

COMMUNICATION

A Conformationally Homogeneous Combinatorial Peptide Library

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In search for a rational way to convert the information encoded in peptide structures into peptidomimetics, major progress could be made by coupling the power of selection methods, now enormously increased in number as a result of the development of combinatorial peptide libraries, with the rational design of structure-inducing templates for the selectable sequences. The availability of libraries of peptides with predetermined structure would enable selection-driven peptidomimetic design, whereby a conformational model for the peptide pharmacophore would be directly derived from the screening, allowing the design of a suitable non-peptidic scaffold to replace the peptide backbone. We describe here the first example of a conformationally homogeneous combinatorial peptide library, which yields ligands with the expected structure upon selection. The library was built by randomising five positions in the α -helical portion of a 26 amino acid Cys₂His₂ consensus “zinc-finger” motif. Since in zinc-fingers metal coordination and folding are coupled, in our library metal-dependent binding represents a built-in control against the selection of structurally undefined sequences. The α -helical library was produced as both fusion with the pVIII protein of filamentous phage and soluble peptides by chemical synthesis, the latter enabling the expansion of the selectable repertoire by the inclusion of non-coded amino acids. The two libraries were independently screened with the same receptor (a monoclonal IgA reactive against the lipopolysaccharide of the human pathogen *Shigella flexneri*), yielding a very similar consensus. In particular, the peptides defined by both methods showed very strong, zinc-dependent binding to the IgA. The geometrical arrangement of the side-chains of the selected peptide pharmacophore was shown by circular dichroism, Co(II)-complex absorption and high-resolution NMR to be structurally invariant with respect to the parent zinc-finger.

Keywords: zinc-finger; synthetic peptide library; filamentous phage; affinity selection; NMR

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Much effort is being devoted to the search for a rational way to convert the information encoded in peptide structures into peptidomimetics (Farmer, 1980; Wiley & Rich, 1993; Olson *et al.*, 1994; Rizo & Gierasch, 1992; Hruby, 1993; Moore, 1994). The current paradigm for peptidomimetic design divides the process into three steps (Moore, 1994): (1) identification of the amino acid side-chains (pharmacophoric groups) that are responsible for agonist / antagonist activity; (2) determination of the spatial arrangement of the pharmacophoric groups (“pharmacophore”); (3) design of a template upon which these groups can be mounted in a way that retains the

spatial orientation of the parent peptide. While the development of combinatorial peptide libraries (Scott & Smith, 1990; Felici *et al.*, 1991; Houghten *et al.*, 1991; Lam *et al.*, 1991; Geysen & Mason, 1993; Gallop *et al.*, 1994) has greatly facilitated the accomplishment of the first step, the rate-limiting factor in the whole process is still step (2), i.e. the establishment of a conformational model for the pharmacophore (Moore, 1994).

To address this problem, we sought to exploit fully the power of selection methods by rationally designing structure-inducing templates for the selectable sequences; the ultimate goal being the

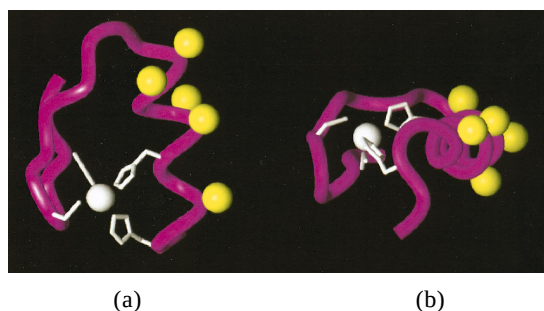


Figure 1. Schematic representation of the zinc-finger peptide library. (a) The selectable positions X^1 - X^2 - X^3 - X^4 - X^5 are shown as yellow spheres centered on the side-chain β -carbon; the zinc atom and the coordinating cysteine and histidine residues are in white. (b) View of the structure along the helical axis.

Peptide synthesis. 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and all the reagents for peptide synthesis, including the solid-phase resin (Polyhype SU-500) were purchased from Novabiochem (Läufelfingen, Switzerland), solvents were from Labscan (Anhydrosan series, Stillorgan, Ireland), other reagents were from Sigma Chemical (St Louis, MO) or Merck (Darmstadt, FRG); all were used as received, without further purification. The synthesis was performed on a Zinsser SMPS 350 synthesizer by Fmoc/t-Bu chemistry (Atherton & Sheppard, 1989) as described (Houghten *et al.*, 1991; Pinilla *et al.*, 1992; Wallace *et al.*, 1994). The complete set (positions X^2 - X^3 - X^4 - X^5) includes 26 amino acid residues; in the final version of the chemical library we used in the X^1 position only 14 residues (Ala, Arg, Asn, Asp, Gln, Glu, His, Lys, Met, Ser, Thr, Tyr, Val and Orn; see Results and Discussion). Overall, we synthesised and screened 118 pools, totalling 6.4×10^6 different peptides. For the incorporation of the X positions we used a mixture of Fmoc amino acids, whose relative concentration was adjusted to obtain close to equimolar incorporation ($\pm 25\%$): Ala (1), Arg (2), Asn (1), Asp (1), Gln (1), Glu (1), His (1), Ile (2), Leu (1), Lys (1), Met (1), Phe (1.4), Ser (1), Thr (2), Trp (1.4), Tyr (1.4), Val (2), Abu (1), Cha (1), Nle (1), Nva (1), Orn (1), Hph (1.4), Fcl (1.4), Fno (1.4), Phg (1.4). The individual peptides corresponding to the consensus derived from the screening were prepared by the same procedures. The purity was 70 to 85%, and >95% after HPLC purification. They all showed the expected mass spectra and amino acid ratios.

Phage library. The phage library was prepared by utilising a 2-gene system. The expression vector pIF4 (a derivative of pC89; Felici *et al.* (1991), containing the pel leader sequence fused to the sequence coding for the mature pVIII; F. Felici, unpublished results) was amplified by inverse PCR with the 2 oligonucleotides: OL59 (5'-GTACATAAGCTTTTACCGCATTCGGGCATTTGTA-GGGATCGTCACCCTCAGCAGCCATAG) and OL60 (5'-TGCATGAAGCTTCTCCAGAAANVMNNSCTGNNNSN-NSCACCAGNNSACCCACACCGGTGACGATCCCGCA-AAAGCGGCC); the resulting DNA fragments were recircularised by cutting with *Hind*III restriction endonuclease and subsequent ligation. A total number of 9.7×10^6 independent clones were selected on ampicillin-containing plates and the phage supernatant was prepared (in LB medium containing 0.1 mM $ZnCl_2$) by superinfection with M13K07 helper phage. The predicted sequence of the resulting zinc-finger-pVIII fusion protein is therefore: NH_2 -AEGDDPYKCEPGKGSFSQK(X^1 , X^2)L(X^3 , X^4)HQ(X^5)-THTGDDPAKAAFDLSQASATEYIGYAWAMVVVIVGAT-IGIKLFFKFTSKAS-COOH, where the zinc-finger peptide

synthesis of a panel of libraries of assembled pharmacophores, each one characterised by a defined structure, and collectively exploring a reasonably large shape space. Each ligand selected from these libraries would directly yield the information necessary to start the last step in the design of a peptide mimic; and systematic screening of the panel would therefore enable selection-driven peptidomimetic design.

We describe here the first example of such a conformationally homogeneous combinatorial peptide library, based on the α -helical geometry, and show that the ligands selected from this library have the expected pharmacophoric structure.

The design of the library took advantage of a natural protein domain, a strategy that has proven successful for the immunoglobulin fold (Gallop *et al.*, 1994; Pessi *et al.*, 1993; Martin *et al.*, 1994). We chose the Cys₂His₂ zinc-finger, one of the most common eukaryotic DNA-binding motifs (Pellegrino & Berg, 1991; Jacobs, 1992), with the residues responsible for specificity mainly located in the α -helical subdomain (Pavletich & Pabo, 1991). These positions are tolerant to sequence variability (Michael *et al.*, 1992), and their potential for a randomise-and-select approach has in fact already been exploited in attempting to find new DNA-binding specificities (Rebar & Pabo, 1994; Jamieson *et al.*, 1994). By moving our focus away from the biological function, we envisaged the possibility of using the zinc-finger scaffold to obtain an all-purpose conformationally homogeneous α -helical peptide library. Structural conservation in the presence of sequence alterations not involving the key conserved amino acids (see below) was suggested by NMR studies of several zinc-finger peptides revealing a common, well defined structure (Frankel *et al.*, 1987; Parraga *et al.*, 1988; Lee *et al.*, 1989; Omichinski *et al.*, 1990), and by preservation of folding in an analogue with multiple Ala substitutions (Michael *et al.*, 1992). A feature of utmost importance for our design is that in zinc-fingers metal coordination and folding are coupled: since the screening may be performed in the presence and absence of metal, the same sequence would be available with the expected pharmacophoric structure (metal-bound form) and with any alternative

sequence is preceded by the 6 amino-terminal residues and followed by residues 3 to 50 of the f1 major coat protein (P at the beginning and G at the end of the insert are common to both sequences). A phage clone displaying the CP1 sequence was constructed in the same way, utilizing the oligonucleotide OL58 (5'-TGCATGAAGCTTCTCCCA-GAAATCCGACCTGGTTAAACACCAGCGTACCCACACCGGTGACGATCCCGCAAAAGCGGCC) instead of OL60, and used as a negative control. The phage library was selected by biopanning, essentially as described (Scott & Smith, 1990; Felici *et al.*, 1991); IgAC5 concentrations of 1 μ M and 0.1 nM were utilised in the first and in the second cycle of selection respectively. Positive clones were identified by plaque assay (Luzzago *et al.*, 1993) and their reactivity was quantitatively determined through ELISA of phage supernatants (Dente *et al.*, 1994).

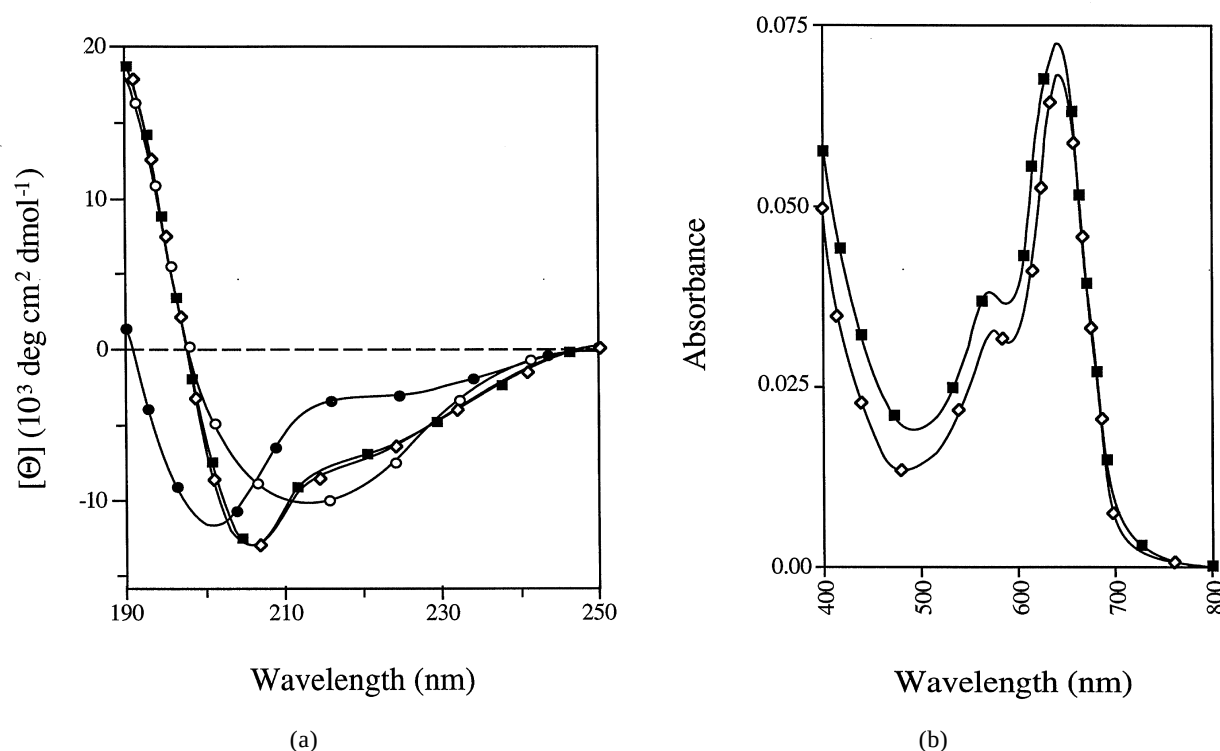


Figure 2. Comparative Circular Dichroism (CD) and Co(II)-complex spectra of CP-1 and the peptide library. (a) CD spectra of CP-1 in the presence (■) and absence (●) of 2 equivalents of ZnCl_2 , the zinc-bound Phe- X^1 - X^2 - X^3 - X^4 - X^5 pool in the chemically synthesized library (○) and the zinc-bound X^1 - X^2 - X^3 - X^4 - X^5 library (◇). The spectra were recorded at 21°C on a Jasco J-710 spectropolarimeter as described (Kim & Berg, 1993); we used a 16 s time constant and a 2 nm/min scan speed, a 0.1 cm cell and a peptide/peptide pool concentration between 50 and 70 μM (buffer: 10 mM Tris-HCl (pH 7.5), ± 2 equivalents ZnCl_2). The X^1 - X^2 - X^3 - X^4 - X^5 library, with all the positions randomised, was synthesised as described in the legend to Figure 1. (b) Co(II)-peptide complex absorption spectra of CP-1 (■) and the X^1 - X^2 - X^3 - X^4 - X^5 library (◇). The spectra were obtained as described (Krizek *et al.*, 1991), using a peptide/peptide mixture concentration of 300 μM (buffer: 100 mM Tris-HCl, 50 mM NaCl (pH 7.5), +1.5 equivalents CoCl_2).

topography (unfolded, metal-free form); in our library, therefore, metal-dependent binding would represent a built-in control against the selection of structurally undefined sequences. Moreover, since both the CD (Kim & Berg, 1993) and the adsorption spectra of peptide-Co(II) complexes (Krizek *et al.*, 1991) are highly characteristic of this fold, we would have an easy means of characterising even a large panel of selected variants.

The library is depicted in Figure 1. The parent sequence chosen is a previously described consensus Cys_2His_2 peptide (CP1; Kim & Berg, 1993; Krizek *et al.*, 1991) with the sequence Pro-Tyr-Lys-Cys-Pro-Glu-Cys-Gly-Lys-Ser-Phe-Ser-Gln-Lys-**Ser-Asp-Leu-Val-Lys**-His-Gln-**Arg**-Thr-His-Thr-Gly (bold-face: residues randomised in the library). The structure is organised around a zinc ion (white) tetrahedrally coordinated by two cysteine residues in the β -sheet and two histidine residues in the α -helix (white), and further stabilised by the hydrophobic packing of three conserved residues in positions 2, 11 and 17; in CP1 the conserved hydrophobics are Tyr2, Phe11 and Leu17. The randomised positions, residues 15, 16, 18, 19 and 22, are defined as X^1 - X^2 - X^3 - X^4 - X^5 , respectively; these residues are the most exposed in the helix

(accessibility 25 to 60%) and cluster on one side of the structure, fully available for interaction with a receptor; the distance between the C^α atoms of X^1 and X^5 is about 12 Å.

The α -helical library was produced both as fusion with the pVIII protein of filamentous phage and as soluble peptides by chemical synthesis. For the chemical library, we used the Positional Scanning format (Pinilla *et al.*, 1992; Wallace *et al.*, 1994), whereby five different sublibraries are prepared, in the form O^1 - X^2 - X^3 - X^4 - X^5 , X^1 - O^2 - X^3 - X^4 - X^5 , X^1 - X^2 - O^3 - X^4 - X^5 , X^1 - X^2 - X^3 - O^4 - X^5 and X^1 - X^2 - X^3 - X^4 - O^5 , where O is a known and X is an unknown amino acid residue. In each randomised position we included all the natural amino acid residues (except Pro, Gly and Cys) and a panel of uncoded residues: 2-aminobutyric acid (Abu), cyclohexylalanine (Cha), norleucine (Nle), norvaline (Nva), ornithine (Orn), homophenylalanine (Hph), 4-chlorophenylalanine (Fcl), 4-nitrophenylalanine (Fno) and phenylglycine (Phg). This enabled us to expand the selectable repertoire and also to probe the tolerance of this naturally evolved structure towards insertion of uncoded amino acid residues.

The availability of soluble mixtures allowed a preliminary screening of structural alterations by

CD and Co(II)-binding experiments on the library pools. While for positions 2 to 5 (residues 16, 18, 19 and 22 in the sequence) no effect was observed for the non-natural substitutions also, a major alteration of both CD and Co(II)-complex spectra was apparent in the majority of pools having hydrophobic residues in position X¹ (residue 15), as shown in Figure 2(a) for the phenylalanine pool. We therefore synthesised a second (and final) version of the library where Phe, Trp, Ile, Leu, Abu, Cha, Nle, Nva, Fcl, Fno, Phg and Hph were not included in this position (X¹), while being present in X²-X³-X⁴-X⁵; the resulting library X¹-X²-X³-X⁴-X⁵ showed unperturbed spectra for all the peptide pools. In the phage library, hydrophobic residues were excluded from the first variant position by the use of the codon NVM in OL60 (G/A/T/C-A/C/G-A/C), which eliminates the residues Phe, Leu, Ile, Met, Val and Trp. The CD and Co(II)-complex spectra of the X¹-X²-X³-X⁴-X⁵ mixture (all positions randomised) are shown in Figure 2(a) and (b), in comparison with the spectra of the parent peptide CP-1: it is apparent that, within the limits of this collective analysis, the structure is preserved in the zinc-finger library. Further details about the structural screening of the library will be reported elsewhere.

The two libraries were independently screened with the same receptor, a monoclonal IgA reactive against the lipopolysaccharide of the human pathogen *Shigella flexneri* (IgAC5; A. Phalipon, unpublished results). This is the first example of the simultaneous use of the two methods, whose relative merits have been discussed (Birnbach & Mosbach, 1992; Gallop *et al.*, 1994). The two selections yielded a very similar consensus: X¹ = His, X² = Phe, X³ = Val/Ser, X⁴ = Gln and X⁵ = His/Arg/Gly for the phage library and X¹ = His, X² = Phe/Fcl/Leu, X³ = Ala/Val/His, X⁴ = Gln/Ser/Ala and X⁵ = His/Arg/Asn/Lys for the synthetic library. In particular, the binders defined by both methods would be X¹ = His, X² = Phe, X³ = Val, X⁴ = Gln and X⁵ = His/Arg (peptides H¹F²V³Q⁴H⁵ and H¹F²V³Q⁴R⁵).

Single peptides corresponding to a subset of the possible sequences defined by the two selections were synthesised and tested by ELISA (Figure 3). The two common peptides show a very strong, zinc-dependent signal, with H¹F²V³Q⁴H⁵ being the best binder of the whole series. Interestingly, Phe-to-Fcl substitution in position X² and a Val-to-Ser substitution in position X³ are well tolerated. On the other hand, a few sequences derived from the screening of each single library (e.g. H¹F²S³Q⁴R⁵ and H¹F²V³Q⁴N⁵) show weaker zinc-dependent binding, which is sometimes accompanied by an increase in the zinc-independent signal. While the former may be simply explained by the substitution of a critical residue (e.g. H⁵), the latter might derive from an increased intrinsic propensity of the corresponding sequence to fold into an α -helix even in the absence of metal. The data illustrate the advantage of simultaneous selection, which enables cross-validation of the consensus

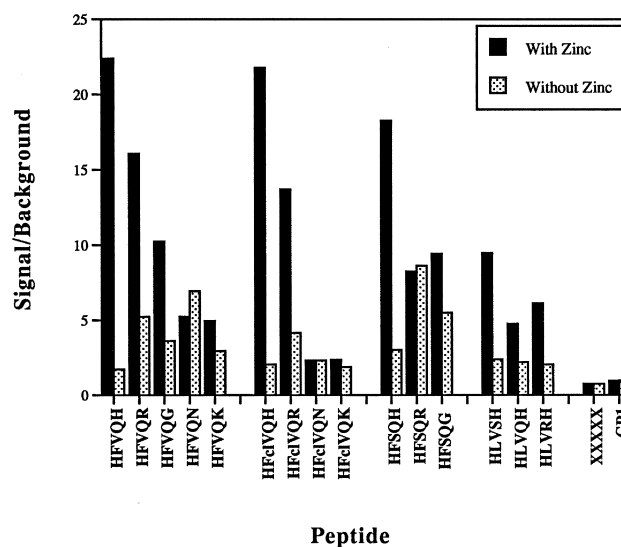


Figure 3. Binding activity of the peptides selected from the phage and synthetic zinc-finger library with IgAC5. Binding activity is defined as the ratio of sample absorbance to background absorbance; the results shown are the mean of duplicate experiments. Controls include CP1 and the X¹-X²-X³-X⁴-X⁵ peptide mixture with all the positions randomised (indicated on the graph as XXXXX). Binding to the IgAC5 was measured by ELISA as follows: Peptide (80 μ M; in either the absence or the presence of 160 μ M ZnCl₂) and IgAC5 (0.43 μ g/ml) were incubated in Tris-buffered saline (TBS) for 1 h at room temperature. The resulting peptide-antibody complexes were detected by adding 100 μ l of the above solution to polystyrene 96-well microplates (Nunc-Immunoplate Maxisorp), which had been coated overnight with 100 μ l of a 1:20,000 dilution of rabbit anti-CP1 antiserum in TBS and blocked with 0.8% BSA in TBS, then incubating for 1 h at room temperature. After washing, bound peptide-antibody complexes were detected by sequential incubation with alkaline phosphatase-conjugated goat anti-mouse IgA α -chain (Sigma; 1:5000 dilution in TBS + 0.8% BSA, 100 μ l/well) and *p*-nitrophenyl phosphate (Sigma 104 tablets; 1 mg/ml in 10% diethanolamine containing 0.5 mM MgCl₂, pH 9.8, 100 μ l/well) at 20°C. Colour development was quantified by measuring the absorbance at 405 nm on a LabSystems MultiSkan microplate reader. The anti-CP1 antiserum was produced by immunization with the synthetic peptide. The synthesis and selection of the synthetic library were performed as for the single peptides (final concentration of the peptide pools in ELISA was 80 μ M); the consensus was derived from the pools giving both high signal in the presence of zinc and low signal in the absence of metal. The ratio between the 2 signals represents a built-in control against the selection of unstructured sequences.

sequences derived from each screening: this effectively reduces the incidence of possible artifacts associated with each method and, more specifically for the chemical library, may help to resolve some ambiguities inherent in the positional scanning format (Pinilla *et al.*, 1992; Wallace *et al.*, 1994). We view this feature, which is at present available for peptide molecules only, as a distinct advantage over other types of chemical libraries (Gordon *et al.*, 1994).

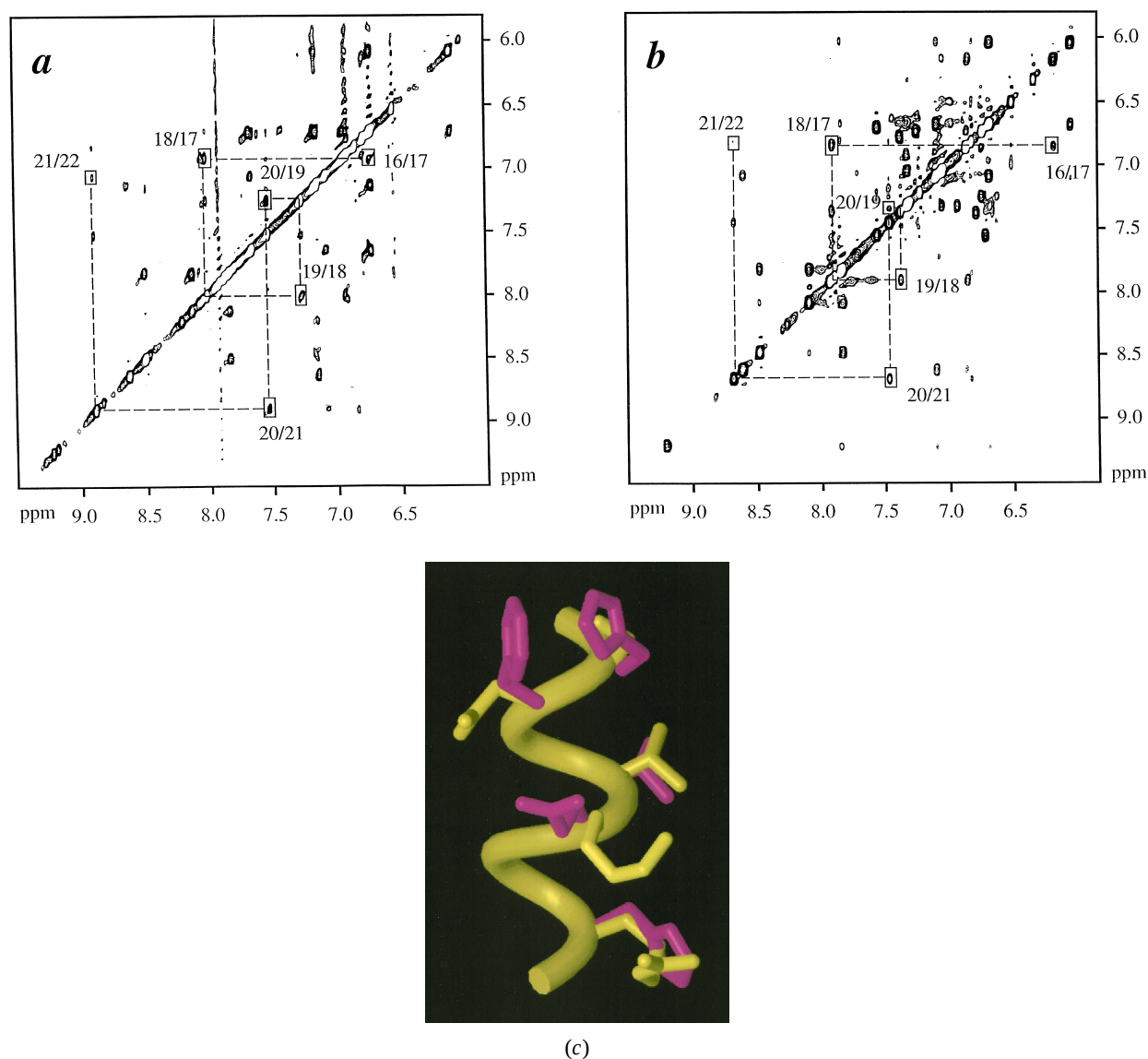


Figure 4. Comparative NMR analysis of the parent (CP1) and selected ($\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$) zinc fingers. *a* and *b*, Selected amide region of the NOESY experiment for CP1 (*a*) and $\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$ (*b*). The pattern of NOEs from residue 16 to 22 is highlighted. *c*, Structure conservation among CP1 and the selected $\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$ zinc-finger is shown for the critical helical region. The ribbon (yellow) represents the α -helix of CP1; the side-chains of the selected residues of $\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$ (His15, Phe16, Val18, Gln19 and His22, magenta) and the corresponding positions in CP1 (Ser15, Asp16, Val18, Lys19 and Arg 22, yellow) are shown after best superimposition of their C^α atoms.

NMR analysis was performed on a Bruker AMX 500. The temperature was kept at 300 K and pH was 6.6 (CP1) and 6.3 (selected $\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$ zinc-finger). The assignment was carried out with the combined use of a COSY, TOCSY (60 ms mixing time) and NOESY (150 and 350 ms mixing time) in H_2O and $^2\text{H}_2\text{O}$. The experiments were performed with a jump-and-return optimised sequence for water suppression. Peptide concentration was 2 mM, in the presence of 2.4 mM ZnCl_2 . The picture showing the comparison between the parent and selected zinc finger (Figure 3) was generated with the program INSIGHT^{tmk}, by using averaged structures obtained by application of the program DIANA^{tmk} to the experimental NMR constraints. Further structural details will be published elsewhere.

All the selected peptides, independently of their relative affinity for the mAb, gave CD and Co(II)-complex spectra indistinguishable from those of CP1 (not shown). To confirm further that the ligands selected from the library are structurally conserved with respect to the parent zinc-finger, we analysed the $\text{H}^1\text{F}^2\text{V}^3\text{Q}^4\text{H}^5$ peptide, together with CP1, by NMR. The spectra gave very similar results and

it was possible to locate the β -strands between residues 2 to 4 and 9 to 11 and the α -helical region between residues 16 and 22. Both molecules show the expected interstrand contacts among αH – αH and HN – αH , which localise the strands between residues 2 to 4 and 9 to 11, and the $^3J_{\text{HN}-\alpha\text{H}}$ coupling constants measured in those regions are >9.0 Hz. The α -helices are clearly characterised by the same

pattern of NOE contacts and the same values for the homonuclear coupling constants. The region 16 to 22, which includes the variant residues, exhibits $^3J_{\text{HN}-\alpha\text{H}}$ coupling constants ≤ 4.6 Hz, typical of a helical conformation. Moreover, the sequential NOE pattern HN–HN (Figure 4a,b) and βH –HN, and the $i - i + 3$ αH –HN and αH – βH can be followed all along these stretches. Conservation is also observed for the packing of the secondary structure elements, and more specifically for Tyr2, Cys4, Cys7, Phe11, Leu17 and His20; these residues, according to the model, form the core of the structure. Figure 4c shows the deduced comparison between the parent structure and the selected pharmacophore.

Taken together, our data are consistent with the notion that the zinc-finger α -helical library is conformationally homogeneous, and that selection from this library will yield not only the sequence but also the overall geometry of the desired ligand. Although this cannot include detailed information on the side-chain conformations in the bound state, which is only derived by structural analysis of the ligand–receptor complex, as far as the peptide backbone is concerned the information is fairly complete. Various examples demonstrate how knowledge of the ligand geometry may enable the direct design of a suitable non-peptidic scaffold with retention of the peptidic side-chains (Wiley & Rich, 1993; Olson *et al.*, 1994; Moore, 1994; Hirschmann *et al.*, 1992a,b; Nolan *et al.*, 1992; McDowell *et al.*, 1994).

The availability of a panel of libraries with predetermined ligand geometry could ultimately lead to selection-driven design of peptidomimetics, a process whereby a first-generation peptide pharmacophore is rapidly derived from screening. This information would then be either directly converted into a first-generation peptidomimetic, or probed and refined by the synthesis of secondary libraries spanning a narrower shape space. Overall, this iterative process would be analogous to traditional drug design, but applied to populations of molecules instead of individuals (Gordon *et al.*, 1994). Since one might expect selection from libraries with a reduced accessible shape space to be intrinsically more difficult than from libraries of linear, conformationally flexible peptides, the successful selection of an IgA ligand that is described here augurs well for this strategy. In all cases where no direct structural information may be used as a starting point for a drug discovery project, the availability of this novel type of library may turn out to be an important advantage.

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