A natural motif approach to protein design: a synthetic leucine zipper peptide mimics the biological function of the platelet factor 4 protein

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Abstract The design of smaller functional mimics of large proteins has long been an important challenge. In this study we use the natural leucine zipper as a structural template to design a 31-residue peptide analog that mimics the function of the larger platelet factor 4 (PF4) protein. The heparin binding activity of PF4 has been introduced into an unrelated leucine zipper sequence only by virtue of incorporating four lysines of PF4. Circular dichroism and binding experiments have shown that the designed leucine zipper peptide adopts a stable helical conformation and shows significant PF4-like heparin binding activity. These results strongly suggest that the lysine residues play an important role in the binding of PF4 to heparin. The de novo generation of the PF4 function in a designed leucine zipper peptide demonstrates that the leucine zipper motif is a useful scaffold for the design of functional peptides and proteins.

Key words: Protein design; Leucine zipper; Platelet factor 4; Heparin binding; Circular dichroism

1. Introduction

The de novo design of peptides and proteins with desired structure and biological function has been an elusive goal. Following the general concept that the amino acid sequence of a protein determines the protein's three-dimensional structure, and that the structure confers the biological function [1], many studies have attempted to understand the path from sequence to structure and function. Extensive progress has been made in elucidating the path from sequence to structure with the studies of de novo designed peptides and proteins [2–5]. With the advent of these designed structural proteins, attempts have also been made to introduce biological functions into these de novo sequences [6–8]. However, since the de novo proteins designed from scratch often fold into the stage of the 'molten globule' which lacks a unique tertiary structure, the functional proteins designed based on the 'molten globule' stage of the de novo sequences are generally much less active than their native counterparts.

In view of the current limitation in the ability to design native-like structures from first principles, an alternative strategy is the natural motif approach in which active or binding sites of a target protein are grafted onto unrelated, pre-existing sequence templates to generate novel biological functions. By exploiting the structural motifs already designed by nature, one skips the uncertain step of going from sequence to structure and thus avoids the problem of the 'molten globule' common to the structures designed from scratch. The use of stable natural motifs with well-defined tertiary structures may offer similar or enhanced biological activity and stability relative to native proteins. One potential application of this natural motif approach is in the design of a smaller peptide that could mimic and dissert the function of a larger protein, an emerging field that has recently attracted extensive attention [9–11]. The use of an unrelated motif, which has little sequence homology to the target protein that it is designed to mimic, provides a simplified system to dissert complex biological functions and test hypothetical models of a particular structure–function relationship. In addition, the natural motifs used in our study are generally small in size (20–60 amino acids) and are amenable to synthetic chemistry. Therefore, they may be valuable intermediate targets for the subsequent development of small nonpeptide mimics.

As a test of the small natural motif approach to functional protein design, we have chosen the heparin binding activity of platelet factor 4 (PF4) as a target function. PF4 is a 70-amino-acid protein that belongs to the family of chemokines [12,13]. In addition to the specific binding to heparin, PF4 has been implicated in a number of biological activities including reversal of heparin anticoagulation [14], reversal of immunosuppression [15], inhibition of angiogenesis [16] and suppression of myeloid progenitor cell proliferation [17]. The structure of PF4 is known and many studies have been carried out to elucidate the residues important for heparin binding. It has been suggested that two pairs of lysine residues in the C-terminal helix of PF4 are important for binding heparin [12].

The natural structural motif we have chosen to design the heparin binding activity of PF4 is the GCN4 leucine zipper. The leucine zipper motif, first described by Landschulz et al. [18], has been shown to consist of a stable dimer of amphiphilic helices that form a coiled-coil [19,20]. Peptide fragments from the GCN4 leucine zipper form a remarkably stable and precise conformation in aqueous solution that has been determined by NMR and crystallography [21,22]. Comparison of the crystal structures for PF4 [23] and GCN4 leucine zipper peptide showed a close similarity in the orientations of the helices (Fig. 1A). This prompted us to hypothesize that the incorporation of the proposed required lysines of PF4 into the leucine zipper motif (which has unrelated function and sequence) may generate a new surface for heparin binding. To test this, a peptide was designed ($PF4_{zip}$) consisting of the sequence for the GCN4 leucine zipper with four lysine residues substituted in positions that mimic the positions of the four required lysines in human PF4 without disrupting the residues involved in forming the leucine zipper (Fig. 1B). In

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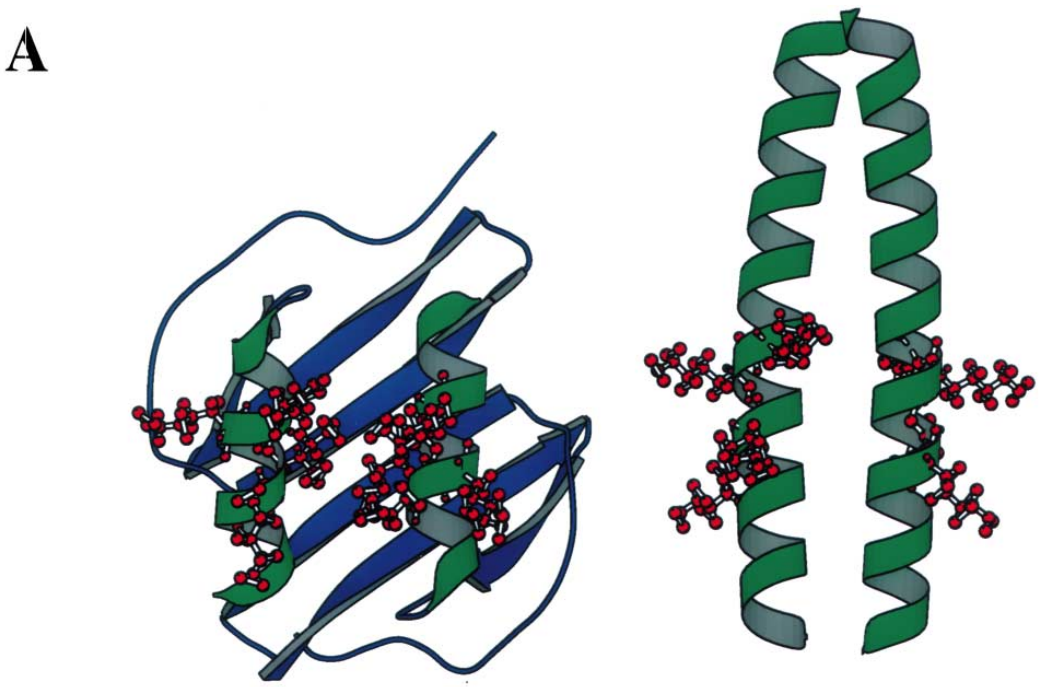
addition, a peptide with the native sequence for the GCN4 leucine zipper ($GCN4_{zip}$) was also synthesized as a control.

2. Materials and methods

2.1. Peptide synthesis

Both $GCN4_{zip}$ and $PF4_{zip}$ were prepared by solid phase peptide synthesis with Fmoc-strategy using a PerSeptive Biosystems 9050 peptide synthesizer (PerSeptive Biosystems, Milford, MA) and Fmoc-Gly-PEG-PS resin (PerSeptive Biosystems, loading = 0.16 mEq/g). The side-chain-protecting groups of Nα-Fmoc amino acids were: Arg, Pmc; Asn, Trt; Asp, OtBu; Cys, Trt; Gln, Trt; Glu, OtBu; His, Trt; Lys, Boc; Ser, tBu and Tyr, tBu (Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, Trt = trityl, OtBu = *tert*-butyl ester, Boc = *tert*-butyloxycarbonyl and tBu = *tert*-butyl ether). The Nα-Fmoc amino acids (4 equiv.) were sequentially added as a preactivated solution of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIPEA) to the resin (660 mg, 0.25 mmol). *N*-Methyl-2-pyrrolidinone (NMP) was used as solvent and 20–50% piperidine in NMP was used to remove the protected Fmoc groups. Upon completion of all synthetic cycles, resin



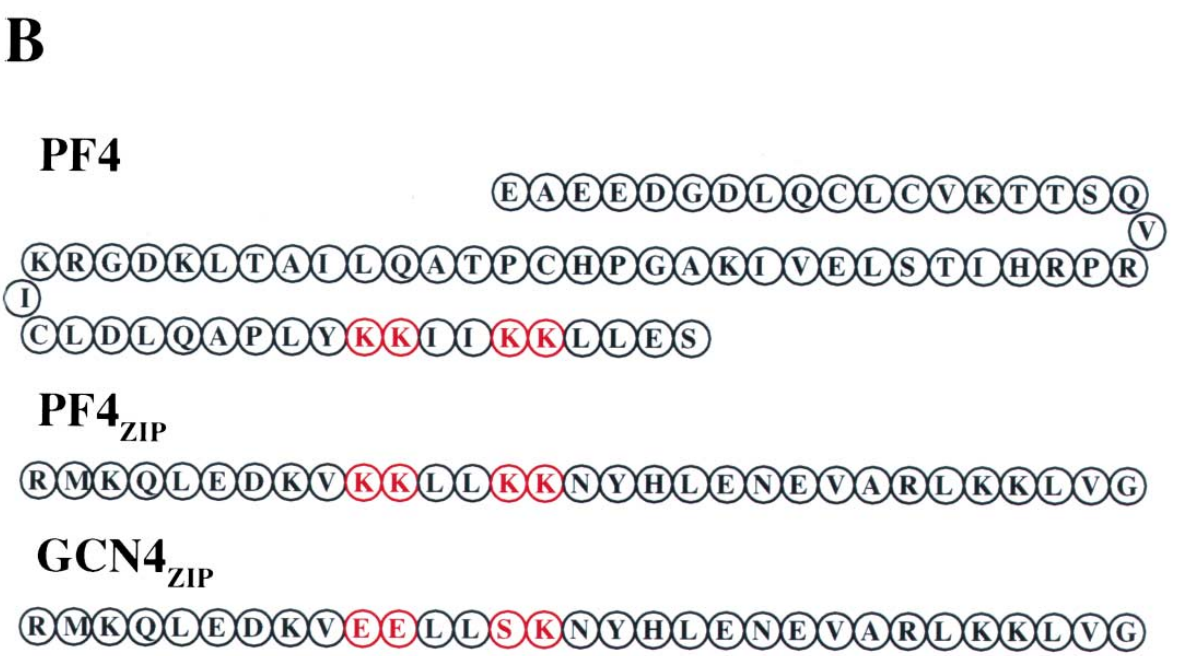


Fig. 1. A: Comparison of the crystal structure of PF4 dimer with the crystal structure of the GCN4 leucine zipper dimer modified to include the lysines proposed to be essential for heparin binding. For clarity, a dimer instead of the actual tetramer of PF4 seen in the crystal structure is shown. The lysine side chains important for heparin binding are highlighted in a ball and stick model. Structures were displayed using the Insight II software package (Biosym, Inc. San Diego, CA). B: Sequences of the designed leucine zipper peptide ($PF4_{zip}$) as compared to the native PF4 protein and the control leucine zipper peptide ($GCN4_{zip}$). Residues altered in $PF4_{zip}$ are shown in red.

was removed from the reaction vessel and treated with reagent K (trifluoroacetic acid (TFA): phenol: thioanisole: ethandithiol: H_2O/10: 0.75: 0.5: 0.25: 0.5) for 2 h at room temperature with gentle stirring. The mixture was then filtered directly into ice-cold ethyl ether. The resulting suspension was transferred into a centrifuge tube and centrifuged for 10 min. at 2000×*g* at room temperature. The supernatant was discarded and the precipitate was resuspended in ethyl ether, and again centrifuged for 5 min. The procedure was repeated twice before the precipitate was dissolved in 5% acetic acid/water and lyophilized. The crude peptide was purified by preparative HPLC using a Dynamax-300Å C18 column (241×22 mm ID, 15 μm spherical packing at a flow rate of 9 ml/min), with UV detection (220 nm). Two HPLC solvents—solvent A (d.i. H_2O/0.1% TFA) and B (acetonitrile/0.1% TFA)—were used in a programmed gradient of 5–25% B over 40 min. (0.5%/min.). The fractions containing the peptide were pooled together and lyophilized.

2.2. Heparin binding assays

Following the procedure of Rucinski et al. [24] ^{125}I-labeled peptides were bound to a heparin–sepharose column in 0.02 M Tris-HCl buffer pH 7.0 containing 0.05 M NaCl. The column was extensively washed with the binding buffer and bound peptide was eluted with a 0.1–2 M NaCl gradient.

2.3. CD spectroscopy

All spectra were recorded using a Jasco J710 circular dichroism spectrometer with a 0.01 cm pathlength quartz cuvette. 40–60 μM peptide solutions were prepared in 3 mM sodium citrate, 3 mM sodium phosphate, and 3 mM sodium borate buffer at pH 6.11. Peptide concentrations were determined by tyrosine absorbance in 6 M guanidine hydrochloride assuming an extinction coefficient at 276 nm of 1500 M^{-1} cm^{-1} [25]. Three scans were acquired and accumulated from 189 to 250 nm, at 0.1 nm resolution. Thermal stability of the peptides were determined by monitoring the change in $[\Theta]_{222}$ as a function of temperature. The temperature was increased at an interval of 2°C with an equilibration time of 2 min. The temperature dependence (T_m) was determined from the minima of the first derivative of $[\Theta]_{222}$ with T^{-1}, where T is in K [26].

3. Results

The heparin binding activity of the designed leucine zipper peptide $PF4_{zip}$ was measured by retention on a heparin–agarose column in the presence of increasing NaCl concentrations.

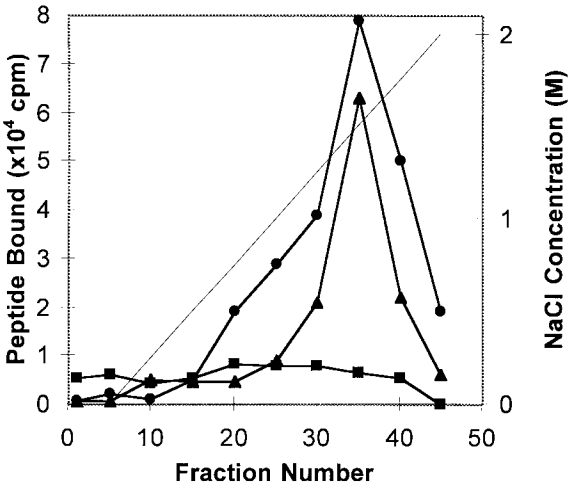


Fig. 2. Heparin binding of the designed leucine zipper peptide ($PF4_{zip}$) (●) as compared to the control leucine zipper peptide ($GCN4_{zip}$) (■) as well as native PF4 (▲). For the assay, ^{125}I-labeled peptides were bound to a heparin–sepharose column in 0.02 M Tris-HCl buffer pH 7.0 containing 0.05 M NaCl. The column was extensively washed with the binding buffer and bound peptide was eluted with a 0.1–2 M NaCl gradient.

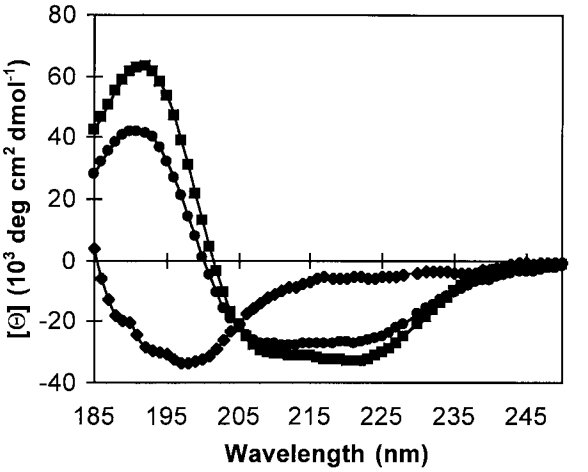


Fig. 3. CD Spectra of $PF4_{zip}$ (●) as compared to $GCN4_{zip}$ (■) and $PF4_{58-70}$ (♦). These spectra were recorded using a Jasco J710 circular dichroism spectrometer with a 0.01 cm pathlength quartz cuvette. 40–60 μM peptide solutions were prepared in 3 mM sodium citrate, 3 mM sodium phosphate, and 3 mM sodium borate buffer at pH 6.11. Peptide concentrations were determined by tyrosine absorbance in 6 M guanidine hydrochloride assuming an extinction coefficient at 276 nm of 1500 M^{-1} cm^{-1}. Three scans were acquired and accumulated from 189–250 nm, at 0.1 nm resolution. As shown, the CD intensity of −33 000 deg cm^2 $dmol^{-1}$ at 222 nm for $GCN4_{zip}$ suggests that the peptide is essentially 100% α-helical [34,35], which is identical to the leucine zipper studied by O'Shea et al. [19]. The CD intensity of −27 000 deg cm^2 $dmol^{-1}$ at 222 nm for $PF4_{zip}$, suggests that the peptide is 80% α-helical. The CD spectrum of the peptide containing the C-terminal helix of PF4 ($PF4_{58-70}$) suggests that the peptide is random coil.

Fig. 2 shows that radiolabeled $PF4_{zip}$ binds with high affinity to heparin-sepharose since it is eluted at about 1.4 M NaCl. Native PF4 also elutes at about 1.4 M NaCl. These data suggest that the designed leucine zipper peptide strongly binds heparin like the native PF4 protein. In contrast, no heparin binding activity is observed in the control native leucine zipper peptide $GCN4_{zip}$ which contains an identical amino acid sequence except for the three modified lysine residues (Fig. 2). These results demonstrate that the remarkable heparin binding activity of the designed leucine zipper peptide is specifically due to the introduction of the lysine residues. Further in vitro functional assay studies have shown that the designed leucine zipper peptide also reverses heparin anticoagulation, as determined by the activated partial thromboplastin time (APTT), while the control leucine zipper peptide does not have such activity (unpublished results).

In order to determine the structural basis for the observed heparin binding activity of the designed leucine zipper peptide, we have carried out circular dichroism (CD) experiments to investigate the solution conformation of this peptide. The CD spectrum of this peptide is shown in Fig. 3. The double minima at 208 and 222 nm and the maximum at 195 nm indicate that the peptide adopts a highly helical conformation in aqueous solution. In addition, the peptide displays a two-state melting profile which is typical for a fully folded native protein (unpublished results). The T_m for $GCN4_{zip}$ is 55°C and 42°C for $PF4_{zip}$. The result for $GCN4_{zip}$ correlates with the observations of O'Shea et al. [19]. These results demonstrate that the designed leucine zipper peptide maintains a native-like helical conformation of the leucine zipper template. To further investigate the importance of this helical confor-

mation for heparin binding activity, we studied the solution conformation of a peptide comprising the C-terminal helix of PF4 ($PF4_{58-70}$). Although this peptide contains the binding domain of PF4 with the four lysines proposed as heparin binding sites, the peptide has very low binding affinity for heparin, eluting at 0.2 M NaCl and is able to reverse heparin anticoagulation only about 20% at relatively higher concentrations. CD spectrum of the peptide shows that it displays a random coil conformation. This explains the loss of binding activity of the peptide and confirms the importance of the helical conformation as a framework for presenting the heparin binding lysine side chains.

In addition to the heparin binding activity, we tested the designed leucine zipper peptide for other PF4-related biological functions in vitro. The designed leucine zipper peptide was shown to have no effect on neutrophil activation (unpublished results). It is known that, while native PF4 does not activate neutrophils, N-terminally modified PF4 containing ELR sequence [27,28] and C-terminal PF4 peptides $PF4_{47-70}$ and $PF4_{58-70}$ do react with IL-8 receptors and activate neutrophils [28]. The lack of ability to activate neutrophils in the designed leucine zipper peptide suggests that residues other than heparin binding lysines may be responsible for neutrophil activation. Taken together, these results support our design concept that, by using unrelated sequences as peptide design templates, the specific targeted function can be maximized while other undesired biological properties can be minimized. Therefore, this approach may have important implications in the development of potential therapeutic agents with specific bioactivity and fewer unwanted side effects.

4. Discussion

In summary we have demonstrated that a structure-based designed synthetic leucine zipper peptide incorporating hypothetical heparin binding sites of four lysine residues displays remarkable biological activity of the native PF4 protein. This result strongly suggests that four lysine residues positioned properly in a helical conformation are essential for heparin binding. The designed leucine zipper peptide may also have important implications for therapeutic development. For example, efficient reversal of heparin anticoagulation is essential for a successful completion of open heart surgery in patients with cardiopulmonary bypass. Routinely, protamine sulfate is being used for this purpose, but this may result in a number of complications [29,30]. Recombinant PF4 appears to be a safer and more efficient drug as shown in a study in rats and baboons. In this regard, the small size and potent activity of the designed leucine zipper peptide makes it an ideal target for using synthetic chemistry to further develop nonpeptide mimetics as potential therapeutic agents.

The small natural motif approach may have general implications on the dissection of protein structure–function and development of small molecular mimics of functional epitopes in a large protein. Smaller peptide fragments containing the functional domains of a large protein are useful models to study the folding and function of the protein. However, when these peptides are synthesized or expressed in isolation from the rest of the protein structure, they often lose their structural integrity and biological activity, as in the case of the PF4 C-terminal peptide ($PF4_{58-70}$). In this study, we have shown that the heparin binding activity of the 70-residue PF4 protein is reproduced with a 31-residue leucine zipper analog. The de novo generation of PF4 function in a much smaller leucine zipper peptide provides a striking example for the utility of the natural motif approach to mimic unrelated large proteins. Recently a systematic approach of using a phage display technique to minimize protein functional domains has been reported [31]. While this strategy requires extensive stepwise random mutagenesis of a protein domain in order to restore its structural integrity and consequently biological activity, the natural motif-based design, as demonstrated in this work can be readily accomplished through a direct incorporation of the active sites of a protein into a preexisting stable structural template, and thus may present an alternative approach to dissect and minimize functional domains of a protein.

Finally the results of this study demonstrate the feasibility of our method in utilizing leucine zipper motifs as templates to design novel biological properties. The designed leucine zipper peptide adopts a stable conformation and possesses significant PF4-like heparin binding activity. Recently others have also reported the use of different natural scaffolds of scorpion toxins for protein engineering [32]. Taken together, these studies suggest that the diverse structural motifs that nature has provided us can be exploited to design new functional peptides and proteins. In addition to protein design, we have also used the leucine zipper as a template system to study the roles of mainchains and sidechains in the stabilization of protein structures [33]. It can be expected that the natural motif approach will continue to be an effective strategy in the studies of protein folding and design.

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