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Structure-Guided Design of a Peptide Lock for Modular Peptide Binders

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Abstract: Peptides play an important role in intermolecular interactions and are frequent analytes in diagnostic assays, also as unstructured, linear epitopes in whole proteins. Yet, due to the many different sequence possibilities even for short peptides, classical selection of binding proteins from a library, one at a time, is not scalable to proteomes. However, moving away from selection to a rational assembly of preselected modules binding to predefined linear epitopes would split the problem into smaller parts. These modules could then be reassembled in any desired order to bind to, in principle, arbitrary sequences, thereby circumventing any new rounds of selection. Designed Armadillo repeat proteins (dArmRPs) are modular, and they do bind elongated peptides in a modular way. Their consensus sequence carries pockets that prefer arginine and lysine. In our quest to select pockets for all amino acid side chains, we had discovered that repetitive sequences can lead to register shifts and peptide flipping during selections from libraries, hindering the selection of new binding specificities. To solve this problem, we now created an orthogonal binding specificity by a combination of grafting from α -catenin, computational design and mutual optimization of the pocket and the bound peptide. We have confirmed the design and the desired interactions by X-ray structure determination. Furthermore, we could confirm the absence of sliding in solution by a single-molecule Förster resonance energy transfer. The new pocket could be moved from the N-terminus of the protein to the middle, retaining its properties, further underlining the modularity of the system.

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Supplementary Material
for
Structure-guided design of a peptide lock for modular peptide binders

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PDB-ID	Protein	Ligand	P3'	P4'	P5'	P6'	P7'	P8'	P9'
1G3J	β -catenin	XTCF3-CBD	D	E	L	I	R	F	K
1I7W	β -catenin	E-cadherin	D	S	L	L	V	F	D
1JDH	β -catenin	HTCF-4	D	E	L	I	S	F	K
1M1E	β -catenin	ICAT	D	V	V	M	A	F	S
1T08	β -catenin	APC	D	T	L	L	H	F	A
3OUX	β -catenin	pLef-1	D	E	M	I	P	F	K

SI Figure 1: Sequence alignment of different natural ligands binding to β -catenin and plakoglobin structures. Amino acids are colored according to their type (red=negatively charged, green=polar uncharged, yellow=hydrophobic, cyan=positively charged and aromatic, orange=aromatic, blue=positively charged). Binding pocket numbers are shown above the respective amino acids.

	11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
LSF	S E L P Q M V Q Q L N S P D Q Q E L Q S A L R K L Q Q I A S G G N E Q I Q A V I D A G
KLSF	S E L P Q M V Q Q L N S P D Q Q E L Q S A L R K L Q Q I A Q G G N E Q I Q A V I D A G
AKLSF	S E L P Q M V Q Q L N S P D Q Q E L Q S A L R K L Q Q I A M G G N E Q I Q A V I D A G
Lock 1 Pocket	S E L P Q M V Q Q L N S P D Q Q E L Q S A L W K L R N I A S G G N E Q I Q A V I D A G
dArmRP PDB-ID: 5AEI	S E L P Q M V Q Q L N S P D Q Q E L Q S A L R K L S Q I A S G G N E Q I Q A V I D A G
	54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96
LSF	A L P A L V Q L L S S P N E Q I L A K A L G A L A N I A S G G N E Q I Q A V I D A G A
KLSF	A L P A L V Q L L S S P N E Q I L A K A L G A L A N I A D G G N E Q I Q A V I D A G A
AKLSF	A L P A L V Q L L S S P N E Q I L A S A L G A L A N I A S G G N E Q I Q A V I D A G A
Lock 1 Pocket	A L P A L V Q L L S S P N E Q I L S S A L G A L S N I A S G G N E Q I Q A V I D A G A
dArmRP PDB-ID: 5AEI	A L P A L V Q L L S S P N E Q I L Q E A L W A L S N I A S G G N E Q I Q A V I D A G A
	97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129
LSF	L P A L V Q L L S S P N E Q I L Q F A L I A L S N I A S G G N E Q
KLSF	L P A L V Q L L S S P N E Q I L Q F A L I A L S N I A S G G N E Q
AKLSF	L P A L V Q L L S S P N E Q I L Q F A L I A L A N I A S G G N E Q
Lock 1 Pocket	L P A L V Q L L S S P N E Q I L Q L A L W A L S N I A S G G N E Q
dArmRP PDB-ID: 5AEI	L P A L V Q L L S S P N E Q I L Q E A L W A L S N I A S G G N E Q
	130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159
LSF	I Q A V I D A G A L P A L V Q L L S S P N E Q I L Q E A L W
KLSF	I Q A V I D A G A L P A L V Q L L S S P N E Q I L Q E A L W
AKLSF	I Q A V I D A G A L P A L V Q L L S S P N E Q I L Q E A L K
Lock 1 Pocket	I Q A V I D A G A L P A L V Q L L S S P N E Q I L Q E A L W
dArmRP PDB-ID: 5AEI	I Q A V I D A G A L P A L V Q L L S S P N E Q I L Q E A L W

SI Figure 2: Sequence alignment of the different design models. Mutated amino acids are colored according to their type (red=negatively charged, green=polar uncharged, yellow=hydrophobic, orange=aromatic, blue=positively charged). Position W159K was not mutated in the AKLSF design as this lysine bound to the C-terminal carboxy group of the AKLSF peptide in the design model and was not involved in sidechain interactions.

A **B**

SI Figure 3: Surface representation of the second-generation lock bound to the LSF (A) or ITW (B) peptide moieties. dArmRP in green and bound peptide in cyan.

SI Figure 4: Determination of kinetic binding constants for the complex of YIII M5 AII mCherry:KRKRKRKRKR-sfGFP. Upper panels: association kinetics at different concentrations of dArmRP with 40 nM KRKRKRKRKR-sfGFP. Lower panels: determination of dissociation rate by mixing complex with excess unlabeled competitor dArmRP. The residual plots of the respective fits (red) are shown below the measured kinetics.

SI Figure 5: Fit of kinetic binding constants for the complex of mCherry-Lock 1:KRKAKRKLSF-sfGFP. Upper panels: association kinetics at different concentrations with 40 nM KRKAKRKLSF-sfGFP as present in the measurement. Lower panels: competition assays for the determination of the dissociation rate. The residual plots of the respective fits (red) are shown below the measured kinetics.

SI Figure 6: Fit of kinetic binding constants for the complex of mCherry-Lock 2:KRKAKRKLSF-sfGFP. Upper panels: association kinetics at different concentrations with 40 nM KRKAKRKLSF-sfGFP as present in the measurement. Lower panels: competition assays for the determination of the dissociation rate. The residual plots of the respective fits (red) are shown below the measured kinetics.

SI Figure 7: Fit of kinetic binding constants for the complex of mCherry-Lock 2:KRKAKRKITW-sfGFP. Upper panels: association kinetics at different concentrations with 40 nM KRKAKRKLSF-sfGFP as present in the measurement. Lower panels: competition assays for the determination of the dissociation rate. The residual plots of the respective fits (red) are shown below the measured kinetics.

SI Figure 8: Recurrence analysis of single particles (RASP) indicates that at least three species are required to fit the transfer efficiency histogram of the $Y_{III}M_5A_{II}:KR_5$ complex. (A) Principle of RASP. In a first step, all fluorescence bursts that fall in an initial transfer efficiency window (E_{initial} , shaded red) are located in the measurement. In a second step, all bursts occurring within 5 ms after the initial bursts are selected and used to construct the light blue transfer efficiency histogram (the “recurrence histogram”). Under these conditions, the probability that the first and the second burst come from the same molecule is $>85\%$. If different molecular species with different transfer efficiency distributions contribute to a histogram, they can be isolated in this way (as long as they do not interchange in those 5 ms). The transfer efficiency histogram of the whole measurement is shown in the background in gray. (B) Burst pairs with E_{initial} in the indicated range (on the right) are selected and the normalized transfer efficiency histogram of the bursts recurring

within 5 ms is plotted in gray. The transfer efficiency window for burst selection was moved from $E_{\text{initial}} = 0.65$ to 1.0 in steps of 0.025. All 15 recurrence histograms are fitted globally with three Gaussian peak functions (green, blue and orange). (C) χ^2 analysis (sum of the squared residuals between histogram and fit) indicates that at least three Gaussian peak functions are required for a good fit of all recurrence histograms.

SI Figure 9: Transfer efficiency histograms of the Y_{III}M₅A_{II}:KR₅ complex (top) and the Lock 2:ITW complex (bottom), same as in Figure 5A. This time, measured transfer efficiency histograms (gray) are compared to a fit (red line) with three populations calculated from PDA assuming intrinsic peak broadening (in addition to shot-noise broadening). The intrinsic distributions of transfer efficiencies are given below the histograms. They share the same peak width and positions, and are shaded in green, blue and orange, respectively. Their fitted mean transfer efficiency E_{fit} is given on the top, their relative contribution to both histograms on the right.

Table 1 - Data collection and refinement statistics for structures. Values in parentheses show the data for the highest resolution shell.

	Lock_1:(KR) ₄ KLSF	Lock_2_(GS) ₆ _KRKRKAKLSF	Lock_2_(GS) ₆ _KRKRKAKITW	internalLock_2_(GS) ₆ _KRKRKLKFKR	internalLock_2_(GS) ₆ _KRKAKITWKR
PDB-ID	6S9L	6S9M	6S9N	6S9O	6S9P
condition	66 % (v/v) MPD	22.5 % (w/v) PEG smear medium	22.5 % (w/v) PEG smear medium	25 % (w/v) PEG smear low	10 % (v/v) Ethylene glycol, 18 % (w/v) PEG smear medium
		0.1 M CaCl ₂ , 0.1 M MgCl ₂ ,	0.1 M CaCl ₂ , 0.1 M MgCl ₂ ,	0.04 M CaCl ₂ , 0.04 M Na-formate	0.1 M MgCl ₂ , 0.1 M KCl
	0.1M HEPES NaOH pH 8	0.1 M Pipes pH 7	0.1 M Pipes pH 7	0.1 M Tris pH 8	
Data collection					
Resolution range	47.72 - 2.1 (2.175 - 2.1)	45.61 - 2.1 (2.175 - 2.1)	48.2 - 2.0 (2.071 - 2.0)	48.45 - 3.17 (3.283 - 3.17)	42.16 - 2.8 (2.9 - 2.8)
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	P2 ₁	C2	C2
Cell dimensions					
a, b, c (Å)	59.35, 80.26, 122.72	55.26, 85.5, 194.03	54.853, 85.474, 193.97	168.7, 82.4, 191.78	140.68 37.41 127.32
α, β, γ (°)	90, 90, 90	90, 96.334, 90	90, 96.323, 90	90, 90.364, 90	90 96.588 90
Total Reflections	472453 (48437)	557467 (46095)	793261 (77346)	307233 (31223)	110212 (9800)
Unique reflections	34965 (3443)	103532 (9974)	118079 (11689)	44419 (4437)	16544 (1601)
Multiplicity	13.5 (14.1)	5.4 (4.6)	6.7 (6.6)	6.9 (7.0)	6.7 (6.1)
Completeness (%)	99.95 (99.94)	98.57 (95.80)	97.98 (97.33)	98.16 (98.28)	98.59 (96.16)
I/σ(I)	6.79 (1.29)	6.84 (1.39)	14.23 (2.49)	5.82 (0.94)	7.12 (1.40)
Wilson B-factor	25.81	25.08	28.08	79.99	50.44
R _{merge}	0.3687 (2.324)	0.2449 (1.127)	0.1263 (0.9605)	0.3067 (1.962)	0.2997 (1.62)
R _{meas}	0.3831 (2.411)	0.2737 (1.274)	0.1373 (1.045)	0.3318 (2.119)	0.325 (1.768)
R _{pim}	0.1034 (0.6378)	0.1191 (0.5763)	0.0597 (0.4045)	0.1251 (0.793)	0.124 (0.6967)
CC _{1/2}	0.991 (0.506)	0.979 (0.618)	0.999 (0.92)	0.993 (0.778)	0.991 (0.637)
Refinement					
R-work	0.2071	0.2318 (0.3378)	0.1868 (0.2849)	0.2373 (0.3499)	0.2183 (0.3486)
R-free	0.2363	0.2677 (0.3509)	0.2189 (0.3007)	0.2921 (0.3847)	0.2689 (0.4082)
RMS(bonds)	0.004	0.018	0.021	0.003	0.004
RMS(angles)	0.94	1.80	1.93	0.53	0.94
Ramachandran plot (%)					
Favoured	99.66	99.78	99.78	98.66	99.25
Allowed	0.17	0.17	0.06	1.24	0.6
Outliers	0.17	0.06	0.17	0.1	0.15
Rotamer outliers (%)	0.21	2.37	2.28	0.75	0.77
Average B-factor (Å ²)	33.68	35.26	34.01	91.45	54.98
Non-hydrogen atoms	4900	14498	14653	15158	4962
Protein	4547	13302	13442	15081	4931
Ligand	56	22	139	56	16
Water	297	1174	1072	21	15

Rosetta Scripts Code for the redesign of Lock 1

XML-File:

```
<ROSETTASCRIPTS>

  <TASKOPERATIONS>
    ProteinInterfaceDesign name=pido repack_chain1=1 repack_chain2=1 design_chain1=0 design_chain2=1 interface_distance_cutoff=10/> task operation that designates
    which residues are designable and repackable at the interface
  </TASKOPERATIONS>

  <FILTERS>
    <Ddg name=ddg scorefxn=talaris2014 threshold=-18.5 repeats=2/> binding energy calculation; an average of two repeats is computed for better numerical accuracy
    <Sasa name=sasa threshold=800 /> Buried surface area upon complex formation
    <Rmsd name=rmsd confidence=0/> confidence=0 means that the filter will be evaluated but not used as an acceptance criterion
    <CompoundStatement name=ddg_sasa> combine filters into a single logical statement
      <AND filter_name=ddg/>
      <AND filter_name=sasa/>
    </CompoundStatement>
  </FILTERS>

  <MOVERS>
    <Docking name=docking score_high=soft_rep fullatom=1 local_refine=1/> Invokes RosettaDock local-refinement (in full-atom) with a soft potential
    <BackrubDD name=backrub partner1=1 partner2=0 interface_distance_cutoff=8.0 moves=1000 sc_move_probability=0.25 scorefxn=talaris2014
    small_move_probability=0.15 bbg_move_probability=0.25/> perturb the backbone of chain2
    <RepackMinimize name=des1 scorefxn_repack=soft_rep scorefxn_minimize=soft_rep minimize_bb=0 minimize_rb=1/>
    <RepackMinimize name=des2 scorefxn_repack=talaris2014 scorefxn_minimize=talaris2014 minimize_bb=0 minimize_rb=1/> Design & minimization at the interface
    <RepackMinimize name=des3 minimize_bb=1/>

    <ParsedProtocol name=design>
      <Add mover_name=des1/>
      <Add mover_name=des2/>
      <Add mover_name=des3/>
      <Add mover_name=backrub/>
      <Add mover_name=des3 filter_name=ddg_sasa/>
    </ParsedProtocol>
    <GenericMonteCarlo name=iterate scorefxn_name=talaris2014 mover_name=design trials=5/>
  </MOVERS>

  <PROTOCOLS>
    <Add mover=docking/>
    <Add mover=iterate/>
    <Add filter=ddg/>
    <Add filter=sasa/>
    <Add filter=rmsd/>
  </PROTOCOLS>
</ROSETTASCRIPTS>
```

Flag-file:

```
-s CR.pdb
-out::pdb_gz true
-correct
-ex1
-ex2
-extrachi_cutoff 5
-ignore_unrecognized_res
-run:constant_seed
-nstruct 200
-jd2:ntrials 10
-parser:protocol flexbb-interfacedesign.xml
-docking:no_filters
```

Rosetta Scripts Code for the redesign of the peptide on Lock 2

XML-File:

```
<ROSETTASCRIPTS>

  <TASKOPERATIONS>
    ProteinInterfaceDesign name=pido repack_chain1=1 repack_chain2=1 design_chain1=0 design_chain2=1 interface_distance_cutoff=10/> task operation that designates
    which residues are designable and repackable at the interface
  </TASKOPERATIONS>

  <FILTERS>
    <Ddg name=ddg scorefxn=talaris2014 threshold=-24 repeats=2/> binding energy calculation; an average of two repeats is computed for better numerical accuracy
    <Sasa name=sasa threshold=950 /> Buried surface area upon complex formation
    <Rmsd name=rmsd confidence=0/> confidence=0 means that the filter will be evaluated but not used as an acceptance criterion
    <CompoundStatement name=ddg_sasa> combine filters into a single logical statement
      <AND filter_name=ddg/>
      <AND filter_name=sasa/>
    </CompoundStatement>
  </FILTERS>

  <MOVERS>
    <Docking name=docking score_high=soft_rep fullatom=1 local_refine=1/> Invokes RosettaDock local-refinement (in full-atom) with a soft potential
    <BackrubDD name=backrub partner1=1 partner2=0 interface_distance_cutoff=8.0 moves=1000 sc_move_probability=0.25 scorefxn=talaris2014
    small_move_probability=0.15 bbg_move_probability=0.25/> perturb the backbone of chain2
    <RepackMinimize name=des1 scorefxn_repack=soft_rep scorefxn_minimize=soft_rep minimize_bb=0 minimize_rb=1/>
    <RepackMinimize name=des2 scorefxn_repack=talaris2014 scorefxn_minimize=talaris2014 minimize_bb=0 minimize_rb=1/> Design & minimization at the interface
    <RepackMinimize name=des3 minimize_bb=1/>

    <ParsedProtocol name=design>
      <Add mover_name=des1/>
      <Add mover_name=des2/>
      <Add mover_name=des3/>
      <Add mover_name=backrub/>
      <Add mover_name=des3 filter_name=ddg_sasa/>
    </ParsedProtocol>
    <GenericMonteCarlo name=iterate scorefxn_name=talaris2014 mover_name=design trials=5/>
  </MOVERS>

  <PROTOCOLS>
    <Add mover=docking/>
    <Add mover=iterate/>
    <Add filter=ddg/>
    <Add filter=sasa/>
    <Add filter=rmsd/>
  </PROTOCOLS>
</ROSETTASCRIPTS>
```


Flag-file:

```
-s CR.pdb  
-out::pdb_gz true  
-correct  
-ex1  
-ex2  
-extrachi_cutoff 5  
-ignore_unrecognized_res  
-run:constant_seed  
-nstruct 200  
-jd2:ntrials 10  
-parser:protocol flexbb-interfacedesign.xml  
-docking:no_filters
```