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A double catgrip mixed L and D mini protein only 20 residues long

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Abstract—Stereochemistry limits but also defines proteins, as conformational constructs stereospecific for poly-L structure. Employed as a variable in sequence, stereochemistry could make proteins customizable in the letters of L and D amino acid alphabet. In proof of concept, we previously demonstrated stereochemical reengineering of canonical β-hairpins as bracelet and boat shaped molecules. Illustrating the prospect for functional design, a 20-residue four-stranded mini-β protein is now customized stereochemically as a canoe shaped molecule. A conformational construct of four side by side hydrogen-bonded strands in alternately $^{L}\beta$, $^{D}\beta$ conformation, joined via Type-II/II′ β-turns, is planned to be preponderantly apolar in β-sheet favoring residues, interspersing two ion pairs, and suitably L and D in sequence. Synthesis followed by MD, NMR, CD, and MALDI-MS studies established the molecule as a canoe shaped fold in water, demonstrable in affinity of alkali and alkaline-earth metal ions as expected given its catgrip like elements. Another success in accomplishing a synthetic miniprotein complex in stereochemistry and stereospecific in conformation, exceptionally small yet functional in metal ion affinity, affirms the value in combined L and D alphabet in programming molecular shapes and functions stereochemically.

1. Introduction

Molecular design is biologically inspired.[1] While much progress has been made in pursuing this inspiration, the gulf between what is currently feasible in stepwise synthesis and what is on promise along protein-folding approach remains large. Foldamer approach is an active interest as well, but is in infancy.[2] In targeting molecules small yet customizable in shape and function, polypeptides are perceptibly disadvantaged; unwieldy in size, unstable in conformation, restricted in fold topology, and therefore conservative in molecular morphology. Indeed, α-helix and β-sheet are the only recognized building blocks limiting the scope of possibilities in protein tertiary structure.[3] Arguably the limit is a biological imperative. The access in ϕ, ψs to largely α_R and β Ramachandran basins,[4] and in regular secondary structure to only α-helix and β-sheet motifs[5] is an imposition of poly-L structure. Stereochemistry limits but also defines protein structure, being in strict control of conformation. The implication is that used as a variable of sequence, stereochemistry could allow engineering of specific shapes and functions in polypeptide structure with the alphabet of combined L, and D residues. Proteins feature structural elements, critical for folding or function, that are with ϕ, ψs in right half of Ramachandran diagram, that is, sterically favored for a D amino acid. Lacking D amino acids, proteins make do, in forming such structures, with either L-amino acid, even though sterically less favored, or glycine, even though sterically flexible and conformationally ill defined. The substitution of a D amino acid in these elements can be energetically advantageous; hence, there has been active interest in field of de novo design in substitution possibilities for a D amino acid, stabilizing native-like folds and nucleating them stereochemically. The approach has a long list of successes, with examples like K^+-selectivity filter,[6] diverse β-turns,[7,8] and higher order folds like β-hairpin,[8–10] even α,β-miniproteins.[11]

We have been pursuing de novo design exploring combined alphabet of L and D amino acids for possible molecules stereochemically programmed in sequence. Allowing L and D option freely in every sequence position will give 2^{30} or $\sim 10^9$ isomeric possibilities in even a 30-residue polypeptide. Given stereospecific choice in ϕ, ψs, a universe of sequentially definable folds, stereospecific for chosen L, D sequences, can be envisaged.

Keywords: De novo protein design; Peptide design; Stereochemical programming; β-Hairpin; Catgrip.
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Illustrating the notion that indeed mixed L and D alphabet will support molecular folds not encountered in nature,[9,12] we reprogrammed canonical β-hairpin as bracelet[13] and boat[14] shaped molecules. The designs were accomplished by mutating pairs of cross-strand neighbor L residues in canonical β-hairpins to D structure, creating specific morphological plans stereochemically. Extending the approach, we now describe similar modification of a canonical four-stranded mini β-sheet protein as a molecule in canoe shaped morphology. Planning all four extended β-strands to be alternately L and D , we aim for a molecule in overall curved morphology, defining catgrips[15] in its edge strands. A 20-residue mixed-L, D peptide is shown to display the specified conformation, and affinity for alkali and alkaline-earth metal ions as expected for its catgrips, describing a synthetic double-catgrip miniprotein exceptionally small and simple.

2. Results

The bracelet[13] and boat[14] shaped molecules were canonical β-hairpins engineered stereochemically to desired molecular-morphological plans. A four-stranded mini β-protein is now targeted for modification as a canoe shaped molecule, and thus as a catgrip mimic. The designed peptide Ac-Lys(1)-^{D}Ala(2)-Val(3)-^{D}Pro(4)-Gly(5)-Leu(6)-^{D}Val(7)-Glu(8)-^{D}Val(9)-Pro(10)-Gly(11)-^{D}Ala(12)-Lys(13)-^{D}Asp(14)-Ile(15)-^{D}Pro(16)-Gly(17)-Val(18)-^{D}Ala(19)-Ile(20)-NH_2 is with four alternating L, D strands in extended conformation joined across three Pro-Gly β-turn elements,[7,8] defining a system of interconnected β-hairpins (Figure S1), with first and last strand as potential catgrips, and two carboxyls in calmodulin-like clustering[16] (Fig. 1), defining another potential cation binding site. The molecule was modeled with in-house graphics software, placing Pro-Gly as β-turns toward the required registry in inter-strand hydrogen bonds. Being extended-β in the strands, the peptide was equipped with largely β-sheet favoring side chains, in a favorable mix of cross-strand interaction. Given its morphological complexity, the modeled fold was submitted to molecular dynamics (MD) in explicit solvent under NVT over 5 ns at 300 K, checking its conformational viability. The results summarized in Figure 2 establish the peptide as a reasonably well-preserved fold in water, with Cα RMSD $\leqslant$ 2.5 Å, but not in methanol.

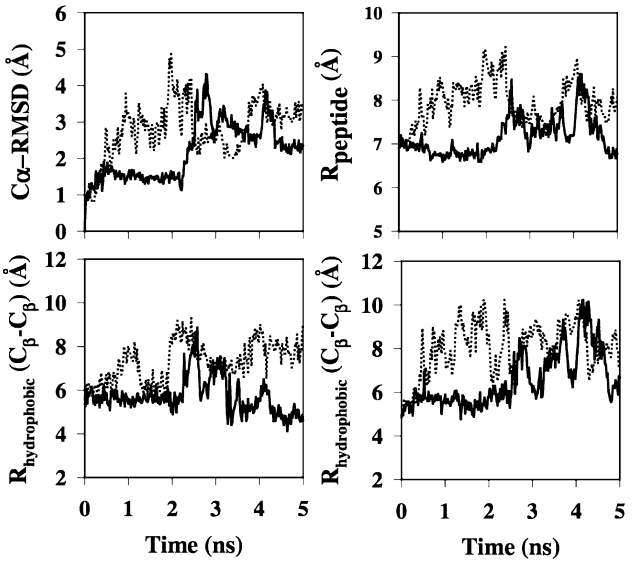


Figure 2. Summary of results from MD at 300 K over 5 ns in water (—) and methanol (···). Cα–RMSD, RMS fluctuation of Cα atoms; $R_{peptide}$, radius of gyration over the peptide; $R_{hydrophobic}$, radius of gyration over Cβ atoms.

In water, the peptide displays mutual clustering (a distance between Cβ atoms $\leqslant$ 6 Å) in cross-strand neighbor side chains of both apolar and polar residues, as noted in Figure 2. Reassured of a possible hydrophobically assisted fold in water, we took up the peptide for synthesis and experimental characterization.

Synthesized by conventional solid phase chemistry, and judged >90% pure (Figure S2), the peptide displayed MALDI-MS peaks at 1970 for M_H^+, 1993 for M_{Na}^+, and 2009 for M_K^+ (Figure S2), against M_{calc} 1969. There was no concentration dependent variation in either chemical shifts or line widths in ^{1}H NMR spectra recorded at 0.2 and 2.0 mM concentration, suggesting the peptide to be free of aggregation in this concentration range in both water and methanol. The peptide displayed solvent dependent ^{1}H NMR spectra, better dispersed in water (Figure S3), suggesting possibility of a better ordering in this solvent than methanol (Figure S4). Other than trace impurities, the peptide displayed a single set of ^{1}H NMR resonances in both water and methanol (Figures S3 and S4), suggesting the absence of isomers or polymorphs in slow exchange over

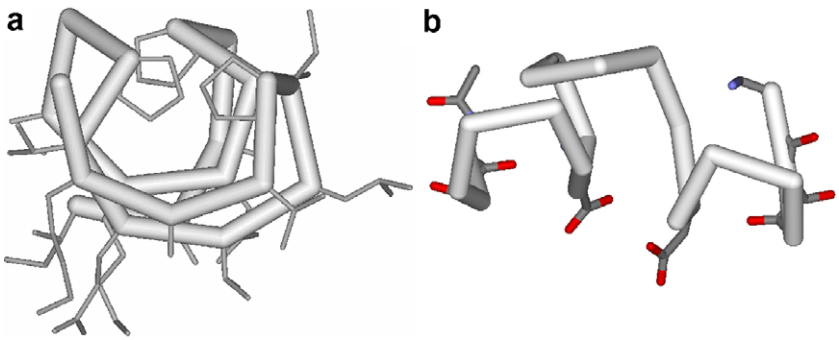


Figure 1. (a) CYANA modeled mean NMR structure for the canoe in water. (b) Molecular model of the canoe highlighting its potential catgrips in N-terminal and C-terminal strands and the calmodulin-like carboxylate cluster in side chains.

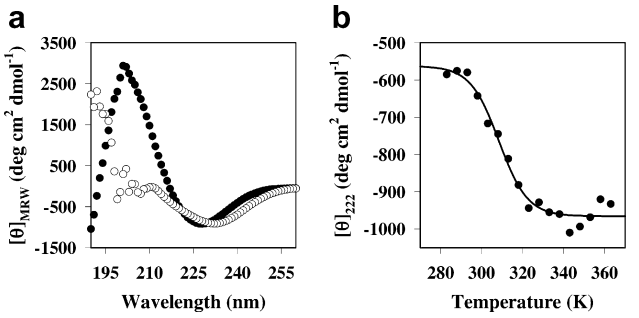


Figure 3. (a) Far UV CD spectra of the peptide (100 μM) in water (pH ~ 5) and methanol at 298 K. (b) CD monitored {$[\theta]_{222}$} thermal melt of peptide (100 μM) in water at pH ~ 5.

NMR time scale. This is noteworthy for the peptide with three stereogenic prolines, capable of *cis–trans* isomers that typically are in slow exchange over NMR time scale,[17] and stereochemically similar to gramicidin-A, an alternating-L, D peptide notoriously polymorphic as detectable with NMR.[18,19]

The peptide displays CD bands that are concentration independent in molar ellipticities in 40–100 μM range (Figure S5), but strongly solvent dependent (Fig. 3a). A broad minimum in 215–235 nm range combined with a strong maximum at ~200 nm appears in water, suggesting possible ordering of β-hairpin like folds in water, for which combination of minimum at ~218 nm and maximum at ~198 nm is taken as diagnostic.[20–22] Displaying absence of such a signature, the peptide could be unordered in methanol. In water, the peptide displays an apparent sigmoidal thermal melt at ~222 nm (Figure 3b) with mid point (apparent T_m) at 315 K, but not the characteristic flat extrema of a real cooperative fold.

^{1}H NMR chemical shifts of the peptide in water (Table S1) were assigned with combined use of TOCSY,[23] NOESY,[24] and ROESY[25] spectra. C$^\alpha$H-chemical shifts are diagnostic of specific secondary structure in peptides and proteins.[26] The peptide displays positive, that is, down field, C$^\alpha$H-chemical shifts ($\Delta\delta_{H\alpha}$) in almost every residue, excluding the residues in turn segments (Figure S6), a characteristic of β-structure.[26] The absolute magnitude of Δδ is rather low (<0.1) in some of the residues, either due to poor ordering, or atypical stereochemical structure of the peptide, or its small size, which could cause most residues to be solvent accessible, an effect minimizing strongly the NMR secondary-structure shifts.[27] In ^{1}H NMR spectra recorded in 288–318 K range in water (data not shown), the peptide displayed no marked differentiation in NH thermal coefficients, to allow identification of the NHs possibly intra-molecularly hydrogen bonded.[28] Validity of NH temperature coefficient as a diagnostic of intra-molecularly hydrogen bonded NHs with small peptides in water has been questioned[29] because of possibility of NH exchange with solvent. The peptide displayed a rich spectrum of NOEs, another diagnostic of conformation. As illustrated schematically in Figure 4, many of the NOEs are in support of ordering of the peptide as a system of inter-connected β-hairpins.[7,8] Specifically, NH–NH/NH–C$^\alpha$H type

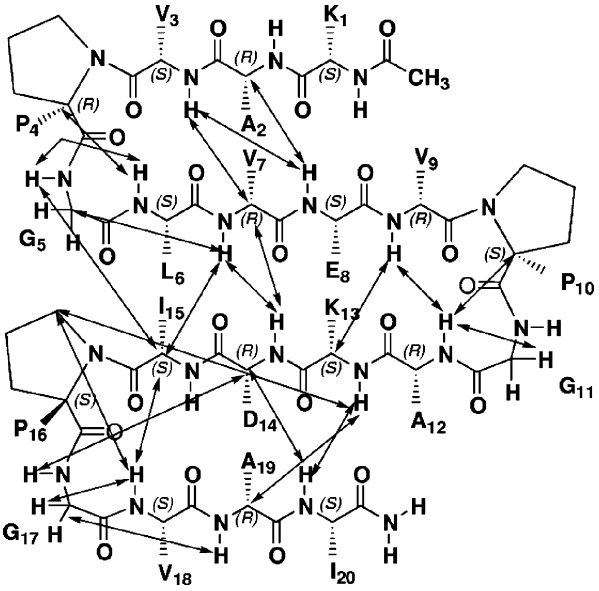


Figure 4. Summary of long-range backbone NOEs observed for the peptide in water, pH ~ 5, at 298 K.

NOEs observed between Gly(5)-Leu(6)/Leu(6)-Pro(4), Val(9)-Ala(12)/Ala(12)-Pro(10), Ala(12)-Gly(11) and Val(18)-Ile(15), Val(18)-Gly(17) are diagnostic of β-hairpin like folds, while NH–C$^\delta$H type NOEs between Lys(13)-Pro(16) and Val(18)-Pro(16) are in evidence of curvature in the peptide placing Pro(16) in proximity of Lys(13) and Val(18). In addition, the peptide also displayed several main chain–side chain and side chain–side chain NOEs in evidence of a possible specific conformation in water. The peptide was taken up for NOE based NMR structure calculation with CYANA.[30] A total of 61 distance constraints (16 intra, 12 short, 18 medium, and 15 long range), calibrated according to NOE volumes, were used in performing the structure calculation. Ten best-fit CYANA[30] calculated NMR structures of the peptide (Figure. S7) displayed mutual mean global backbone root mean square deviation (RMSD) of 0.13 ± 0.05 Å and global heavy atom RMSD of 0.81 ± 0.23 Å, excluding N- and C-terminal residues. The energy-minimized average NMR structure (Fig. 1a) displayed RMSD 2.2 Å with the most populous conformer of the peptide in water detected with MD (Figure. S8).

In its NMR structure, the peptide features two putative catgrips[15] defined in amino terminal C=Os belonging to CH_3CO and Ala(2), and carboxy terminal C=Os belonging to Gly(17) and Ala(19) (Fig. 1b). The elements are in good conformational and geometrical congruity with a representative set of protein catgrips as noted in Table 1.[31–36] Several metal salts in ~300 mM concentration displayed no effect on the position of CD bands of the peptide in water; but a small change in ellipticity (Figs. 5a and 6a) in inverse correlation with ionic radii of the metal ions ($Na^+ < K^+ < Cs^+$) was noted (Fig. 5b). The metal salts evoke a dramatically contrasted response from the peptide in methanol (Fig. 6b); even $CaCl_2$, an alkaline-earth metal salt, with no effect on the peptide CD in water (Fig. 6a), evokes a

Table 1. Summary of conformational and geometrical features in catgrips of canoe against the catgrips in a representative set of proteins

Protein, PDB	Catgrip segment $(i-1, i, i+1)$	ϕ, ψ of the segment (degrees)	$\angle$ O_{i-1}, O_i, O_{i+1} (degrees)	Distance between O_{i-1} and O_{i+1} (Å)	Catgrip structure
Annexin, 2RAN[31]	98, 99, 100	(−79, −14), (−102, 157), (79, −142)	58	3.78	
Fibrinogen, 1FZA[32]	322, 323, 324	(−140, 158), (−122, 161), (−141, 108)	38	2.57	
Thermitase, 2TEC[33]	87, 88, 89	(−133, 146), (54, −130), (−111, 177)	62	3.88	
Subtilisin Carlsberg, 1AF4[34]	79, 80, 81	(−149, 152), (72, −132), (−116, −177)	58	3.91	
Subtilisin E, 1SCJ[35]	79, 80, 81	(−127, 165), (56, −127), (−117, −168)	59	3.81	
Mesentericopeptidase, 1MEE[36]	79, 80, 81	(−133, 165), (51, −128), (−111, −169)	57	3.73	
Canoe	Ac, 1, 2	(−160, 112), (140, −143)	62	4.20	
	17, 18, 19	(−62, −36), (−94, 88), (125, −143)	50	3.92	

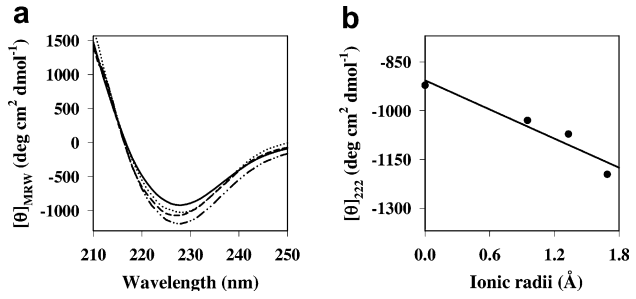


Figure 5. Salt effects in the peptide CD. (a) Effect of ~300 mM alkali (···, Na), (---, K), (··–··–, Cs) chloride salts on CD of the peptide (100 μM) in water (—). (b) Correlation plot ($R^2 = 0.92$) of the changes in molar ellipticity at 222 nm versus ionic radii of the alkali metal ions.

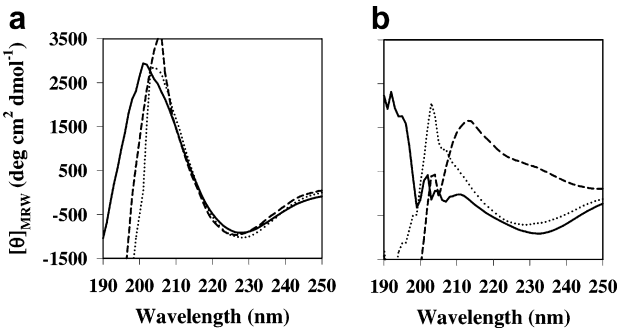


Figure 6. Effects of alkali (···, Na) and alkaline (---, Ca) chloride salts (~300 mM) on CD spectra of the peptide (100 μM) in (a) water (—) and (b) methanol (—).

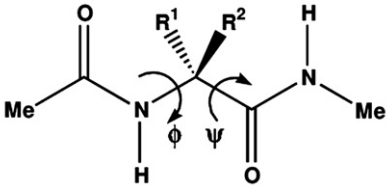
strong response from the peptide in methanol (Fig. 6b). The metal salts (~300 mM CsCl and $CaCl_2$) also influence ^1H NMR spectrum of the peptide in a solvent dependent manner, showing no observable effect on the peptide in water, but a strong dispersal of ^1H chemical shifts in methanol (Figures S9, S10, and S11). Combined evidence of MD, NMR, and CD suggests that the peptide could be poorly ordered but experiencing a metal-salt induced ordering in methanol; the ordering may involve metal ion mediated organization of a cationophoric species; however, no specific characterization of this effect has been undertaken. All observed metal salt effects are only detectable in a large molar excess of metal salts (~300 mM); hence, the peptide could be low in affinity and poor in specificity of metal ions.

In MALDI-MS[37] in presence of CsCl, the peptide displays four singly charged ions (Figure S12), corresponding to protonated molecular ion $M_{obs} + H^+$ at *m/z* 1970, and cation-associated singly charged ions, $M_{obs} + Cs^+ - 1H^+$ at *m/z* 2103, $M_{obs} + 2Cs^+ - 2H^+$ at *m/z* 2235, and

$M_{obs} + 3Cs^+ - 3H^+$ at *m/z* 2366. Clearly, the peptide picks up one, two, and three Cs^+ ions with loss of protons to a singly charged metal–peptide complex. In MALDI-MS with equimolar KCl and CsCl, the peptide displays apparent preference for Cs^+ over K^+ (Figure S13), reflecting metal–peptide complexes in 1:1, 2:1, and 3:1 ratio. In MALDI-MS with $CaCl_2$, the peptide displays two ionic species (Figure S14), the protonated molecular ion $M_{obs} + H^+$ at *m/z* 1970 and the 1:1 peptide–Ca^{2+} complex at *m/z* 2008, corresponding to $M_{obs} + Ca^{2+} - 2H^+$.

3. Discussion

Peptide conformations are sterically defined.[38] The fundamental steric effect is that of side chains in N–Cα (ϕ-torsion) and Cα–CO (ψ-torsion) bond rotations.[4]



(a) R^1, R^2 = H; Gly (b) R^1, R^2 = Me; Aib
(c) R^1 = H, R^2 = Me; L-Ala (d) R^1 = Me, R^2 = H; D-Ala

Scheme 1. Dipeptide unit illustrating its stereogenic center and N–Cα (ϕ) and Cα–CO (ψ) bond torsions.

Side-chain sterics will permit mainly ${}^{L}\alpha_R$, ${}^{L}\beta$ type ϕ, ψs or ${}^{D}\alpha_L$, ${}^{D}\beta$ type ϕ, ψs (Scheme 1, Fig. 7) depending on stereochemistry. Selecting between stereochemically permitted options with chemical effects in side-chain structures, especially in short peptides, remains a challenge. Further constraining rotational freedom across N–Cα and Cα–CO can help; the effects widely explored include those of an additional Cα methyl in alanine[39] and a Cα–Cβ double bond in phenylalanine.[40] Conformationally constrained, α-aminoisobutyric acid (Aib) and $\Delta^{\alpha,\beta}$-dehydrophenylalanine are powerful inducers of conformation. A contrasted de novo approach seeking expansion rather than contraction of conformation space in polypeptide structure, increasing rotatable bonds in backbone, with β- and γ-amino acid monomers, has been explored.[41–43]

Due to mutual exclusivity of sterically permissible ϕ, ψs in L and D residues (Fig. 7), mixed-L, D peptide folds could be stereospecific, like poly-L α-helix (poly-${}^{L}\alpha_R$) and β-sheet (poly-${}^{L}\beta$) motifs, and alternating-L, D β-helices (alternating ${}^{L}\beta$, ${}^{D}\beta$), single- or double-stranded,[44] channel[45] or pore[46] motifs of gramicidin-A (Fig. 8). Other than alternating-L, D nano-tubes,[47] and β-turns[7,8] explored for de novo design,[8–11] there are no well-defined conformational constructs complex in L and D structure, except a hexapeptide,[48] and the bracelet[13] and boat[14] shaped molecules. Adding one more example, a canoe shaped molecule has now been designed, as a catgrip unusually small and simple.

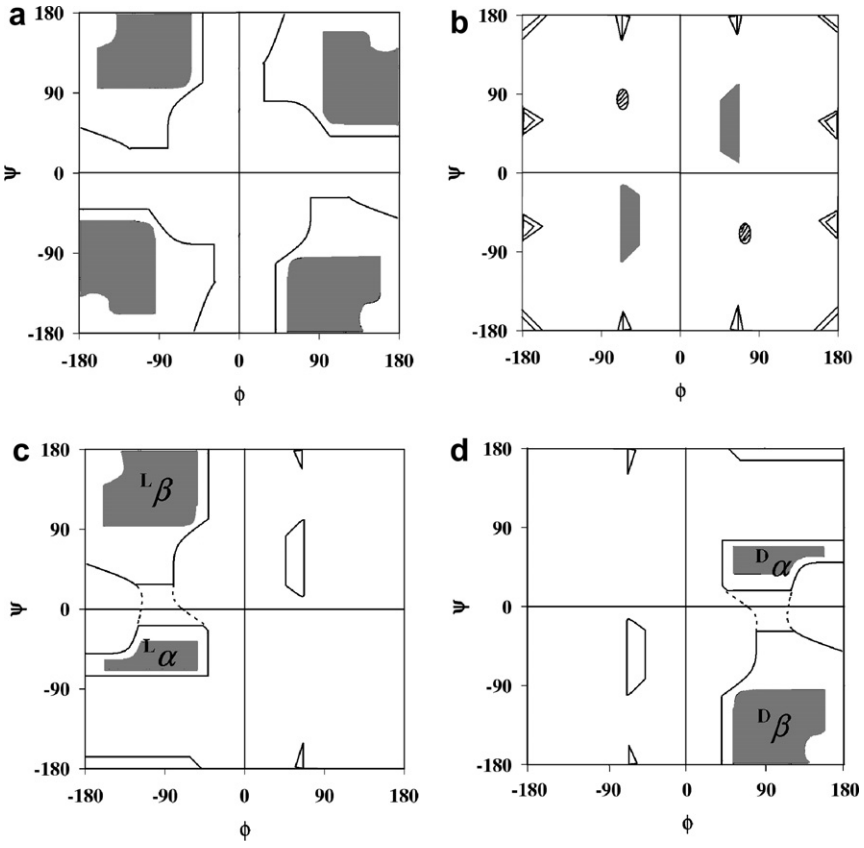


Figure 7. Steric effects in ϕ, ψ dihedrals. Schematic representation of Ramachandran space for Gly (a), Aib (b), ^{L}Ala (c), and ^{D}Ala (d), illustrating symmetry and stereospecificity in conformation depending upon the substituent and stereochemistry in inter-peptide linkage.

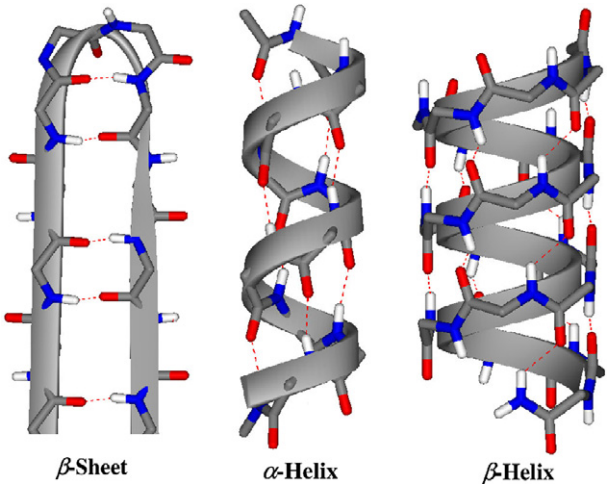


Figure 8. The stereospecific folds of invariant chain stereochemistry. The β-sheet and α-helix motifs are poly-L, while the β-helix motif is alternately L, D.

The β-hairpin has been in intense scrutiny as a β-sheet prototype[49] and a receptor.[50] Pleated due to cross-strand neighbor side chains alternately up and down, but relatively flat with practically no inter-strand tilt, we harnessed type II and II′ β-hairpins,[8,49] with Pro as first corner residue,[51] as the canonical prototypes in our design effort.[13,14] Employing ᴰPro-Gly and ᴸPro-Gly as tight turns, we approach, respectively, type II′ and II β-hairpins, that are, respectively, poly-ᴸβ and poly-ᴰβ type in cross-strand conformation. Mutually non-superposable mirror-image relatives, the type II and II′ β-hairpins, are identical ᴸβ, ᴸβ and ᴰβ, ᴰβ in the cross-strand neighbor residues. Interchanging cross-strand neighbor pair between ᴸβ, ᴸβ and ᴰβ, ᴰβ type creates a 90° crossover between main-chain and side-chain elements; accordingly, the β-hairpin could be modified as bracelet[13] and boat[14] shaped molecules, and now as canoe shaped molecule (Fig. 9). The molecules were fitted with mainly β-sheet favoring residues, in suitable mix of cross-strand neighbor interactions.[52] Residue propensity is an important consideration in β-sheet stability and design. β-Branched residues like Ile and Val are strong inducers of β-sheets and were harnessed in our design as sheet directing residues, also enforcing Xxx-Pro amide bond to *trans* geometry, maximizing both lateral and diagonal cross-strand hydrophobic interactions, contributing in β-sheet stability. Interactions between ionized side chains, called ion pairs, can also be important in stability of conformation.[52] Thus, two pairs of Lys-Glu residues were placed as cross-strand neighbors for possible stabilization of the hairpins by ion-pair interaction.[52a]

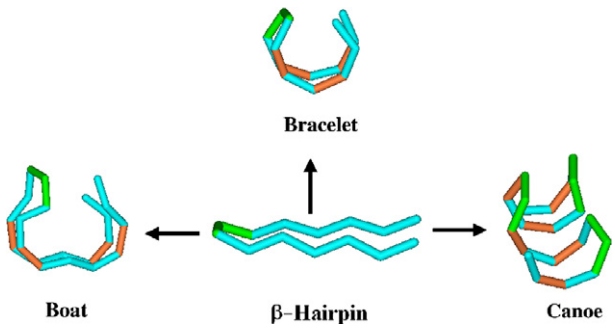


Figure 9. Stereochemical reprogramming of canonical poly-L β-hairpin to bracelet, boat, and canoe shaped folds by changeover of specific pairs of cross-strand neighbor residues from ᴸβ, ᴸβ–ᴰβ, ᴰβ structure. Tight turn elements are color-coded green, while the residues mutated in configuration are color-coded red.

Combined evidence of MD, CD, NMR, and MALDI-MS establishes that the planned sequence construct is a miniprotein in canoe shaped morphology in water, demonstrable in affinity of alkali and alkaline-earth metal ions as expected for its pair of catgrips. The peptide also features a calmodulin-like carboxylate cluster,[16] a potential additional site for cation binding. The potential cationophore sites are distinctive in the charge characteristics, an important consideration in cation preference according to Pearson's HSAB principle.[53] The apparent preference of the peptide for softer Cs^+ in both solution and gas phase implies the catgrips may be its primary cationophores. The peptide associates with three metal ions according to MALDI-MS; even the harder Asp(14), Glu(8) carboxylate cluster (Fig. 1b) may participate in cation binding in a calmodulin[16] type interaction. In fact, it is possible that the harder alkaline-earth Ca^{2+} interacts preferentially in this manner.[54]

Stereochemistry limits but also defines proteins, as conformational constructs stereospecific for poly-L structure. Used as an alphabet, L and D structure could allow proteins to be molecules stereochemically customizable in shape and function. As proof of concept, we previously demonstrated stereochemical reengineering of canonical β-hairpin as bracelet and boat shaped molecules. Illustrating the prospect for functional design, a 20-residue four-stranded mini-β protein has now been customized as a canoe shaped molecule. The execution of another morphological plan complex in configuration and stereospecific in conformation is in affirmation of generality cum versatility of the proposed design approach. Short stretches with residues alternating across opposite halves of Ramachandran diagram occur in proteins, as elements of unusual structural or functional importance; type II and II′ β-turns,[7] catgrip,[15,31–36] anionnest,[55] and K^+-selectivity filter[56] are examples. The elements are logical targets for stereospecific design as mixed-L, D constructs, as has been exemplified in successful design of β-turns,[8] all β-mini protein,[10] K^+-selectivity filter,[6] and now of the catgrip mimic unusually small and simple.

Computational optimization of protein cores[57] and receptor sites[58] has revolutionized de novo design. In a conceptual breakthrough of practical importance, proteins are now engineered independently in main chain, the structural scaffold, and side chains, the functional interface. Predicting folds from sequences remains difficult, but inverse design of side chains against a given fold has been tackled;[59] the approach was extended to de novo design by Mayo and coworkers[60] and to

functional design by Hellinga and coworkers.[61] Designing a scaffold and selecting a complementary set of side chains, Baker and coworkers accomplished inverse design of the first truly artificial protein.[62] Extending the evolving design paradigm,[9,12,62] we propose stereochemical programming of scaffold as a novel concept in de novo protein design.

4. Experimental

4.1. Molecular dynamics (MD) simulation

MD was performed in explicit solvent under NVT (constant in number of particles, volume, and temperature) in a cubic box with periodic boundary. The force-field[63] gromos-96 43a1 was implemented in GROMACS[64] on Intel P-IV processor operating in Linux 8.0. Numerical integrations were performed in step size of 2 fs. Bonds were constrained with *SHAKE*[65] to the tolerance 0.0001. Non-bonded pair list cut-off was 1.4 nm with shift function. The residues of D-configuration were introduced in GROMACS library under guidance of its developers. Ionization states were adjusted to pH $\sim$ 5 and the termini were protected for congruence with experiment. Simple point charge (SPC) water model[66] was used while the methanol model was used as provided in GROMACS. Solvent density was set to the value corresponding to 1 atm at 300 K.[67] Solute and solvent were coupled independently to Berendsen bath at 300 K, to the coupling time constant 0.1 ps. Placed in center of a periodic box large enough to accommodate $\sim$1.5 nm solvent layer on each side, the solute was energy minimized to 100 kJ mol^{-1} nm^{-1} tolerance with steepest-descent, first in absence then presence of solvent. Solvent was relaxed after position restraining the solute. Production MD was initiated and thereafter the trajectory was sampled at 10 ps intervals.

4.2. Peptide synthesis

All suitable protected amino acids were from Novobiochem (Switzerland). The synthesis was performed manually on Rink Amide AM resin using standard Fmoc chemistry and TBTU/HOBT or HOBT/DIC as coupling reagent. Monitored with Kaiser and chloranil tests, each coupling typically required about 6–8 h. Fmoc was deblocked with 30% Piperidine–DMF. N-Terminus was acylated (–NHCOCH$_3$) with Ac$_2$O/DIPEA/DMF, 1:2:20. The cleavage of the final peptide and the deprotection of side chains was accomplished together with reagent-K (TFA/H$_2$O/phenol/thioanisole/EDT in a ratio of 82.5:5:5:5:2.5). The filtrate from resin was purged with N$_2$, precipitated with cold anhydrous diethyl ether and lyophilized to a dry powder. The final purification of the peptide was accomplished with HPLC over RP-18 (10 μm, 10 mm × 250 mm; Merck) eluting with ACN/H$_2$O/0.1% TFA/0–100% gradient.

4.3. Circular dichroism (CD)

CD measurements were on JASCO J-810 CD spectropolarimeter calibrated with d_{10}-camphoursulfonic acid.[68] Working solutions 100 μM in peptide and 300 mM in alkali/alkaline metal salts were prepared gravimetrically. Data were collected at 298 K in 0.2 cm path length quartz cell in 190–260 nm range with 2 nm bandwidth. Scanning at 100 nm/min with 1.0 s time constant in 1 nm steps, five scans were averaged and smoothened after baseline correction for solvent. Variable temperature experiments were in 283–363 K range in water at pH $\sim$ 5 with 2 min equilibration at each temperature. The observations in millidegrees were converted to residue ellipticity [θ_{MRW}] with a reported relation.[68]

4.4. Nuclear magnetic resonance (NMR)

NMR experiments were on VARIAN INOVA 600 MHz and Bruker AVX 500 MHz instruments, at 298 K in 90% H$_2$O/10% ^{2}H$_2$O or 100% CD$_3$OD with 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as internal reference. The peptide was $\sim$2–3 mM at pH $\sim$ 5. Salt concentration was $\sim$300 mM. Solvent was suppressed with presaturation or WATERGATE sequence. The data were processed on SGI with VNMR and XWIN-NMR or on Windows with FELIX. Typically, sine-squared window function phase shifted by 70° was applied in both dimensions with the data zero filled to 2 K × 1 K or to 4 K × 4 K before Fourier transformation. Phase sensitive TOCSY[23] was with 80 ms mixing time, 32 scans in 512 experiments. NOESY[24] was with 300 ms mixing time, 64 scans in 512 experiments. ROESY[25] was with 200 ms mixing time, 64 scans in 512 experiments. Structure calculation was performed with CYANA-2.1.[30] D-Amino acid residues were introduced in CYANA-2.1 library under guidance of the developer.

4.5. MALDI-mass measurements (MS)

Mass spectra were recorded in MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time Of Flight) mode on a duly calibrated AXIMA-CFR Kratos instrument. Positive ions were detected in linear/reflectron mode. Exact molecular mass was determined for possible characterization of peptide: metal ion stoichiometry.[37] The salt and peptide were, respectively, 300 mM and 100 μM in all measurements. Under the condition employed, the coordinated solvent was stripped off from the metal ions.

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Supplementary data

Data not shown for Molecular modeling, MD, CD, NMR, and MALDI-MS studies, as a part of the main manuscript are available online.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.030.

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