phosphorylase a

Glycogen phosphorylase a (Pa) from rabbit muscle catalyzes the removal and phosphorylation of the terminal glucose residue of glycogen to generate glucose-1-phosphate (Table 1). Peptides affinity-selected for Pa were isolated using method 4 and seven peptides that fell into three sequence homology clusters were identified (Table 2). The

Table 3

Enzyme inhibition using synthetic peptides.

Enzyme	Peptide	Substrate	Titration curves (rate versus [peptide])	Secondary plots (K_i app versus [S])	K_i (nM)	Proposed mechanism
TyrRS	Tyr-1	Tyrosine	Simple	Linear; slope = y-int/K_m	80	C
		ATP	Simple	Hyperbolic; limit = 0.65 μM	60	M
	Tyr-2	Tyrosine	Sigmoidal	Unclear	ND	ND
		ATP	Sigmoidal	Hyperbolic; limit = 4 μM	500	ND
	Tyr-3	Tyrosine	Simple	Linear; slope = 0	500	NC
		ATP	Simple	Linear; slope = 0	800	NC
	Tyr-4	Tyrosine	Sigmoidal	Linear; slope = 0	300	NC
		ATP	Sigmoidal	Linear; slope = 0	500	NC
	Tyr-5	Tyrosine	–	–	dni	–
		ATP	–	–	dni	–
	Tyr-6	Tyrosine	–	–	dni	–
		ATP	–	–	dni	–
ProRS	Pro-3	Proline	Sigmoidal	Hyperbolic; limit = 3 μM	500	M
		ATP	Sigmoidal	Hyperbolic; limit = 1.2 μM	400	M
β-Glucosidase	Glu-3	PNPG	–	–	dni	–
	Glu-4	PNPG	Sigmoidal	Linear; slope = 0	400	NC
	Glu-8	PNPG	Simple	Linear; slope = 0	4000	NC
Hexokinase	Hex-3	Glucose	Simple	Parabolic; limit = 16 μM	900	C
		ATP	Simple	Hyperbolic	4000	M
	Hex-7	Glucose	Sigmoidal	Parabolic	80	C
		ATP	Sigmoidal	Linear; slope = 0	100	NC
Glycogen phosphorylase a	Pa-2	Glycogen	ND	ND	30,000	ND
	Pa-7	Glycogen	ND	ND	10,000	ND
Alcohol dehydrogenase	ADH-1A	Ethanol	Simple	Hyperbolic; limit = 1.4 μM	100	M
		NAD	Simple	Linear; slope = y-int/K_m	800	C
	ADH-4C	Ethanol	Sigmoidal	Linear; slope = y-int/K_m	300	C
		NAD	Simple	Linear; slope > y-int/K_m	500	ND
Carboxypeptidase B	CP1	Hipp-Arg	–	–	dni	–

Summary of enzyme inhibition studies using synthetic peptides. Experiments for secondary plots was conducted with saturating concentrations of fixed substrate. For hexokinase, the observation of parabolic K_i,app versus [S] for glucose excludes any simple mechanism. For alcohol dehydrogenase, the sigmoidal inhibition determined for ethanol and anomalous linear, secondary plot for NAD excludes any simple mechanism. C, competitive; NC, noncompetitive; M, mixed; dni, did not inhibit; ND, not determined.

relative affinity of each peptide for Pa was evaluated using phage titrations (Table 2) and two of the highest affinity were chosen for synthesis and kinetic evaluation. Enzyme inhibition studies with synthetic peptides, Pa-2 and Pa-7, showed that both were effective in inhibiting the enzyme activity with K_i values in the 10–25 μM range.

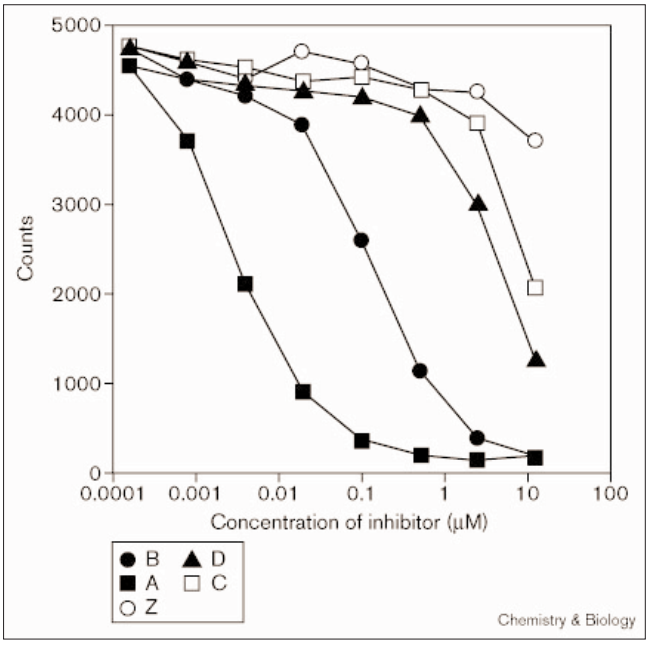
Carboxypeptidase B

Carboxypeptidase B (CPB) is a metalloprotease that cleaves proteins adjacent to arginine and lysine residues (Table 1). Phage affinity selection on CPB immobilized by method 4 identified three peptides, two of which shared extensive sequence homology. The phage had low affinity for CPB as determined by affinity titrations (Table 2). The highest affinity peptide, CP-1, was synthesized for kinetic analysis, which indicated that the peptide was not an inhibitor of enzyme activity (Table 3).

Detection of TyrRS inhibitors using synthetic peptides

The second goal of this study was to evaluate the use of these peptides as probes to detect inhibitors. We chose two peptides specific for TyrRS (Tyr-1 and Tyr-4) that had different inhibition mechanisms, as determined by kinetic evaluation, and tested their ability to detect known TyrRS inhibitors. Inhibitors A and B in the test set had been shown previously to be competitive inhibitors with respect to both tyrosine and ATP (data not shown). We used three different detection systems, scintillation proximity, time-resolved fluorescence and fluorescence polarization, to determine whether the binding of these peptides was sensitive to the presence of an inhibitor. The scintillation proximity and time-resolved fluorescence methods use immobilized target protein, whereas the fluorescence polarization assay is a homogenous assay with the target, peptide and compound in solution (see the Materials and methods

Figure 2



Dose-response curves in the binding assay using the Tyr-1 peptide and inhibitors. The peptide binding assay using time-resolved fluorescence for detection was carried out as described in the Materials and methods section. Compounds A–D are described in Table 4. To show the specificity of inhibition in the peptide binding assay, Compound Z, a specific inhibitor for ProRS that does not inhibit TyrRS in a charging assay, was included as a negative control.

section). The concentration of inhibitor that reduced peptide binding to 50% of the maximal signal (EC_{50}) was then compared with the IC_{50} values for the same inhibitors determined by the standard biochemical charging assay for TyrRS activity, which monitors the transfer of [^{3}H]-tyrosine to $tRNA^{Tyr}$ (Figure 2 and Table 4). Each assay format detected all of the inhibitors tested, even those with potencies in the micromolar range. The absolute EC_{50} value in the binding assay was dependent on the concentration of target protein present (data not shown) and under the conditions used here, in most cases, the binding assay yielded potencies comparable to those found with the functional assay. This correlation was unexpected in that the binding assay is carried out in the absence of substrates, whereas the functional assay contains all substrates. Both peptides were sensitive probes for the detection of these inhibitors, even though peptide Tyr-4 was a noncompetitive inhibitor (Table 3). This assay was used to screen a 640-member library (LOPAC, RBI; see the Materials and methods section) for compounds that would disrupt the interaction between Tyr-1 and TyrRS. With the exception of known inhibitors added as controls, none of the 640 compounds were found to be inhibitors, indicating that the detection of inhibitors using this assay is not disrupted by a vast majority of small molecules and does not, therefore, suffer from a high rate of nonspecific disruption. These results indicate that peptides identified using phage display can be used in competitive binding assays to detect inhibitors of enzyme function, and the mechanism by which the peptides bind to the target do not *a priori* exclude their use as an effective tool to detect inhibitors.

Discussion

To utilize effectively the rapidly expanding list of useful targets in the drug-discovery process, new techniques and tools are needed that accelerate the identification of inhibitors without relying on functional assays. The starting point for this study was the premise that peptides that bound to proteins would bind preferentially to functional sites and that these peptides could be used to identify inhibitors of the target proteins function. We chose a variety of biochemically well-characterized enzymes from a broad range of classes and used phage display to isolate peptides that bound to each enzyme.

A majority of the peptides tested inhibited the function of the enzyme for which they were affinity selected. The peptides that bound to these targets showed a clustering of similar residues within the sequences, suggesting that the peptides adopt a similar structure when binding to the enzyme and probably, therefore, bind to the same sites. Data from competition experiments between synthetic peptides and phage displaying related sequences were consistent with this hypothesis. Surprisingly, in several instances where multiple sequence homology clusters to the same target were identified, peptides with unrelated sequences effectively competed with some of the peptide-displaying phage. This implies that the peptides are binding to overlapping sites and provides strong evidence for the preferential selection of peptides that bind to a limited number of sites on the target. When these peptides were tested for their ability to inhibit biochemical activities of the various enzymes, 13 of 17 were found to be effective inhibitors. If we confine the analysis to peptides derived from sequence clusters and exclude outliers, 12 of 14 peptides were found to inhibit the activity of 6 out of 7 enzymes.

Kinetic analysis of the peptides did not reveal a general trend in the mechanism of inhibition by the inhibitory peptides. Titration curves of rate versus inhibitor concentration at a fixed substrate concentration were often non-first order, suggesting that target inhibition was not simple mechanistically. The peptides for hexokinase and alcohol dehydrogenase displayed competitive kinetics with respect to at least one of the substrates, suggesting that they are binding to the substrate-binding sites of these enzymes. Further evidence that the peptides are targeting functional sites came from using the phage themselves as reagents. In the case of ProRS, peptide Pro-3 showed complex inhibition kinetics (non-first-order dose-response curves, mixed inhibition), but the binding

Table 4

Detection of inhibitors with biochemical and peptide binding assays.

Inhibitor	MW	cLogP	Compound class	Charging assay	Flash plate assay		Time-resolved fluorescence assay	Fluorescence polarization assay
				IC_{50} (mM)	EC_{50} (mM)		EC_{50} (mM)	EC_{50} (mM)
					Tyr-1	Tyr-4	Tyr-1	Tyr-1
A	508.51	−1.63	Aminoacyl intermediate mimic	0.04	0.03	1	0.008	0.02
B	516.76	4.53	Aromatic pyrazole acid	0.20	0.2	0.08	0.1	0.2
C	391.25	3.16	Pyrazole amide	0.6	8.0	2.0	≅10.0	ND
D	494.35	5.01	N-Sulfonyl acyl hydrazide	3.0	7.0	2.0	6.0	2

Comparison of biochemical charging assay and peptide binding assay. ND, not determined.

of the parent phage to the target could be prevented by addition of a competitive inhibitor of the enzyme. Similarly, phage binding to β-glucosidase could be prevented by incubation of the immobilized enzyme with conduritol epoxide, even though peptides Glu-4 and Glu-8 were both noncompetitive inhibitors of the enzyme. The binding of phage displaying peptide Tyr-2 to TyrRS could be blocked by a competitive inhibitor even though the inhibition mechanism of this peptide was unclear. The ability to identify peptides that inhibit the activity of such a variety of targets indicates that this method of identifying surrogate ligands is a powerful tool that can be applied across a diverse range of enzyme classes.

These observations provide strong evidence that the peptides interact with functional sites on the enzymes and not randomly with the protein surface. Although the mechanism by which this selective targeting occurs is unclear, an explanation could lie in physical properties of functional sites. They are generally pockets in the protein surface with a higher composition of arginine, histidine, tryptophan and tyrosine residues than other surface exposed regions of proteins [17]. The functional sites of even vastly different proteins share, therefore, some common characteristics. Crystallographic and thermodynamic data have been used to suggest that a large part of the binding energy for enzyme–substrate interactions is derived from the displacement of water molecules from these cavities [18–20], and it is possible that these same forces drive the binding of peptide ligands identified using phage display. One piece of information consistent with this idea is the high content of residues with bulky hydrophobic groups, especially tryptophan, in all of the peptides described in this work. In spite of their similar compositions, the peptides are exquisitely specific for their cognate target protein (i.e. none of the peptides cross-reacts with other target proteins). This could indicate a common driving force in the binding of these peptides, whereas the specificity for each target could come from the spacing of these residues as well as other amino acids in the sequence.

An important aspect of the approach described here is that a functional assay is not required to identify these sites. We tested the activity of each of the targets in this study to show that they were in an active conformation prior to the start of each selection. This was done because we found that affinity selections carried out on targets that were inactivated (either by binding to plastic or over biotinylation) did not yield any specific binding phage. Presenting the target protein in different ways (see the Materials and methods section) often identified the same phage that bound a given target and was not a critical factor in the successful identification of specific binding phage. The critical aspect of this approach, therefore, is to present the target in an active conformation. If gentle immobilization conditions are used, such as limited biotinylation, knowledge of the activity of the target protein is not required to identify peptide ligands that bind specifically. This raises the possibility of using this technique to identify functional sites on proteins of unknown function. Work to assess the utility of this approach on less characterized proteins is in progress.

One important implication of these phenomena is that peptides derived from phage display can be valuable tools aiding the identification of small-molecule inhibitors. Using synthetic peptides that bound to TyrRS, we showed that a simple competitive binding assay could be used to detect inhibitors with a wide range of potencies. This was true for peptides that were either competitive or noncompetitive inhibitors of TyrRS, indicating that, at least in this case, the inhibition mechanism for the peptide does not exclude its use as an effective screening tool. We formatted the assay using three different detection methods (scintillation proximity, time-resolved fluorescence and fluorescence polarization), showing the versatility of this approach. Peptide ligands isolated by phage display can, therefore, be used sensitive probes for the detection of inhibitors.

Significance

Formatting an assay suitable for high-throughput screening is a difficult task, even when there is detailed

knowledge of the protein being studied. This task becomes even more difficult when the protein is not well characterized. We have shown that peptides isolated using phage display can be used to format assays for enzyme targets. We have found that many (13 of 17) peptides identified using phage display target functional sites on enzymes, the same sites to which effective therapeutics must be targeted. We have also shown that these peptides can be used in a simple competitive binding assay to identify small-molecule inhibitors of enzyme function. The assay is easy to format, flexible enough to be used with a number of detection systems and readily adaptable to automation. These characteristics make it ideal for high-throughput screening of large libraries of compounds. This assay can be used in the same format for enzymes with a wide variety of activities. This simplifies and accelerates the process of screen development and inhibitor identification for a wide range of enzyme targets.

Materials and methods

Materials

Immulon 4 plates (96 well), were purchased from Dynatech. Streptavidin, bovine serum albumin, conduritol epoxide, yeast alcohol dehydrogenase and rabbit muscle glycogen phosphorylase a were purchased from Sigma. Streptavidin-coated paramagnetic particles were from Promega. Anti-M13 antibody coupled to horseradish peroxidase was obtained from Pharmacia. Long-chain-long-chain-N-hydroxysulfosuccinimidobiotin (Sulfo-NHS-LC-LC-biotin) and 2-[4′-hydroxyazobenzene]-benzoic acid (HABA) were purchased from Pierce. Porcine carboxypeptidase B and yeast hexokinase were obtained from Boehringer Mannheim. Recombinant *H. influenzae* tyrosyl-aminoacyl tRNA synthetase was isolated from an over expressing *E. coli* strain as a glutathione-*S*-transferase fusion and *E. coli* prolyl-aminoacyl tRNA synthetase was isolated from an overexpressing *E. coli* strain. *Agrobacterium faecalis* β-glucosidase was a generous gift from Steve Withers (University of British Columbia). Peptides were synthesized by AnaSpec. The LOPAC library of compounds was purchased from RBI/Sigma (Natick, MA).

Biotinylation of target proteins

Target proteins were biotinylated with sulfo-NHS-LC-LC-biotin to achieve 2–5 moles of biotin per mole of target protein following the manufacturer's protocol. The extent of biotinylation was determined using HABA according to the manufacturer's instructions.

Affinity selection of phage-displayed peptides

Affinity selection of peptides was conducted essentially as described [21]. The proteins were analyzed before affinity selection by SDS–PAGE for purity and K_m, k_{cat} determinations for the relevant substrates were also carried out to ensure that the catalytic activity of the targets was in reasonable agreement with published values. For targets that were biotinylated, activity assays were conducted before and after biotinylation. Phage displaying peptides that bound to the targets were selected using protein immobilized by the following methods.

Method 1. Target protein was immobilized directly on the plastic surface of a 96-well plate. Target protein (0.5–1 μg/well) was incubated in a 96-well plate overnight at 4°C in 0.1 M $NaHCO_3$, pH 8. The wells were blocked with 0.1% BSA in 0.1 M $NaHCO_3$, pH 8 prior to the addition of phage libraries.

Method 2. The target protein was biotinylated and captured on streptavidin-coated paramagnetic particles. Biotinylated-target protein (0.5–1 μg) was bound to streptavidin paramagnetic particles. Excess biotin-binding sites were blocked with biotin prior to adding the phage libraries.

Method 3. Biotinylated-target protein and phage were incubated together in solution prior to capturing the target-phage complex on streptavidin-coated paramagnetic particles. Biotinylated target protein (0.5–1 μg) was incubated in solution with the phage libraries for affinity selection. The target–phage complex was then captured on streptavidin paramagnetic particles.

Method 4. The target protein was biotinylated and captured on streptavidin-coated 96-well plates. Excess biotin-binding sites were blocked with biotin prior to the addition of the phage libraries.

After three or four rounds of selection, individual phage were tested for binding to the target and several unrelated proteins.

Phage libraries

Phage libraries were either PHD7 and PHD12 (New England Biolabs) that display either 7- or 12-residue peptides, a random 12-mer library kindly supplied by Brian Kay (University of Wisconsin, Madison), or random 11-residue peptides fused to gene III in which the central residue of each library was fixed with a different residue. Each library had a complexity between 5×10^8 and 2×10^9. The construction and comparison of these libraries with random libraries will be described elsewhere.

Phage ELISA assays

Following affinity selection, eluted phage were plated and individual plaques were picked and amplified overnight in 2× YT. The isolates were tested in phage ELISAs as described previously [21]. In brief, targets were immobilized in microtiter wells, phage were added and allowed to bind, the plates were washed and a horse radish peroxidase (HRP)-linked antibody directed against the major coat protein of the phage was used to detect phage bound to the target. HRP was detected using 2,2′-azinobis(3-ethylbenzthazolinesulfonic acid) (ABTS) a substrate and reading the absorbance at 405 nm. Phage producing a strong signal at 405 nm that was dependent on the presence of the target were plaque purified, amplified and tested for binding to target protein and a series of unrelated proteins in a phage ELISA to verify specificity of binding.

Sequencing of phage-displayed peptides

Single-strand DNA was prepared using the Qiagen M13 single-strand prep kit. Automated DNA sequencing was conducted by the Sequencing Facility at the University of North Carolina, Chapel Hill.

Phage affinity titrations

The relative affinities of the selected phage were determined for each target by conducting phage ELISAs using serial dilutions of phage on a single target protein concentration. An 11-point, twofold dilution series from 50 μl was used in the titrations. Background signals of target protein exposed to antibody but no phage were subtracted from each value. Phage titers were determined, and the absorbance readings from the phage ELISAs were plotted as a function of plaque forming units per assay. Absorbance readings at 405 nm produced by 1×10^9 pfu were all in a linear responsive range and these are presented in Table 2 to judge relative affinities of the displayed peptides.

Phage:peptide competition assays

Phage:peptide competitions were conducted by pre-incubating the immobilized target protein with 100 μM synthetic peptide for 20 min. Phage from a fresh overnight culture were added without a wash step and the phage ELISA was conducted as described above.

Enzyme activity characterization

TyrRS. The activity of the TyrRS from *H. influenzae* was monitored by charging tRNA with [^{3}H]-tyrosine followed by trichloroacetic acid (TCA) precipitation essentially as described previously [22].

Pro-RS. The activity of the Pro-RS from *E. coli* was monitored by charging tRNA with [^{3}H]-proline followed by TCA precipitation essentially as described previously [22].

β-Glucosidase. Reactions were monitored by following the cleavage of p-nitrophenyl-β-D-glucopyranoside by measuring the absorbance at 405 nm as previously described [23].

Hexokinase. Hexokinase activity was followed by using a coupled assay with glucose 6-phosphate dehydrogenase and monitoring the reduction of $NADP^+$ at 340 nm as described in the manufacturer's data sheet.

Carboxypeptidase B. Carboxypeptidase B activity was assayed by measuring the cleavage of Hippuryl–arginine or Furyl-acryloyl–alanine–lysine at pH 7.5 in the presence of 100 mM NaCl [24].

Glycogen phosphorylase a. Enzymatic activity was monitored spectrophotometrically using a coupled enzyme assay with phosphoglyceromutase and glucose-6-phosphate dehydrogenase. Following hydrolysis of glycogen by glycogen phosphorylase, the resulting glucose-1-phosphate was converted to glucose-6-phosphate by phosphoglyceromutase. The resulting glucose-6-phosphate then serves as a substrate for glucose-6-phosphate dehydrogenase along with NADP. The conversion of NADP to NADPH is monitored at 340 nm resulting in an increase in absorbance upon hydrolysis of glycogen.

Alcohol dehydrogenase. Alcohol dehydrogenase activity was determined by monitoring the reduction of NAD^+ at 340 nm.

Enzyme inhibition studies

Inhibition studies were performed using the synthetic peptides described in the text. This was done by holding one substrate concentration constant at 10× the K_m concentration, while varying the concentrations of peptide, and varying the other substrate concentration around K_m. Initial velocity data were then fitted to the various inhibition patterns using GraFit.

Peptide binding assay

Flash plate assay format. TyrRS, immobilized on Flashplates (New England Nuclear), was preincubated in the presence of various concentrations of inhibitors before the addition of biotinylated Tyr-1 or Tyr-4 peptides. The level of TyrRS–peptide interaction was monitored by adding streptavidin labeled with ^{35}S and allowing it to bind to the TyrRS–peptide complex. The amount of ^{35}S-streptavidin-biotin-peptide–target complex in each well was determined by aspirating the buffer, followed by scintillation counting.

Time-resolved fluorescence method. Polystyrene high protein binding 384-well plates (Costar) were coated with 0.25 μg streptavidin in 20 μl 0.1 M $NaHCO_3$ per well and blocked with 1% BSA. The plates were washed 3× with TBST (10 mM Tris–Cl, pH 8, 150 mM NaCl, 0.05% Tween-20), 0.5 pmol TyrRS per well in 20 μl TBST was added and allowed to bind, followed by three washes with TBST. Compounds (20 μl in TBST) were added to each well and incubated at room temperature for 30 min prior to addition of 2 pmol 4:1 peptide/streptavidin-europium conjugate in 5 μl TBST containing 50 mM biotin. The plate was incubated at room temperature for 1 h and washed 3× with TBST. 30 μl europium enhancement solution (Wallac) was added to each well and fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) using a < 400 nm excitation filter and a 620 nm emission filter. The europium labeled streptavidin-biotinylated peptide conjugate was prepared by adding 4 pmol biotinylated peptide per pmol streptavidin. After incubation on ice for 30 min, the remaining biotin binding sites were blocked with biotin prior to addition to the assay plate.

Fluorescence polarization method. To each well of a 384-well polystyrene plate (low protein binding, Costar), 10 μl of each compound in TBST was added to 5 μl of 200 nM TyrRS. After incubation at room temperature for 30 min, 5 μl of 40 nM oregon-green-labeled peptide was added to each well and the plate was placed on a rotating mixer at room temperature for 1 h. Fluorescence polarization readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) using a 485 nm excitation filter and a 520 nm emission filter.

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