Exploring the repeat protein universe through computational protein design

TJ Brunette1,2, Fabio Parmeggiani1,2, Po-Ssu Huang1,2, Gira Bhabha3, Damian C. Ekiert4, Susan E. Tsutakawa5, Greg L. Hura5,6, John A. Tainer5,7 & David Baker1,2

A central question in protein evolution is the extent to which naturally occurring proteins sample the space of folded structures accessible to the polypeptide chain. Repeat proteins composed of multiple tandem copies of a modular structure unit are widespread in nature and have critical roles in molecular recognition, signalling, and other essential biological processes. Naturally occurring repeat proteins have been re-engineered for molecular recognition and modular scaffolding applications. Here we use computational protein design to investigate the space of folded structures that can be generated by tandem repeating a simple helix–loop–helix–loop structural motif. Eighty-three designs with sequences unrelated to known repeat proteins were experimentally characterized. Of these, 53 are monomeric and stable at 95 °C, and 43 have solution X-ray scattering spectra consistent with the design models. Crystal structures of 15 designs spanning a broad range of curvatures are in close agreement with the design models with root mean square deviations ranging from 0.7 to 2.5 Å. Our results show that existing repeat proteins occupy only a small fraction of the possible repeat protein sequence and structure space and that it is possible to design novel repeat proteins with precisely specified geometries, opening up a wide array of new possibilities for biomolecular engineering.

In repeat proteins, the interactions between adjacent units define the shape and curvature of the overall structure. While in nature the sequences of these units generally differ, stable repeat proteins with identical units have been designed for several families and, for leucine-rich repeats, customized units have allowed for the control of curvature and design of new architectures. To our knowledge, all designed repeat protein structures to date have been based on naturally occurring families. These families may cover all stable repeat protein structures that can be built from the 20 amino acids or, alternatively, natural evolution may only have sampled a subset of what is possible.

To explore the range of possible repeat protein structures, we generated new repeat protein backbone arrangements and designed sequences predicted to fold into these structures. Designs were entirely de novo; they are not based on naturally occurring repeat proteins. The well-packed repeating structures that can be obtained from a simple helix–loop–repeat unit are limited to straight rods, and hence we focused on the helix–loop–helix–loop unit from which repeat proteins with a wide diversity of curvatures can be generated. The lengths of the two helices were varied between 10 and 28 residues, and the lengths of the two turns from 1 to 4 residues. Starting conformations for four tandem repeats of each of the 5,776 (19 × 19 × 4 × 4) independent combinations of helix and loop lengths were generated by setting the backbone torsion angles to ideal helix values for helices and extended chain values for loops. Rosetta Monte Carlo fragment assembly was carried out to generate compact structures; each Monte Carlo move was made at the equivalent position in each repeat to preserve symmetry. Rosetta design calculations were then used to identify low-energy amino acid sequences with good core packing. At each step in the Monte Carlo-simulated annealing design process, a position is picked at random, and the current residue is replaced by a randomly selected amino acid and side-chain conformation (rotamer); a detailed all-atom energy function is then evaluated. Identical substitutions were carried out in each unit to maintain sequence identity between the four repeats; exposed hydrophobic residues in the N- and C-terminal repeats were switched to polar residues in a second round of sequence design to increase solubility. All steps in the design process were completely automated, and the calculations were carried out without manual intervention. Designs with low energies and complementary core side-chain packing were identified, and for the amino acid sequence of each of these designs, multiple independent Rosetta de novo folding trajectories were carried out starting from an extended chain. The structures and energies of the sampled conformations map out an energy landscape for each protein.

Designed helical repeat proteins (DHRs) for which the design model had much lower energy than any other conformation sampled in the

1Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA. 1Institute for Protein Design, University of Washington, Seattle, Washington 98195, USA. 2Department of Cellular and Molecular Pharmacology, UCSF, San Francisco, California 94158, USA. 3Department of Microbiology and Immunology, UCSF, San Francisco, California 94158, USA. 4Molecular Biophysics & Integrated Biomaging, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. 5Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, USA. 6Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA. 7Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195, USA.

*These authors contributed equally to this work.
Figure 3 | Characterization of designed repeat proteins. a. Design success rate. Values for the subset with disulfide bonds are in parentheses. b. Results on six representative designs. Top row, design models. Middle row, computed energy landscapes. Energy is on y axis (r.e.u., Rosetta energy unit) and r.m.s.d. from design model on x axis. All six landscapes are strongly funnelled into the designed energy minimum. Bottom row, circular dichroism spectra collected at 25 °C (red), 95 °C (blue; high dynode voltages reduce measurement accuracy below 200 nm) and back to 25 °C (black). The proteins do not denature within this temperature range (m.r.e., mean residue ellipticity; deg cm$^2$ dmol$^{-1}$ residue$^{-1}$).
of structures can be generated by tandem repeating a simple helix–loop–helix–loop unit. In contrast, native helical repeat proteins span a much narrower range of helical parameters (Fig. 2b, colours indicate different families) with very few straight (high \( r \), low \( \omega \)) or highly twisted (low \( r \), high \( \omega \)) geometries.

We selected for experimental characterization 83 designs spanning the range of \( \alpha \)-helix and loop lengths and overall helical architectures; 26 of these contain disulfide bonds. BLAST searches against the NCBI databases yielded no hits with \( E \) values better than 0.0001 for 49 of the designs, and none of the hits found for the remaining designs were to

Figure 4 | Crystal structures of 15 designs are in close agreement with the design models. Crystal structures are in yellow, and the design models in grey. Insets in circles show the overall shape of the repeat protein. The r.m.s.d. values across all backbone heavy atoms are: 1.50 Å (DHR4), 1.73 Å (DHR5), 1.30 Å (DHR7), 2.28 Å (DHR8), 1.79 Å (DHR10), 2.38 Å (DHR14), 1.21 Å (DHR18), 0.87 Å (DHR49), 1.33 Å (DHR53), 0.93 Å (DHR54), 1.54 Å (DHR64), 0.67 Å (DHR71), 1.73 Å (DHR76), 1.04 Å (DHR79), 0.65 Å (DHR81). Hydrophobic side chains in the crystal structures (red) are close to those in the designs (grey) (Extended Data Fig. 5). The designed disulfide bonds are formed in the structures of DHR4 and DHR7 but not in the structures of DHR5 and DHR18 due to slight structural shifts relative to the design models.
annotated repeat proteins. HHSEARCH comparisons of the designed repeat units to naturally occurring repeat families in Pfam yielded no hits with an E value better than 0.0005 (Supplementary Information Table 4). For each of the designs, we obtained a synthetic gene encoding an amino-terminal capping repeat, two internal repeats, and a carboxy-terminal capping repeat including a six-histidine tag. The proteins were expressed in *Escherichia coli* and purified by affinity chromatography. Of the 83 designs, 74 were expressed in a soluble form and had the expected α-helical circular dichroism spectrum at 25°C, and 72 were stably folded at 95°C (Supplementary Experimental Data). Fifty-three of these (64% of the original experimental set) were monomeric by analytical size-exclusion chromatography coupled to multi-angle light scattering; DHR49 and DHR76 were dimeric in solution. Structure stabilization with disulfide bonds did not systematically improve expression, solubility, or folding (Fig. 3a), probably because the designs are already very stable without disulfide bonds. Representative data on six of the designs are shown in Fig. 3b; the data on all 55 proteins is provided as Supplementary Experimental Data.

We solved the crystal structures of 15 of the designs (Fig. 4) with resolutions between 1.20 Å and 3.35 Å. The design models closely match the crystal structures over both the protein backbone (Cα root mean square deviations (r.m.s.d.) from 0.7 Å to 2.5 Å) and the hydrophobic core side chains (Fig. 4 and Extended Data Fig. 5); crystal structure and design model side chains are in red and grey respectively. As is evident from the extended models in the Fig. 4 insets, the designs have different overall shapes: for example, DHR10 is linear and untwisted, DHR18 is linear and twisted, DHR8 forms a spiral and DHR81 is a flat toroid. The accuracy of the design models was sufficiently high that all of the crystal structures but DHR5 could be solved by molecular replacement. These repeat proteins are among the largest crystallographically validated protein structures designed completely de novo, ranging in size from 171 residues for DHR49 to 238 residues for DHR64. The crystal structures illustrate both the wide range of twist and curvature sampled by our repeat protein generation process and the accuracy with which these can be designed.

To characterize the structures for proteins that were reticent to crystallization and analyse all 55 proteins in solution, we used small-angle X-ray scattering (SAXS)27–30. We collected SAXS profiles for each of the designs, and compared them to scattering profiles calculated from the design models and from crystal structures. For 43 of the designs, the radius of gyration, molecular weight, and distance distributions computed from the SAXS data29 corresponded to those computed from the crystal structures but DHR5 could be solved by molecular replacement. The observed level of control over the repeating helix–loop–helix–loop architecture shows that computational protein design has matured to the point of providing alternatives to naturally occurring scaffolds, including graded and tunable variation difficult to achieve starting from existing proteins. We anticipate that the 44 successful designs described in this work (Extended Data Fig. 9), and sets generated using similar protocols for other repeat units, will be widely useful starting points for the design of new protein functions and assemblies.

Naturally occurring repeat protein families, such as ankyrins, leucine-rich repeats, TAL effectors and many others, have central roles in biological systems and in current molecular engineering efforts. Our results suggest that these families are only the tip of the iceberg of what is possible for polypeptide chains: there are clearly large regions of repeat protein space that are not sampled by currently known repeat protein structures. Repeat protein structures similar to our designs may not have been characterized yet, or perhaps may simply not exist in nature.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Crystal structures have been deposited in the RCSB Protein Data Bank with the accession numbers 5CWG (DHR49), 5CWK (DHR53), 5CWL (DHR54), 5CWM (DHR64), 5CWN (DHR71), 5CWO (DHR76), 5CWG (DHR81) and 5CWQ (DHR81). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.B. (dabaker@uw.edu).
SAXS. SAXS data on size-exclusion–chromatography-purified protein were collected at the SIBYLS 12.3.1 beamline at the Advanced Light Source, LBNL.28,42,43 Scattering measurements were performed on 20-μl samples and loaded into a helium-pumped sample chamber, 1.5 m from the Mar165 detector. Data were collected on both the original gel filtration fractions and samples concentrated 2–8× from individual fractions. Fractions before the void volume and concentrator eluates were used for buffer subtraction. Sequential exposures (0.5, 1, 2, and 5 s) were taken at 12 keV to maximize the signal-to-noise ratio with visual checks for radiation-induced damage to the protein. The data used for fitting were selected for having higher signal to noise ratio and lack of radiation-induced aggregation. In case of concentration dependence, the lowest concentration was used. Models for SAXS comparison were obtained by adding the flexible C-terminal tag present in the constructs to the original designs and the crystal structures, generating 100 trajectories for each starting model by Monte Carlo fragment insertion.22 The results were clustered in Rosetta with a cluster radius of 2 Å and the cluster centres were used for comparison to the experimental data. We used FOXS44,45 to calculate scattering profiles from cluster centres and fit them to the experimental data. The quality of fit between models and experimental SAXS data are usually assessed by the χ value, which, however, suffers from over-fitting in case of noisy data sets and domination of the low region of the scattering vector (q) on the value.27 To avoid artificially low values that represent false positives, we instead used volatility ratio (VR) as primary metric for fit in the range of 0.001 Å−1 < q < 0.25 Å−1. VR values of models with available crystal structures range from 0.7 to 2.3 (Supplementary Information Table 15). VR = 2.5 was selected as upper threshold to consider a design as validated by SAXS. An in-depth evaluation of SAXS curves including mass, radius of gyration, Porod number and probability distribution is described in detail in Supplementary Information.

Model profiles for VR similarity maps were obtained with a standardized fit protocol by the Rosetta–DHR36 folding funnel and SAXS data are shown in Supplementary Information Table 5. Vr values of models with available crystal structures range from 0.7 to 2.3 (Supplementary Information Table 15). VR = 2.5 was selected as upper threshold to consider a design as validated by SAXS. An in-depth evaluation of SAXS curves including mass, radius of gyration, Porod number and probability distribution is described in detail in Supplementary Information.

Additional details and discussions on computational design methods, DHR description, experimental characterization, crystallization and SAXS are provided as Supplementary Information.
Extended Data Figure 1 | Computational protocol for designing *de novo* repeat proteins. **a**, Flowchart of the design protocol. The green box indicates user-controlled inputs, the grey boxes represent steps where protein structure is created or modified, and the white boxes indicate where structures are filtered. **b**, Low-resolution backbone build. **c**, Quick full-atom design (grey) improves the backbone model (red). The superposition in the middle highlights the structural changes introduced. **d**, Structural profile: a 9-residue fragment is matched against the Protein Data Bank repository for structures within 0.5 Å r.m.s.d. The sequences from these structures are used to generate a sequence profile that influences design. **e**, Packing filters were used to discard designs with cavities in the core, illustrated as grey spheres.
Extended Data Figure 2  |  Repeat space explored and model discrimination across design stages. a–c, Percentage of models accepted at backbone building or centroid (a), design (b) and \( ab \) initio (c) stages. Models are divided according to secondary structure length. The combination of loop 1 and loop 2 lengths is indicated on top. \( x \) and \( y \) axis indicate helix 1 and helix 2 lengths, respectively. The fraction of models in the bin that passed the selection stage is indicated in the side bar. Generally, one-residue loops and large differences between helix lengths reduce the number of selected models. d, Distribution of radius and twist of models in the three stages. e, Number of models passing design stages (log scale). From \( \sim 2.8 \) million structures, 761 are accepted.
Extended Data Figure 3 | Model validation by in silico folding. To assess folding robustness seven sequence variants were made for each design. a–g, Illustrate the energy landscape explored by Rosetta ab initio. In red are the protein models produced by ab initio search, in green by side-chain repacking and minimization (relax). Models in deep global energy minima near the relaxed structures are considered folded. The variant with highest density of ab initio models near the relax region was chosen for experimental characterization (blue box). h, Jalview sequence alignment of the first 100 residues of the variants. The yellow bar height indicates sequence conservation, while the black bar represents how often the consensus sequence occurs.
Extended Data Figure 4 | Distribution of DHR axial displacement ($z$) and twist ($\omega$). Parameters for repeat protein family representatives were extracted as described in the Supplementary Information. The DHR models are the 761 proteins validated by in silico folding.
Extended Data Figure 5 | Superposition between single internal repeats (second repeat) of designs (grey) and crystal structures (yellow). Aliphatic and aromatic side chains are in red and cysteines are in orange. DHR7 and 18 show intra-repeat disulfide bonds while DHR4 and 81 form inter-repeat cystines. DHR5 does not form the expected S–S bond. Core side chains in design recapitulate the conformation observed in the crystal structures. Even when the backbone is shifted (for example, DHR5, 8, 15), rotamers are by large correctly predicted.
Extended Data Figure 6 | Structural validation by SAXS. a, \( V_r \) values for the fit of SAXS profiles to design models, in dark grey, and crystal structures, in yellow. For 43 designs, models are within the range defined by crystal structures. DHR49 and DHR76 form dimers in solution and the models employed the configuration observed in the crystal structures. Designs showing aggregation on the scattering profiles, including DHR5 for which the structure was solved, were not included in this figure.

b, c, Pairwise \( V_r \) similarity maps\(^{30}\) of 43 design models. b, Experimental-to-model profile similarity (b) and model-to-model profile similarity (c). Models that are similar to each other show correlation off-diagonal in c, and the same pattern is observed when compared to experimental data in b. The order of display was obtained by clustering the original designed models by structural similarity. The ability to reproduce characteristic patterns within a large set of designs indicates that the models are capturing the relative structural similarities between proteins in solution. The scores are colour coded with red indicating best agreement and white lack of agreement.
Extended Data Figure 7 | Designs are stable to chemical denaturation by guanidine hydrochloride (GuHCl). Circular-dichroism-monitored GuHCl denaturant experiments were carried for two designs for which crystal structures were solved (DHR4 and DHR14), two with overall shapes confirmed by SAXS (DHR21 and DHR62), and two with overall shapes inconsistent with SAXS (DHR17 and DHR67). In contrast to almost all native proteins, four of the six proteins do not denature at GuHCl concentrations up to 7.5 M. Both designs not confirmed by SAXS were extremely stable to GuHCl denaturation and hence are very well-folded proteins; the discrepancies between the computed and experimental SAXS profiles may be due to small amounts of oligomeric species or variation in overall twist.
Extended Data Figure 8 | Structural similarity between DHRs and repeat protein families. DHRs cluster separately from existing repeat proteins. DHRs are equally distributed between right-handed and left-handed repeats, as referred to the repeat handedness, in contrast to known α-helical repeat proteins, which are mostly right-handed. This result indicates that the handedness observed in known families is not an intrinsic limitation of repeat proteins structures. Repeat handedness, as defined by Kobe and Kajava, indicates the rotation of the main chain going from the N- to the C-terminal around the axis connecting the repeat centres of mass. The structural similarity tree was built using pairwise comparison as measured by TM-score.
Extended Data Figure 9 | Extended versions of models validated by SAXS and crystallography. DHRs were characterized as containing four repeats but the number of internal repeats can be increased without additional design steps. Extended models highlight the differences in twist and radius between the validated designs.