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Identification of Avidin and Streptavidin Binding Motifs Among Peptides Selected from a Synthetic Peptide Library Consisting Solely of D-Amino Acids*

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Abstract: Peptides consisting solely of D-amino acids (D-peptides) as opposed to their L-counterparts (L-peptides) are resistant towards proteolytic degradation in the organism and may therefore be useful in future efforts to develop new stable peptide-based drugs. Using the random synthetic peptide library technique several L- and D-peptides, capable of binding to both avidin and streptavidin, were found. The L-peptides contained the previously described HPQ/M motifs, and among the D-peptides three binding motifs could be identified, of which the most frequently found one contained an N-terminal aliphatic hydrophobic amino acid (V, L or I) and an aromatic amino acid (Y or F) on the second position. At the third position in this motif several different amino acid residues were found, although N was the most frequent. Peptides representing two of the D-motifs were synthesized as well as peptides containing the HPQ/M motifs, and their binding properties were examined. Although the D-peptides were originally selected using avidin they also inhibited binding between immobilized biotin and soluble streptavidin as well as avidin. The IC_{50} of some of the peptides were approximately 10^5 times higher than the IC_{50} for biotin but some had a lower IC_{50} than iminobiotin. The D-peptides, which were originally selected from the library using avidin, could also inhibit the binding between streptavidin and biotin. Likewise, L-peptides selected from a library screened with streptavidin, could inhibit the binding of both streptavidin and avidin to immobilized biotin. Furthermore, the D-peptide, VFSVQSGS, as well as biotin could inhibit binding of streptavidin to an immobilized L-peptide (RYHPQSGS). This indicates that the biotin-like structure mimicked by these two seemingly very different peptides may react with the same binding sites in the streptavidin molecule.

Keywords: Synthetic peptide libraries; D-amino acids; L-amino acids; streptavidin; avidin; D-peptides; drug discovery

Abbreviations: Avidin-HRP, HRP labelled avidin; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HRP, horse radish peroxidase; PEG, polyethylene glycol; PBS, phosphate buffered saline; PTH-amino acids; phenylthiohydantion amino acids; PyBOP, benzotriazol-1-yloxy-tris(pyrrolidino)phosponium hexafluorophosphate; streptavidin-HRP, HRP labelled streptavidin; streptavidin-AP, alkaline phosphatase labelled streptavidin.

INTRODUCTION

Peptides composed solely of D-amino acids (D-peptides) as opposed to L-peptides are resistant towards proteolytic degradation in the organism and may therefore be useful in future efforts to develop new stable peptide-based drugs for oral use. During recent years several screening techniques have been described for producing and screening large libraries of compounds for their ability to bind different biomolecules. The so-called peptide library techniques can be divided into two groups: the synthetic and the microbiological libraries. The synthetic

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libraries contain a large number of randomly synthesized peptides, which can either be in soluble form [1] or still persist on the solid phase [2, 3] during the subsequent screening procedure. The biological libraries are constructed by introducing random DNA sequences into bacterial phages in such a way that the different peptides encoded by the DNA are presented on the surface of the phage particles [4]. There are advantages and disadvantages associated with both techniques, but one clear benefit of the synthetic libraries is the possibility of incorporating unnatural amino acids, such as D-amino acids, during the synthesis.

In the synthetic peptide library technique proposed by Furka *et al.* [2] and Lam *et al.* [3], the most important improvement, compared with the previously described methods, was the approach of having only one type of peptide sequence on each bead. This enables direct sequencing of the peptide on a single bead. In the method described by Lam *et al.* [3], a randomized synthetic peptide library (in the following just designated a library) was made by several repeated couplings of activated amino acids to resin beads. Between each coupling step, all the resin beads were pooled, mixed and subsequently divided into as many reaction vessels as the number of different amino acids used. The result was that all peptides on a given bead had the same sequence, and if sufficient amounts of resin beads were used, theoretically all possible combinations of amino acids would be represented in the library.

Here we demonstrate the use of such libraries consisting of either D-amino acids or L-amino acids for screening of peptide ligands to streptavidin and avidin. Several avidin and streptavidin binding peptides were found in both libraries, and three D-peptide binding motifs could be identified. Peptides representing two of these motifs were synthesized and binding inhibition studies were performed.

MATERIALS AND METHODS

Chemicals

Acetonitrile (SuperGradient, Far UV), DMF (HPLC grade) and TFA (analytical grade) were from Lab-Scan Ltd (Dublin, Ireland). PyBOP, Fmoc-protected amino acids and KA resin were from Novabiochem (Bad Soden, Germany). Tentagel resin was from Rapp Polymere (Tübingen, Germany). All other chemicals were of analytical grade from Sigma (Dorset, UK) unless otherwise specified. The DMF was freshly distilled under reduced pressure before use.

Synthesis and Deprotection of Peptide Libraries

The following Fmoc-protected amino acids were used in D- or L-form in 5 molar excess for synthesis of the peptide libraries: alanine, arginine (Pmc), asparagine (Trt), aspartic acid (*t*Bu), glutamine (Trt), glutamic acid (*t*Bu), glycine, histidine (Trt), isoleucine, leucine, lysine (Boc), methionine, phenylalanine, proline, serine (*t*Bu), threonine (*t*Bu), tryptophan, tyrosine (*t*Bu) and valine. Cysteine was omitted from the library in order to avoid disulphide bridges between peptides and to avoid technical difficulties during the subsequent sequencing reaction. Synthesis was performed using conventional Fmoc [5] and PyBOP chemistry [6]. The resin (Tentagel S-NH_2(S30 132) 2 g, 8.9×10^5 beads/g, 0.24 meq/g) was divided into 19 portions and transferred to separate funnels (2 ml) with glass filters. In each glass funnel coupling reactions were performed separately for each of the 19 amino acids, L- or D-form. The different Fmoc-amino acids (0.13 mmol of each) were dissolved in DMF (430 μl), containing PyBOP (0.13 mmol, 0.066 g), 1-Hydroxybenzotriazol (0.13 mmol, 0.019 g) and diisopropylethylamine (0.13 mmol, 0.044 ml). After 4 h each portion of the resin was washed three times with DMF (2 ml) whereupon piperidine in DMF (20%, 1 ml) was added. After 30 min, the deprotection of the Fmoc groups was ensured by measuring the absorption at 315 nm in an aliquot diluted in DMF. The resin was subsequently washed three times with DMF (20 ml) and once with DMF overnight (20 ml). Next day the 19 portions of resin were pooled and mixed thoroughly. Couplings and randomizations were repeated four times as described above. After the completed synthesis, the libraries were deprotected using a cocktail of TFA (8.15 ml), H_2O (0.5 ml), thioanisol (0.5 ml), phenol (0.5 g), ethanedithiol (0.25 ml) and triisopropylsilane (0.1 ml). After 3 h the cocktail was removed, the library was washed ten times with DMF (20 ml), and subsequently washed once with methanol (20 ml) which was finally gradually displaced with PBS (20 ml, 0.1 M phosphate, 0.5 M NaCl, pH 7.2).

Amino Acid Analysis of the Peptide Libraries

Aliquots of beads from the peptide library were hydrolysed overnight in 6N HCl, derivatized with ortho-phtaldialdehyde and analysed on a Waters amino acid analyser [7].

Screening of the Peptide Libraries

Each library was washed three times for 10 min with washing buffer (PBS containing 1% Triton X-100) whereupon the resin was blocked with 0.1% gelatine in washing buffer (20 ml). After 1 h the library was washed three times with washing buffer (20 ml).

Screening of the L-library. Alkaline phosphatase labelled streptavidin (streptavidin-AP) (D 396, DAKO, Ejby, Denmark) (5 ng/ml) in dilution buffer (washing buffer containing 0.5% BSA and 0.01% phenol red) was added to the L-library. After 1 h the library was washed three times with washing buffer (20 ml) and subsequently incubated with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (B-8503, Sigma; 163 μg/ml) and Nitro Blue Tetrazolium (N-6876, Sigma; 327 μg/ml) both dissolved in Tris buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$). When an appropriate colour intensity had developed, the library was washed twice with PBS (20 ml) and distributed in 9 cm Petri dishes in a single layer. The most intense blue beads were removed with a 100 μl micropipette under a stereo microscope (Olympus SZ 40 PT) capable of 17–40 times magnification.

Screening of the D-library. Autofluorescent false positive beads were initially removed from the library using the stereo microscope, fitted with filters for FITC detection (excitation 470 nm, emission 510 nm). From a library containing aproximately 1.8 million beads about 250 greenish autofluorescent beads were removed. They were selected on the basis of a subjective evaluation of whether they would interfere with subsequent screenings with the fluorescent labelled proteins. The fluorescence was often unevenly distributed throughout the beads with, for instance, a core of fluorescence. Subsequently the library was incubated in a 30 ml glass funnel for 2 h with FITC conjugated avidin [8] (10 μg/ml in dilution buffer) and then washed three times with washing buffer (20 ml). The library was then distributed in Petri dishes, and the positive beads, which appeared as bright green fluorescent, were removed with a micropipette. The library was subsequently collected in a 30 ml funnel, washed twice with guanidin hydrochloride (20 ml, 8 M, pH 2.0) and three times with washing buffer (20 ml) before it was incubated with FITC conjugated streptavidin (RPN 1271, Amersham, Berks, UK) (7 ml, 10 μg/ml) in dilution buffer. The positive beads were removed as described above.

Amino Acid Sequencing of Peptides

The selected beads were washed thoroughly with guanidin hydrochloride (8 M, pH 2.0) and single beads were placed directly in an Applied Biosystems Sequencer, model 470A or 477A using the programme provided by the company. Identification of the PTH-amino acids were done on-line with an Applied Biosystems, model 120A, PTH amino acid analyser.

Synthesis of Soluble Peptides

The following peptides were synthesized on an automatic peptide synthesizer, Novasyn Crystal, using conventional Fmoc and PyBOP chemistry on a cleavable resin (NovaSyn KA): three L-peptides, YHPQA, YHPMA and QSFGLLDPKLCY, and four D-peptides, VYNSVSG, VFSVQSG, LTSSFSG and RNWIASGS. The peptides were cleaved from the resin and deprotected by adding a cocktail of TFA (9.5 ml) and ethanedithiol (0.5 ml) and by incubating for 2 h. The peptides were precipitated from the supernatant with diethylether (25 ml) redissolved in TFA (10 ml) and reprecipitated twice with diethylether. Subsequently they were dissolved in water containing acetic acid (10 ml) and lyophilized. Some peptides were further purified by reverse-phase HPLC (Merck, Darmstadt, Germany), using a LiChrosorb RP 18 column (7 μm, 25 × 250 mm) and an elution gradient ranging from 0 to 90% of acetonitrile (buffer A – 99.9% H_2O, 0.1% TFA; buffer B – 90% acetonitrile, 9.9% H_2O, 0.1% TA) over 50 min. All peptides were finally analysed by HPLC using a Lichrosorb RP18 column (5 μm, 4 × 125 mm) in the same gradient (Table 1). A total amino acid analysis was performed as described for the libraries (Table 1).

Liquid-phase Inhibition Experiments

Microtitre plates (Maxisorp, Nunc A/S, Roskilde, Denmark) were biotinylated by addition of a solution of biotinylated BSA [8] (100 μl/well, 50 μg/ml in 0.1 M carbonate buffer, pH 9.6) to each well and subsequent incubation for 2 h. After washing three times with washing buffer (200 μl/well) possible free sites on the plates were blocked with 1% BSA in washing buffer (200 μl/well). After 2 h the plates were washed again as described.

Table 1 Amino Acid Analysis of Soluble Peptides

	YHPQA	YHPMA	L[a]	VYNSVSG	LTSSFSG	VFSVQSG	D[b]
Ala	1.00	1.01	–	–	–	–	0.97
Arg	–	–	–	–	–	–	0.95
Asx	–	–	1.02	1.06	–	–	0.97
Cys	–	–	0.85	–	–	–	–
Glx	1.02	–	0.98	–	–	1.02	–
Gly	–	–	1.03	1.10	1.00	1.07	1.21
His	0.98	0.99	–	–	–	–	–
Ile	–	–	–	–	–	–	0.86
Leu	–	–	3.05	–	0.98	–	–
Lys	–	–	1.02	–	–	–	–
Met	–	1.01	–	–	–	–	–
Phe	–	–	0.99	–	0.97	0.89	–
Pro	1.01	1.03	1.03	–	–	–	–
Ser	–	–	1.00	2.06	3.07	2.09	2.03
Thr	–	–	–	–	0.98	–	–
Trp	–	–	–	–	–	–	0[c]
Tyr	0.99	0.97	1.03	0.88	–	–	–
Val	–	–	–	1.91	–	1.93	–
HPLC[d]	96%	97%	95%	100%	100%	100%	99%

[a] Control L-peptide (QSFGLLDPKLCY).
[b] Control D-peptide (RNWIASGS).
[c] Probably due to some chemical modification of the Trp side chain, it could not be identified in the amino acid analysis. The sequence containing Trp on third position were, however, subsequently confirmed by automated Edman sequencing of this control peptide.
[d] Purity of peptides analysed by HPLC on a Licrosorp RP 18 column.

Inhibition of Streptavidin–Biotin Binding by the L-Peptide YHPQA Using Varying Amounts of Peptide and Streptavidin. The following chess board titration was performed in the biotinylated microtitre plates prepared as described above. Twofold dilutions of horse radish peroxidase streptavidin (streptavidin–HRP, RPN 1231, Amersham) in concentrations ranging from 250 to 4.0 ng/ml (50 μl per well) were added to twofold dilutions of the L-peptide YHPQA in concentrations ranging from 450 to 0.44 ng/ml (50 μl per well). After an hour of incubation at room temperature the plates were washed three times with washing buffer, and a solution of the chromogenic substrate orthophenyl diamine (1 mg/ml) and hydrogen peroxide (1 μl/ml, 35%) in citrate/phosphate buffer (10 ml, 0.035 M citrate, 0.075 M phosphate) was added (100 μl/well). The colour development was stopped with 1 M H_2SO_4 (150 μl/well) after about 2 min and the absorbance at 492 nm was measured in an automatic ELISA reader (Dynatech MR 5000).

Inhibition of Biotin/Streptavidin or Biotin/Avidin Binding by Synthetic Peptides Using Constant Amounts of Streptavidin- or Avidin-HRP. Serial dilutions of either biotin (Sigma, B 4501), iminobiotin (Sigma I-4632), YHPQA, YHPMA, QSFGLLDPKLCY (L-peptides), VYNSVSG, VFSVQSG, LTSSFSG or RNWIASGS (D-peptides) were added to the biotinylated microtitre plates. Solutions of either avidin–HRP (20 μl, 200 ng/ml, P347, DAKO) or streptavidin–HRP (20 μl, 80 ng/ml) were subsequently added to each dilution. After one hour of incubation, washing and detection were performed as described above. The IC_{50} was determined as the concentration of biotin, iminobiotin or peptide needed to inhibit half of the binding of avidin or streptavidin to the immobilized biotin molecules.

Inhibition of Streptavidin/RYHPQ Binding by the Biotin-like Synthetic L- and D-peptides. The L-peptide of the sequence RYHPQSGS was synthesized as described above using a non-cleavable resin. The solid-phase bound peptide was subsequently deprotected as described for the libraries. After washing 10 times with washing buffer the resin was blocked with 0.1% gelatine in dilution buffer for 1 h and washed again twice. Aliquots of 30 μl were distributed in 1 ml Eppendorf tubes and incubated with streptavidin–HRP diluted 1:500 in dilution buffer and an excess (>1 mg/ml) of different peptides or biotin for 2 h.

Directly biotinylated resin beads, non-modified resin beads and a sample of beads from a peptide library were included as controls. After 10 times washing with washing buffer the resins were transferred to new Eppendorf tubes, all liquid was removed and 100 μl orthophenylenediamine dihydrochloride substrate and H_2O_2 in citrate/phosphate buffer (described above) were added. The colour development was stopped after 30 min by addition of 150 μl of 1 M H_2SO_4, and the supernatants were transferred to microtitre plates and measured in the ELISA reader.

RESULTS

Synthesis of Peptide Libraries

Two randomized peptide libraries were synthesized manually, a pentamer L-library and a pentamer D-library, which also contained the linker amino acid sequence, SGS, at the C-terminus. Apart from the amino acids K, N, D, Q, E, G, S in the D-library and K, N, D, Q, E in the L-library the average amounts of the different amino acids were very similar (Figure 1) with variation coefficients of 10 and 15%, respectively. The increased amounts of K were artifarctual and due to unknown compounds being released from the resin itself (data not shown). The increased amounts of S and G in the D-library were due to the presence of the SGS-linker on all beads. N plus D as well as Q plus E was determined as D and E respectively. Therefore, as expected the amounts of these two amino acids were found to be about twice as high as the other amino acids in the libraries.

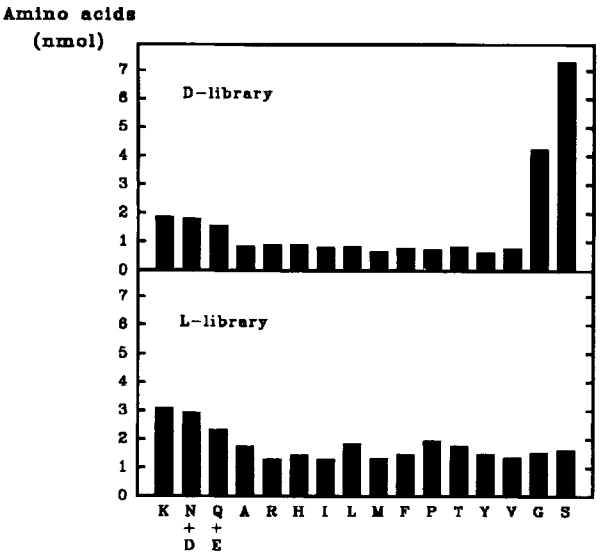

Fig. 1 The result of the amino acids analysis of the D- and L-libraries.

Table 2 Peptide Sequences from Beads Selected with Streptavidin or Avidin

L-peptides[a]	D-peptides[b]			
RYHPQ	VYNSV	VFSVQ	LTLSF	VQSQW
YHPQ[c]	VYNMV	VFSVQ	LTSSF	VQTDW
DNHPQ	VYNEI	VFLIV	ITISL	
LWHPQ	IYNFT	VFMII		
WIHPQ	VYHLA	LFIIV		
THPMV	VYMIQ	VFIFH		
WHPME	LYEIW	VFNVY		
	IYLKY	VFDH		
		ILFEH		

[a] Sequences of peptides selected from the L-library using HRP-labelled streptavidin.
[b] Sequences of peptides selected from the D-library using HRP-labelled avidin.
[c] The hole in this sequence probably was due to some chemical modification affecting the identification of the amino acid by HPLC.

Screening of the Peptide Libraries

Screening of the L-library. The L-library was screened with streptavidin–AP, 32 positive beads were selected and the peptides on the 9 most intensively blue coloured beads were sequenced. The two streptavidin binding motifs, which have been described previously by several groups [3, 9, 10] containing either the amino acid sequence HPQ or HPM were also identified in this library. The HPQ-motif was found on five beads and the HPM-motif on two beads. In four out of the five peptide sequences, HPQ appeared at the C-terminus (Table 2).

Screening of the D-library. Fifty-five positive beads were removed from the pentamer D-library after screening with FITC-labelled avidin. In 22 of these (Table 2) three different binding motifs could be identified (Table 3). The motif most frequently found contained an N-terminal aliphatic hydrophobic amino acid (V, L or I), a small aromatic amino acid (Y or F) on the second position and several different amino acids at the third position, although N was most frequently found (Table 3). Out of 17 peptide sequences, 7 contained a carbonyl group in the sidechain of the amino acid at the third position. The last two positions in the peptides had no obvious restrictions. Another motif had an aliphatic hydrophobic (L or I) in the first position, T in the second position, S in position four and no restraints in

Table 3 Peptide Binding Motifs

D-peptide motifs[a]				
V I L	Y F	N	X	X
L I	T	X	S	X
V	Q	S T	X	W

[a] Motifs found by screening the D-library with HRP-labelled avidin.

positions three and five. The last putative motif, although only two homologous sequences were found, had V in the first position, Q in the second position, no certain restrictions at the third and fourth position and W on the fifth position. The three motifs were found on 17, 3 and 2 beads, respectively. When streptavidin was subsequently used for screening the D-library, 44 positive beads were removed. Twenty-eight of these were sequenced, but no binding motifs could be identified, although there seemed to be a preference for S in the first position and G in the second position (data not shown).

Liquid-phase Inhibition Experiments

The two L-peptides synthesized were examined for their ability to inhibit the binding of HRP-labelled streptavidin to immobilized biotin. The inhibition of the streptavidin/biotin binding by the two peptides was dose-dependent and specific. This is illustrated in Figure 2 for the peptide YHPQA.

Using a constant amount of streptavidin–HRP the inhibitory capabilities of the different peptides were examined in more detail (Figure 3). The resulting IC_{50} values are shown in Table 4. The L-peptide YHPQA seemed to bind equally well to streptavidin and avidin whereas YHPMA was at least a 100 times poorer ligand of these molecules. YHPQA bound to avidin with a strength in the same order of magnitude as iminobiotin. This analogue of biotin seemed, however, to be a poorer ligand to streptavidin (Table 4).

All the D-peptides selected using avidin were also able to bind to avidin in a liquid phase. Interestingly, they also bound to streptavidin (Figure 3C and F). VYNSVSG possessed the strongest inhibitory capacity and was comparable to the L-peptide YHPQA and both peptides were at least as potent as iminobiotin (Table 4). The D-peptides VFSVQSG and LTSSFSG

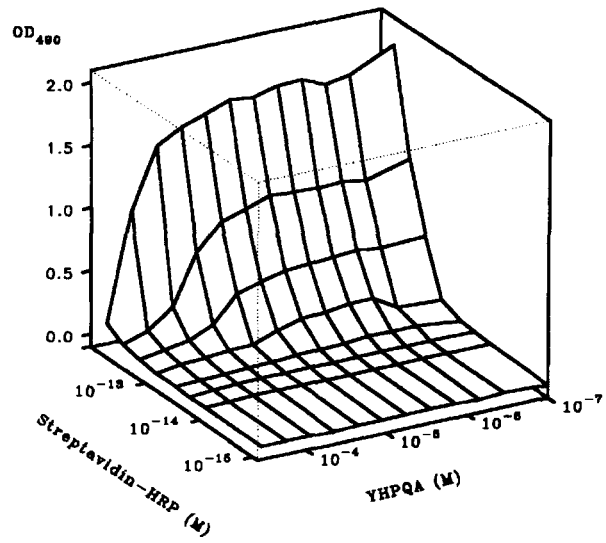

Fig. 2 Inhibition of the binding of streptavidin to immobilized biotin by the L-peptide YHPQA using different streptavidin and peptide concentrations.

had IC_{50} values in the same order of magnitude as the L-peptide YHPMA with regard to avidin binding. VFSVQSG and LTSSFSG, however, bound better to streptavidin than YHPMA did.

Since the Tentagel resin used contained a 3 kDa PEG linker, the peptides VYNSVSG, VFSVGSG and LTSSFSG were also synthesized with a C-terminal PEG moiety of that MW. This had, however, no major effect on the binding properties of these peptides (data not shown).

Inhibition of Solid-phase Streptavidin/RYHPQ Binding

The L-peptide RYHPQSGS was synthesized on a noncleavable resin and subsequently incubated with streptavidin–HRP, peptides and biotin. The D-peptide VFSVQSGS as well as the D-peptide YHPQA and biotin were all able to inhibit the solid-phase binding of streptavidin to the immobilized L-peptide RYHPQSGS (Figure 4). No significant inhibition was observed with the negative control L-peptide QSFGLLDPKLCY. With the amount of peptide used, the binding of streptavidin to a directly biotinylated resin was also inhibited by VFSVQSGSK and YHPQA, although not to the same extent (48 and 78% inhibition, respectively; data not shown).

DISCUSSION

With the random peptide synthesis technique originally described by Furka *et al.* [2] and Lam and Lebl

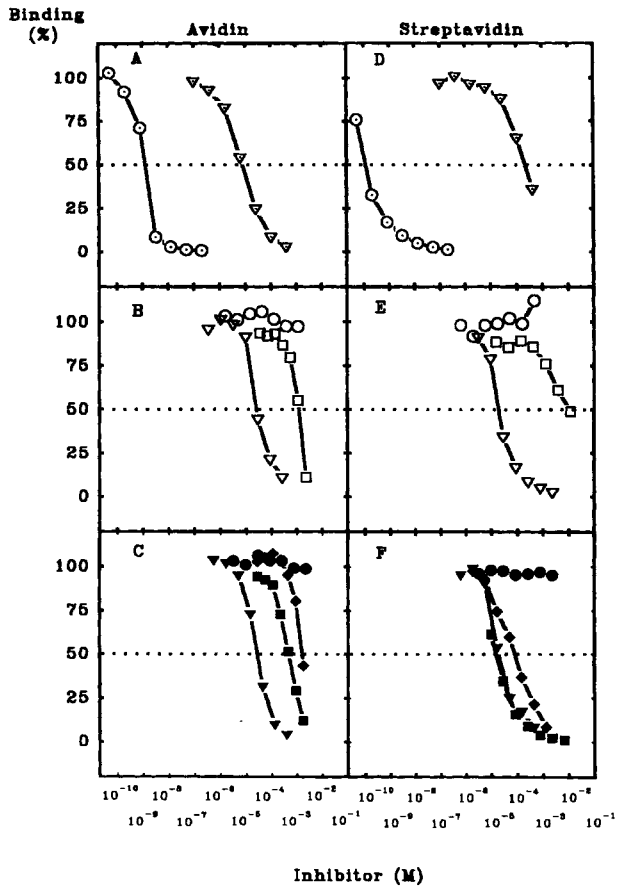

Fig. 3 Inhibition of the binding of avidin (A, B and C) and streptavidin (D, E and F) to immobilized biotin using L-peptides YHPQA (▽), YHPMA (□), QSFGLLDPKLCY (○), D-peptides VYNSVSG (▼), LTSSFSG (■), VFSVQSG (◆), RNWIASGS (●); biotin (⊙) and iminobiotin (▽).

Table 4 IC_{50} Values for Peptides and Biotin/Iminobiotin Inhibiting the Binding Between Biotin and Avidin or Streptavidin[a]

Inhibitor (M)	Avidin (40 ng/ml)	Streptavidin (16 ng/ml)
Biotin	1×10^{-9}	1×10^{-9}
Iminobiotin	9×10^{-6}	2×10^{-4}
YHPQA	4×10^{-5}	2×10^{-5}
YHPMA	1×10^{-3}	1×10^{-2}
QSFGLLDPKLCY	No inhibition	No inhibition
VYNSVSG	3×10^{-5}	2×10^{-5}
VFSVQSG	4×10^{-4}	8×10^{-5}
LTSSFSG	1×10^{-3}	2×10^{-5}
RNWIASGS	No inhibition	No inhibition

[a] IC_{50} values for different peptides, biotin and iminobiotin for inhibition of the binding between solid-phase biotin and HRP-labelled avidin or streptavidin.

[1] it is possible to identify several binding motifs during the same screening. This is due to the parallel approach in which every amino acid sequence is screened concurrently, whereas the convergent approach used in soluble peptide libraries [1] often leads to identification of only one motif in the selected peptides. Here we have been able to confirm the streptavidin binding peptide motifs previously described by others [3, 9]. Furthermore, we have been able to identify and characterize several new and entirely different streptavidin and avidin binding motifs which solely contain D-amino acids. This is among the first reports describing the use of synthetic peptide libraries for identification of D-peptides capable of binding to biomolecules.

In order to obtain 19^5 randomly synthesized peptides a fundamental requirement would be that all the couplings of each of the amino acids are highly efficient and that a sufficient number of beads are present during the reactions. Still, if these require-

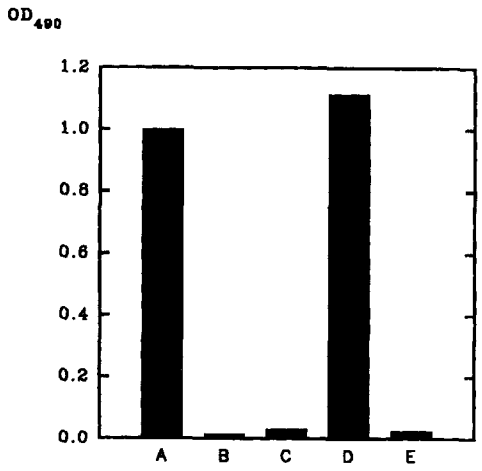

Fig. 4 Inhibition of the binding of streptavidin to the solid-phase bound L-peptide, RYHPQSGS by PBS only (A), the D-peptide VFSVQSG (B), the L-peptide YHPQA (C), the control L-peptide QSFGLLDPKLCY (D) and biotin (E).

ments are fulfilled, it is not likely that all theoretically possible peptide sequences would be represented in the library. Here we have used a total amino acid analysis as a quality control of the random synthesis. The analysis showed a fairly equimolar composition of the different amino acids used with acceptable variation coefficients of 10 and 15%, in the D- and L-libraries, respectively. Although this indicates a reasonable randomized synthesis of the peptides in the libraries, the quality of the different peptides with regard to unwanted side reactions, isomerizations etc. has to be established by other methods.

The HPQ/M motifs found in the L-library when screening with streptavidin were identical to the binding motifs described previously [3, 9]. These motifs should theoretically be found on 2166 (6×19^2) different beads in an ideal, random pentamer library if all positions of the motifs were allowed. This seems, however, not to be the case since only 32 positive beads were found. This is in accordance with Schmidt and Skerra [1], who described a profound dependency of the amino acid residues flanking the binding motif. Therefore, the positive beads found in our L-library probably represent the strongest streptavidin binding peptides.

The HPQ-motif was found on five out of seven of the sequenced beads and the HPM-motif only on two beads. Furthermore, in four out of the five sequences, HPQ appeared in the C-terminus of the peptide. This higher incidence of HPQ among these peptides as well as among other previously published streptavidin binding peptides [3] could be explained by a difference in the affinity to streptavidin of the peptides containing these two motifs, since the peptide containing the HPQ motif (YHPQA) was found to be a much better ligand to streptavidin (Table 4).

The liquid phase binding assays showed that YHPQA and YHPMA inhibited binding of soluble streptavidin as well as avidin to immobilized biotin, although both peptides were selected using streptavidin. This is not in agreement with Lam and Lebl who described different binding motifs of avidin and streptavidin, respectively, and no cross reactivity between these [10]. This was shown by examining the binding of soluble avidin and streptavidin to peptides immobilized on resin beads. Our inhibition assays were performed with peptides in solution. It remains unclear, however, why this should lead to the observed discrepancy.

The peptide library screening technique offers the possibility of screening for ligands consisting of unnatural amino acids. We used the streptavidin/biotin system to search for a D-peptide ligand with binding capacity for streptavidin and/or avidin. A pentamer D-amino acid peptide library was screened with both molecules and a major motif could be identified on 17 out of 22 beads during the initial screening with avidin (Table 2). This motif preferably contained V at the first position, Y or F on the second position and frequently N at the third position (Table III). Interestingly the exact same sequence (VFSVQ) was found on two different beads although this theoretically should not be possible in an ideally randomized library. The amino terminus seemed to be important in this D amino acid motif in contrast to the HPQ and HPM peptides, where the motifs were mostly situated at the C-terminus. Also two minor putative motifs could be identified on three and two beads respectively.

No motifs could be identified when subsequently screening the same D-library with streptavidin. A reason could be that all beads capable of binding strongly to streptavidin were removed from the D-library during the initial avidin screening. If that was the case one would expect that the avidin selected D-peptides could also be recognised by streptavidin. This was studied further in the liquid phase binding assay establishing that the three avidin selected D-peptides representing two of the three binding motifs were all able to bind to avidin as well as to streptavidin (Figure 3). Therefore, it seems that streptavidin and avadin also share a similar specificity for D-peptides as could be demonstrated for the L-peptides.

Both the D- and the L-peptides bind to avidin and streptavidin with comparable affinities (Table 4).

Compared with iminobiotin all the peptides bind 1–100 times weaker to avidin whereas YHPQA and VYNSVSG bind about 10 times better to streptavidin than do iminobiotin. However, none of the L- or D-peptides is nearly as good as the natural ligand, biotin. Because of the difference in concentration of avidin and streptavidin (2.5 times), the IC_{50} values for the two proteins are not directly comparable. These concentrations were chosen as the minimal amounts which could elicit a satisfactory signal in the ELISA.

Although it could be shown that both the L- and D-peptides could compete with biotin for binding, this did not imply that they could also compete with each other for binding to streptavidin. The inhibition experiment using a solid-phase immobilized L-peptide containing the HPQ motif clearly demonstrated, however, that the L-peptides and the D-peptides compete for the same binding site on streptavidin (Figure 4). This suggests that both these peptide motifs somehow are able to mimic the biotin molecule. The amino acids in the avidin/streptavidin binding peptide motifs found in the L- and D-libraries were very different in terms of hydrophobicity and charge. It seems therefore difficult to envision how they both manage to mimic the biotin molecule, which perhaps reflects the difficulties of predicting an equally potent D-ligand from a known L-peptide sequence. By comparing the three-dimensional structure of the L-peptides and D-peptides when bound to streptavidin it should be possible further to elucidate the nature of the binding. The structure of the L-peptide FSHPQNL bound to streptavidin has already been solved [2].

It has been shown previously that L- and D-forms of the same peptide can sometimes bind to the same receptor [13, 14]. Also the reverse enantiomer form of an L-peptide may be able to bind and even exert a biological activity *in vitro* and *in vivo* [5]. However, the affinity of the D-peptides is generally lower than the L-peptides. The experiments described here demonstrate that it is possible to translate an L-peptide to a D-peptide using the peptide library technique. The D-motif had an entirely different and non-predictable composition and importantly, the D-peptides bound to the receptor with equal affinity as the L-peptides. A similar approach was published recently by Lam *et al.* [16] who found a D-peptide ligand for a monoclonal anti-β-endorphin antibody. The sequence of β-endorphin recognized by this antibody is YGGF whereas the sequence of the D-peptide was WTGGY. The affinity and possible biological activity of this peptide was not reported. It should theoretically be possible, however, to transfer this strategy to other systems in order to find stable D-peptide ligands which also are biologically active.

The avidin/streptavidin binding D-peptides can be viewed as a model system for using the peptide library for drug discovery. The IC_{50} values of the synthetic peptides described here are, however, generally lower than for iminobiotin, which has a K_d of 10^{-6} M at pH 7.2. This is not a sufficient affinity for a drug candidate, but it may be possible to perform iterative screening in other peptide libraries in order to increase the binding affinity. It would also be possible to include other unnatural amino acids than the D-forms as well as other chemical 'building blocks' in the libraries in order to find future new lead compounds.

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