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Novel avidin and streptavidin binding sequences found in synthetic peptide libraries

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Abstract Synthetic resin-bound peptide libraries made of protein L-amino acids have been synthesized. During screening of the libraries, peptides that bind to avidin have been identified containing a novel motif with two histidines separated by one residue. A sub-library was synthesized and screened, and new critical residues appeared surrounding the two histidines. Additionally peptide libraries made of D-amino acids have been screened with avidin and streptavidin and novel motifs have been found.

Key words: Synthetic peptide library; Sub-library; Avidin; Streptavidin; Molecular recognition

1. Introduction

The peptide libraries made up of millions of random peptide sequences are a valuable tool in{20}the study of interactions between proteins or peptides and their targets (e.g. hormone receptor, or antibody). The information we get from the ligand libraries may help us to understand such interactions at the molecular level, and especially in the field drug discovery the knowledge of the structure–activity relationship of a molecule is of crucial interest in the search for potential drugs.

Several ways of producing and screening complex mixtures of peptide sequences exist. Both biological and chemical methods are currently being used to find novel sequences that bind to a receptor of interest. For reviews see [1,2]

Furka et al. proposed the 'portioning, mix and recombine' procedure[3] and the nature of this procedure is the synthesis of a unique peptide sequence on each resin bead. This was further developed to the 'one bead–one peptide' library by Lam et al. [4] who generated a complex library and isolated binding sequences by a staining technique that visualized the beads carrying the active peptides. The positive beads were removed from the rest of the library and the sequence of the binding peptide was deduced by micro sequencing. After screening, the library could be reused with another receptor. This solid-phase library has been further developed to include non-sequenceable building blocks by the parallel synthesis of a sequence that encodes the nonpeptidic ligand [5–8].

We have used the 'portioning, mix and recombine' procedure to generate synthetic peptide libraries and have screened these libraries with various biotinylated receptors of biological interest followed by addition of avidin conjugated to either horseradish peroxidase or alkaline phosphatase to visualize binding peptide ligands by conventional ELISA techniques. However, peptides may of course not only bind to the receptor of interest but potentially also to various other sites at the receptor–biotin–avidin peroxidase complex. Thus Lam et al. reported that when a pentapeptide library was screened with streptavidin conjugated to alkaline phosphatase, peptides with the motif HPQ and a few with HPM binding to streptavidin were isolated [4].

Here we report the finding of peptides made by L- or D-amino acids with novel motifs that are able to bind to avidin or streptavidin.

2. Materials and methods

2.1. Materials

Protected amino acids were obtained from Milligen and Novabiochem and Rink amide linker: (*p*-[(*R,S*)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid was obtained from Novabiochem. TentaGel resin was obtained from Rapp Polymere (Tübingen, Germany). BSA, avidin–peroxidase, avidin–alkaline phosphatase, peroxidase, streptavidin–alkaline phosphatase, BCIP, NBT and DAB were obtained from Sigma. Maxisorb from Nunc (Denmark) and G-10 Sephadex from Pharmacia, Denmark.

2.2. Instrumentation

Amino acid analysis was performed using Waters picotag and Waters 501 pump connected to WISP 712. Sequencing was performed on an ABI 477 pulsed-liquid protein sequencer or Procise™. Waters 600E equipped with Waters 990 photodiode array detector was used for analytical HPLC on a C18 column (Delta-Pak 100Å 15 μm, Millipore).

2.3. Synthesis and screening of resin-bound peptide libraries

For the production of peptide libraries the 'portioning, mix and recombine' procedure was used as described [3]. Polyethylene syringes served as reactions vessels throughout the synthesis and the final TFA-deprotection. The resin was divided into 19 aliquots and the protein L-amino acids except cysteine were used. Fmoc-protected amino acids (5 eq) were coupled overnight except for the 1st and 3rd coupling which was reduced to 2 h. The amino acids were either coupled as their

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DAB, diaminobenzidine; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; EDT, ethandithiol; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; NBT, nitro blue tetrazolium; OPD, *o*-phenylenediamine; PBS, phosphate-buffered saline; Pfp, pentafluorophenyl; SPDP, 3-(2-Pyridyldithio)propionic acid *N*-hydroxy-succinimide ester; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate); TFA, trifluoracetic acid.

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Pfp-esters or coupled in situ with TBTU-strategy [9]. Capping was not included in the synthesis. Removal of the Fmoc group was accomplished with 25% piperidine in DMF for 30 min. The side chain protecting groups were removed with 82.5% TFA, 5% anisole, 5% H_2O, 5% EDT, 2.5% thioanisole at room temperature for 2.5 h. The resin was then washed with ethanol and diethyl ether and dried under vacuum. The synthesis of sub-library XXHXHXX (2.5×10^6 beads) was performed as previously described, but in position X cysteine, histidine, arginine and aspartic acid were not included.

Typically screenings were done by incubating 3–4 ml resin (equivalent to $1.5–2 \times 10^6$ beads) with biotinylated receptor in PBS-T (1 M NaCl, 1% BSA and 0,2% (w/v) Tween-20). Subsequently the resin was washed in PBS-T and incubated with avidin–peroxidase (diluted 1:10,000) or streptavidin–alkaline phosphatase (1:20,000). The substrates BCIP/NBT or DAB/H_2O_2 were added and typically 50–500 beads were stained with varying intensities. Only the most intensely stained beads were sequenced.

Peptide VANAASIVHVHL conjugated to BSA (SPDP coupling) was immobilized to Maxisorb overnight in $NaHCO_3$ pH = 9.5. A peptide, LALEGSLQKR, conjugated to BSA and immobilized to Maxisorb, served as control. Serial dilutions of avidin–peroxidase and peroxidase were incubated for 60 min with immobilized peptides, washed with PBS and color was developed by the addition of OPD in Citrate buffer pH = 5.5.

3. Results and discussion

In previous reports by Lam et al. [4,10] motifs were found when streptavidin or avidin were screened in pentapeptide libraries made of the protein L-amino acids excluding Cys. Peptides with the binding motif HPQ were found and shown to bind in the biotin binding pocket of streptavidin but they are unable to bind to avidin. Crystal structures of streptavidin binding to a HPQ peptide reveal that some of the residues in streptavidin that participate in biotin binding also interact with the HPQ peptide but overall the peptide utilizes the binding determinants in the biotin-binding site in an alternative way [11]. Lam et al. also reported that when avidin was screened in a pentapeptide library the motifs HPYP- or HP-P- were found and peptides with this motif binds specifically to avidin and not to streptavidin [10].

Instead of pentapeptide libraries we used decapeptide libraries in screening studies with various biotinylated receptors and chose avidin–peroxidase, avidin–alkaline phophatase or streptavidin–alkaline phosphatase as tags for visualization of the beads. During several screening experiments with biotinylated receptors and avidin–peroxidase we identified many peptides with the -HXH- motif and these sequences are listed in Table 1. One of these sequences, VANAASIVHVHL, was arbitrarily selected and conjugated to BSA for further studies and ELISA measurements of the conjugate showed binding to avidin peroxidase but not to peroxidase alone demonstrating binding to avidin (Fig. 1). Residues surrounding the two histidines are not clearly defined in Table 1 and therefore a new library (sub-library) was constructed. This sub-library (XXHXHXX-bead) was synthesized only with the two histidines fixed and then surrounded by random amino acids (cysteine, histidine, arginine and aspartic acid were not included in position X).

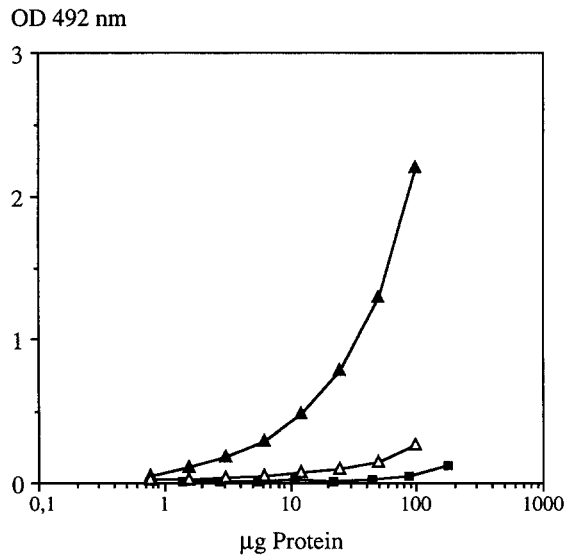

Fig. 1. ELISA results of incubation with avidin–peroxidase conjugate (-▲-) and peroxidase (-△-) to peptide VANAASIVHVHL (target peptide) conjugated to BSA and coated to maxisorb. Binding of avidin–peroxidase (-■-) to peptide LALEGSLQKR (control peptide) conjugated to BSA and coated to maxisorb.

Among the ca. 2.5×10^6 million beads about 200 were stained and 8 were sequenced. The sequences found are listed in Table 2. It is very clear that only aliphatic residues surrounding the two histidines are preferred with especially isoleucine dominating, and this is very interesting since the structures of isoleucine, leucine and valine are so similar. The HXH sequences in Table 1 do not reveal that aliphatic and notably isoleucine are important residues and this clearly demonstrates that sub-libraries are very useful for identifying additional residues, e.g. new information is obtained that was not possible in the first library

Table 1
Sequences that interact with avidin-peroxidase

Group 1 (Aliphatic)	Group 2 (Aromatic)	Group 3 (Asn or Gln)
VAGEVIVHVHMF	AWMWHWHVNVV	PVIAMLHQHV
VANAASIVHVHL	YMYHFHWWIF	AEAQIHQHITK
AKVHVHMMQVQ	VAFHFHAEYGFF	AMNHQHVWLNF
VAVHLHVNYEDM	RRFIHFHYEQ	AFMAWHQHQIM
AVHLHVINNWH	YVWAHFHFLF	VANVQESHQHMQ
MYQTIHIHIH	AFILNHFHWIQ	
LNISHIHINQ	AAAVVMMQHYH	ALLLIVHNHLV
VAIHAHVAWHEP	VADSHWHAFEAG	VHNHFNQVFQ
IKDHVHLTYT		AYTAWEIHNHI
ALYIQDQDHMHM		ARYLTTIHNHF
VAWFINHLHEVE		AGFQYMHNHYF
ASDVHLHQQMG		

The HXH motif identified in decapeptide libraries with the formats: V-A-X_{10}-resin, A-X_{10}-resin, X_{10}-resin.

due to incomplete representation of random sequences. It, however, also shows that finding a consensus sequence in the first library is crucial if sequences are to be optimized by the sublibrary refinement method.

It is remarkable that the avidin binding motif HPYP found in a pentapeptide library [10] was not discovered in our decapeptide libraries and in fact we have so far never sequenced a HPYP motif in our octa- or decapeptide libraries. A likely explanation may be that during our screenings a biotinylated receptor was used which may cause blocking of the biotin binding site. This is in agreement with the fact that binding of HPYP to avidin can be blocked by free biotin [10] while we have not been able to inhibit binding of HXH to avidin by biotin pointing to binding *outside* the avidin-binding site in biotin.

In addition to the libraries made of L-amino acids we have synthesized an octapeptide library made exclusively of D-amino acids and screened it in the *absence* of a biotinylated receptor with avidin–alkaline phosphatase or streptavidin–alkaline phosphatase.

The results of screening the octa all-D library with avidin is shown in Table 3. The motifs found, -rwf-, -kw-g-, k--wyp, y---gy, are very different from the motifs found in all-L peptide libraries, (HPYP/HP-P, absence of biotinylated receptor) in that notably tryptophan, tyrosine, lysine, arginine are essential residues and furthermore, the peptides cannot bind avidin when free biotin is added indicating that they bind *in* or *near* the biotin binding site.

The results of screening the octa all-D library with streptavidin is shown in Table 4. The motifs found, wy--a--- or wh--a---, are in agreement with the previous report by Lam et al. [12] for an all-D pentapeptide library screened with streptavidin (wy--a), and tryptophan and tyrosine are again essential residues though no similarity to the avidin motifs exists. Peptides with the motif w(y/h)--a--- are binding *in* or *near* the biotin binding site in streptavidin because binding can be inhibited by free biotin.

In conclusion we have used two simple lineary peptide libraries made of protein L-amino acids excluding Cys of either exclusively L- or D-amino acids and screenings led to the identification of novel binding motifs to avidin or streptavidin. Since the motifs are significantly different, it is not surprising that they are specific, e.g. binding motifs found with avidin do not bind streptavidin and vice versa.

Table 2
The sequences found in screening the sublibrary XXHXHXX-resin with avidin–peroxidase

--HIHII	-IHIHI-
YIHIHII	WIHIHIV
WIHIHII	VIHIHIV
WVHIHII	MIHIHIV
	TIHIHIV
	MIHIHIM

Table 3
Sequences that interact with avidin

Group 1	Group 2	Group 3
fdhmkrwf	gkwngfpr	ypnrgypr
adylhrwf	akwngpdw	yfwkgyei
ngymarwf	kwigpenw	ytwkgyhf
ldqrwfgr	ykwvygpf	whgykgfp
qrwfpnwp	vgkwdwyp	
	skvawypp	
	hknewypm	
	gswypds-	

Found in the screening of an all-D octapeptide library.

Table 4
Sequences that interact with streptavidin

Group 1	Group 2	Group 3
whtyamas	wysmahnp	twwmeavr
whkhasgr	wyknafae	inwtsyah
nwhhsaqw	wydkayvk	
whhqaiws	wyhdairi	
whrfsvns	wyqeaemd	

Found in the screening of an all-D octapeptide library.

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