

Design and characterization of a protein fold switching network

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To better understand how amino acid sequence encodes protein structure, we engineered mutational pathways that connect three common folds (3 α , β -grasp, and α/β -plait). The structures of proteins at high sequence-identity intersections in the pathways (nodes) were determined using NMR spectroscopy and analyzed for stability and function. To generate nodes, the amino acid sequence encoding a smaller fold is embedded in the structure of an ~50% larger fold and a new sequence compatible with two sets of native interactions is designed. This generates protein pairs with a 3 α or β -grasp fold in the smaller form but an α/β -plait fold in the larger form. Further, embedding smaller antagonistic folds creates critical states in the larger folds such that single amino acid substitutions can switch both their fold and function. The results help explain the underlying ambiguity in the protein folding code and show that new protein structures can evolve via abrupt fold switching.

There have been remarkable advances recently in the ability to predict the tertiary structure of a protein from its primary amino acid sequence^{1,2} as well as to design amino acid sequences that encode stable, unique protein structures³. It is also well-established, however, that some proteins have a propensity for two completely different, but well-ordered, conformations^{4–12}. Better insight into the ambiguity of the protein folding code would lead to a better understanding of how proteins evolve, how mutation is related to disease, and how function can be annotated to sequences of unknown structure^{13–27}. If the protein folding code were truly understood, it would be possible both to predict and design proteins that undergo profound switches in conformation. There has been significant progress in understanding natural proteins that switch folds¹¹ and predicting natural fold-switching proteins from amino acid sequence data²⁵. Designing proteins at the interface between different folds has been possible^{7,28–30} but still presents a formidable challenge. It has been particularly challenging to design monomeric proteins that switch fold without a change in quaternary structure, and a better understanding is needed about how a

very limited subset of intra-protein interactions can tip the balance from one fold and function to another^{29,31,32}.

Our goal here was to engineer monomeric proteins that are in a critical state between two distinct folds. To do this we chose three well-studied protein folds and designed a series of sequences such that each sequence is compatible with two sets of native interactions. Two of these folds are from *Streptococcal* Protein G which contains two types of domains that bind to serum proteins in blood: the G_A domain binds to human serum albumin (HSA)^{33,34} and the G_B domain binds to the constant (Fc) region of IgG^{35,36}. The third protein is S6, a component of the 30S ribosomal subunit of *Thermus thermophilus*^{37–41}. For simplicity, the S6 fold is referred to as an S-fold, the G_A fold as an A-fold, and the G_B fold as a B-fold. These proteins share no significant sequence homology and are representative of three of the ten most common folds: the S-fold is a thioredoxin-like α/β plait; the A-fold is a homeodomain-like 3 α -helix bundle; and the B-fold is a ubiquitin-like β grasp⁴².

Figure 1 depicts a network of high-identity sequence intersections (nodes) that connect the three folds. The arrows in Fig. 1 show a

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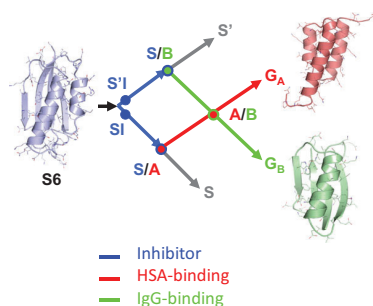


Fig. 1 | Overview of engineered nodes in the S6, G_A , and G_B networks. S6 is the origin sequence in the engineering process. SI and S'I are separate nodes and are loop-size variants of the S-fold, both having protease inhibitor functions. The SI branch of the mutational path leads to a node with the A-fold and HSA binding function. The S'I branch of the path leads to a node with the B-fold and IgG binding function. The S/A node (blue and red circles) includes proteins S_{A1} , S_{A2} , A_1 , and A_2 . The S/B node (blue and green circle) includes proteins S_{B3} , S_{B4} , S_{B5} , B_3 , and B_4 . The A and B paths themselves intersect at an A/B node (green and red circles) at which A- and B-folds are nearly iso-energetic and bifunctional. The S and S' branches continue and connect with many other natural sequences in the α/β plait super-fold family.

network originating with the natural S6 sequence. Circles represent nodes in the network at which structural and/or functional switches occur. The SI and S'I nodes are branch points and lead down diverging sequence pathways, one leading to a node with the A-fold (S/A) and one to a node with the B-fold (S/B). Intersecting mutational pathways lead from S/A to the native G_A protein and S/B to the native G_B protein. At this intersection (A/B), an A-fold switches to a B-fold.

Proteins around the A/B node have been extensively characterized in our earlier work^{29,31,32}. Here we determine that both G_A and G_B can switch into a third fold (α/β -plait) and show that these three folds and four functions (HSA-binding, IgG-binding, protease inhibition, and RNA-binding) can be connected in a network that avoids unfolded and functionless states. We describe how these nodes were engineered, determine key structures using NMR spectroscopy, and analyze stability and binding function. The ability to design and characterize nodes connecting three common small folds suggests that fold switching may be an intrinsic feature of the protein folding code and is important in the evolution of protein structure and function.

Results

Designing a functional switch from ribosomal protein to protease inhibitor

The S6 ribosomal protein is structurally homologous to subtilisin protease inhibitors known as prodomains (Fig. 2a, b)^{43,44}. Prodomain-type inhibitors have two binding surfaces with the protease. One surface comprises the last nine C-terminal amino acids of the inhibitor which bind in the substrate binding cleft of the protease (Fig. 2b). A second, more dynamic surface is formed between two subtilisin helices and the large surface of the β -sheet in the α/β -plait topology of the inhibitor (Fig. 2b)^{45–47}. As a result, the S6 protein could be converted into a subtilisin inhibitor protein of the same overall fold (denoted SI) by replacing its nine C-terminal amino acids with residues optimized to bind in the substrate binding cleft of subtilisin. This replacement results in new contacts between the SI β -sheet and the subtilisin surface helices (Fig. 2b).

The SI-protein is 99 amino acids in length and has a 10 residue loop between β_2 and β_3 . However, there are many natural variations in the length of loops in the conserved α/β -plait topology⁴⁸. Therefore, we also engineered a 91 amino acid version of the S-fold (denoted S'I), which resembles the topology of natural prodomain inhibitors (Supplementary Fig. 1). Specifically, the S'I inhibitor has a longer loop connecting β_1 to α_1 and a shorter turn connecting β_2 to β_3 (Fig. 2b).

The SI and S'I proteins were expressed and purified by binding to a protease column⁴⁹. The CD spectra were compared to the native S6 protein (Supplementary Fig. 1). Inhibition constants (K_i) were measured using an engineered RAS-specific subtilisin protease and the peptide substrate QEEYSAM-AMC⁴⁹. SI and S'I inhibit the RAS-specific protease with K_i values of 200 and 60 nM, respectively (Supplementary Table 1). The details of the competitive inhibition assay are described in the “Methods” section. The results demonstrate that a ribosomal protein can be converted into a protease inhibitor with minor modification (and without a fold switch). In addition, however, the SI and S'I proteins also facilitated engineering subsequent switches to new folds and functions by linking each of the S-, A-, and B- folds to easily measured binding functions: protease inhibition (S or S'-fold); HSA-binding (A-fold, Fig. 2e)⁵⁰; and IgG binding (B-fold, Fig. 2f)⁵¹.

Designing fold switches

In previous work, we created sequences that populate both A- and B-folds by threading the A-sequence through the B-fold, finding a promising alignment, and then using phage-display selection to reconcile one sequence to both folds^{29,52,53}. Here the approach is conceptually similar, except that we use Rosetta⁵⁴ as a computational design tool to test compatible mutations rather than phage display. The design process is as follows:

- Thread the A- or B- sequence through both SI and S'I-fold types.
- Identify alignments that minimize the number of catastrophic interactions.
- Design mutations to resolve unfavorable interactions in clusters of 4–6 amino acids using Pymol⁵⁵ and energy minimize using Rosetta-Relax⁵⁴.
- Optimize protein stability in the S-fold by computationally mutating amino acids at non-overlapping positions. Repeat energy minimization and evaluation with Rosetta-Relax.
- To reduce uncertainties involved in computational design, conserve original amino acids whenever possible.

There is no reason to assume that this method is optimal. We are just applying a practicable scheme for engineering sequences compatible with two sets of native interactions and then evaluating structure, stability, and function. Initial designs were refined based on structural analysis with NMR, thermodynamic analysis of unfolding, and functional analysis using binding assays, as described below. All designed proteins were expressed in *E. coli* and purified to homogeneity as described in the “Methods” section.

Designing a switch from α/β -plait protease inhibitor to 3 α HSA-binding protein

Alignment of the 56 amino acid HSA-binding, A-fold with the 99 amino acid SI-fold and subsequent mutation to resolve catastrophic interactions produced low-energy switch candidates denoted S_{A1} and A_1 . The exact sequence of A_1 is embedded in S_{A1} at positions 11–66 such that the α_1 helices are structurally aligned (Fig. 3a, Supplementary Fig. 2A). Their final computational models were generated by Rosetta using the Relax application. The Relax protocol searches the local conformational space around an experimentally determined structure and is used only to evaluate whether the designed mutations have favorable native interactions within that limited conformational space. The designed models of S_{A1} and A_1 are very similar in energy compared to the respective relaxed native structures (Supplementary Fig. 3 and Source data files).

Structural analysis of A_1 and S_{A1}

Overall, the 3 α -helical bundle topology of A_1 is very similar to the G_A parent structure from which it was derived⁵⁶. The sequence-specific chemical shift assignments for A_1 (Fig. 3b) were utilized to calculate a 3D structure with CS-Rosetta (Fig. 3c, Table 1). Our previous studies

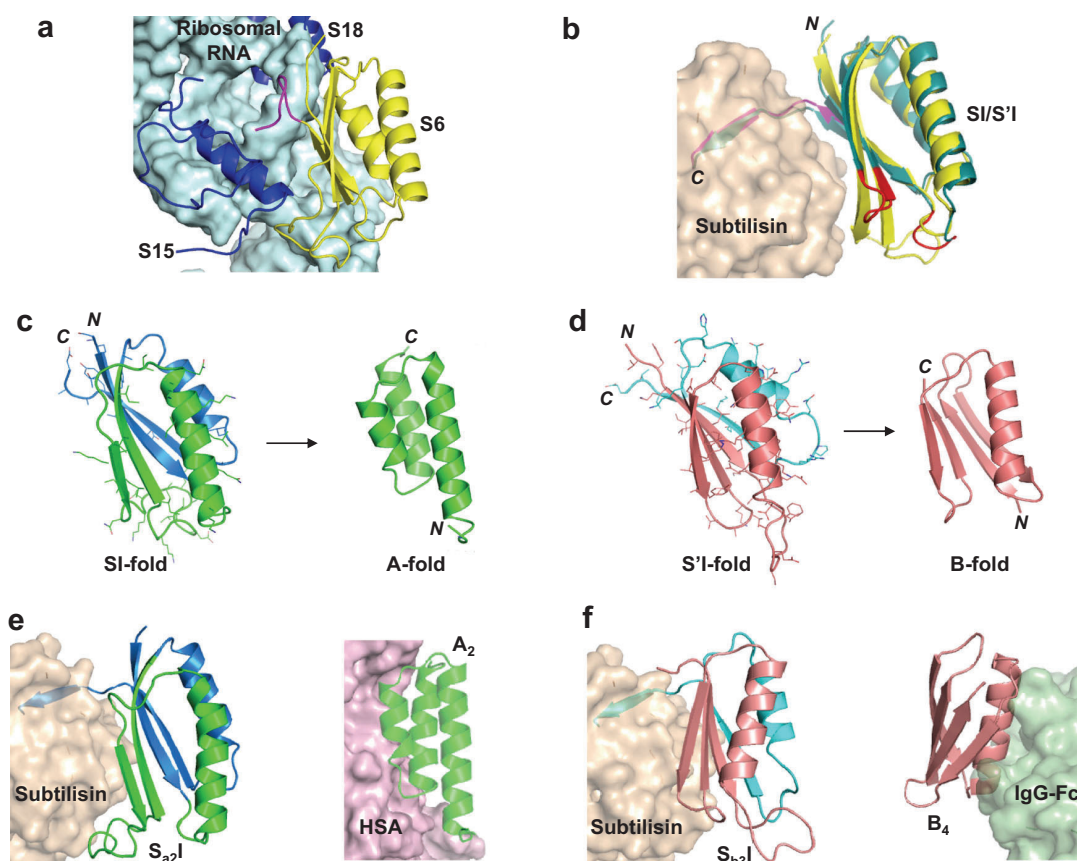


Fig. 2 | Summary of switches in structure and function. **a** Structure of the S6 protein (yellow), RNA (light blue), and S15 and S18 proteins (blue) in the 30S ribosome (PDB 1FKA [<https://doi.org/10.2210/pdb1FKA/pdb>], ref. ⁴³). C-terminal amino acids of S6 are in magenta. **b** Subtilisin (wheat) is shown in complex with a model of the SI-inhibitor (yellow). The C-terminal nine amino acids of SI are shown in magenta. These positions were mutated in native S6 to generate affinity for subtilisin. The S'I-inhibitor (teal) is also shown with the altered loops in red. The subtilisin used in the modeling was the engineered RAS-specific protease. **c** The S_{a1} protein (blue and green) was generated from SI by mutating the 45 positions (mutant side chains shown with sticks). Deletion of C-terminal amino acids (blue)

switches S_{a1} into the A-fold (green). **d** The S_{b3} protein (rose and cyan) was generated from S'I by mutating the 67 positions (mutant side chains shown with sticks). Deletion of C-terminal amino acids (cyan) or point mutation will switch S_{b3} into a B-fold (rose). **e** Model of $S_{a2}I$ (green and blue) bound to subtilisin (wheat). Model of A_2 (based on A_1 structure) bound to HSA (violet). The HSA complex used PDB 2VDB (ref. ⁵⁰) as the template. **f** Model of S_{b3} (rose and cyan) bound to subtilisin (wheat). Model of B_4 (rose) bound to Fc (mint). The Fc complex used PDB 1FCC (ref. ⁵¹) as the template. The subtilisin used in the modeling and inhibition measurements was the engineered RAS-specific protease PDB 6UAO (ref. ⁴⁹).

indicated close correspondence of CS-Rosetta and de novo structures for A- and B-folds with high sequence identity⁵⁷. The N-terminal residues 1–4 and the C-terminal residues 53–56 are disordered in the structure, consistent with ^1H - ^{15}N steady-state heteronuclear NOE data (Fig. 3e). Likewise, S_{a1} has the same overall $\beta\alpha\beta\alpha\beta$ -topology as the parent S6 structure (Fig. 3d, Table 2). The backbone chemical shifts (Fig. 3b) were used in combination with main chain inter-proton NOEs (Supplementary Fig. 4) to determine a three-dimensional structure utilizing CS-Rosetta (PDB 7MN1). The conformational ensemble shows well-defined elements of secondary structure at residues 2–10 (β_1), 16–32 (α_1), 40–44 (β_2), 59–67 (β_3), 73–81 (α_2), and 86–92 (β_4). The principal difference from the native structure is that the β_2 -strand is seven amino acids shorter in S_{a1} than in S6. Heteronuclear NOE data show overall consistency with the structure, indicating that the long loop between the β_2 - and β_3 -strands from residues 45–58 is more flexible than other internal regions of the polypeptide chain (Fig. 3e).

Comparison of A_1 and S_{a1} structures

Although the 56 amino acid sequence of A_1 is 100% identical to residues 11–66 of S_{a1} , a significant fraction of the backbone undergoes changes between the two structures. Most notably, while the α_1 helices in both A_1 and S_{a1} are similar in length, the regions corresponding to

the α_2 and α_3 helices of A_1 form the β_2 and β_3 strands of S_{a1} (Fig. 4a). Core amino acids in the α_1 -helix of A_1 correspond with residues that also contribute to the core of S_{a1} . However, the α_1 -helix in S_{a1} contacts an almost entirely different set of residues (Fig. 4b). For example, amino acids L51, Y53, and I55 in the C-terminal tail of A_1 do not have extensive contact with α_1 but the corresponding residues in S_{a1} (L61, Y63, and I65) form close core interactions with α_1 as part of the β_3 -strand. Most of the other core residues contacting the α_1 -helix of S_{a1} are outside the 56 amino acid region coding for the A_1 fold. These include F4, V6, I8, and L10 from the β_1 -strand; A67 from the β_3 -strand; V72, L75, and L79 from the α_2 -helix; and V85 from the loop between the α_2 -helix and the β_4 -strand. Two additional residues, V88 and V90 (β_4) also contribute significantly to the core but do not contact α_1 . Thus, except for the original topological alignment of the α_1 -helices, the cores of the 3α and α/β -plait folds are largely non-overlapping. In total, approximately half of the residues participating in the S_{a1} core are not present in the A_1 sequence.

Energetics of unfolding for A_1/S_{a1}

Far-UV CD spectra were measured for S_{a1} and A_1 and their thermal unfolding profiles were determined by measuring ellipticity at 222 nm versus temperature (Fig. 5 and Supplementary Fig. 5). S_{a1} has a

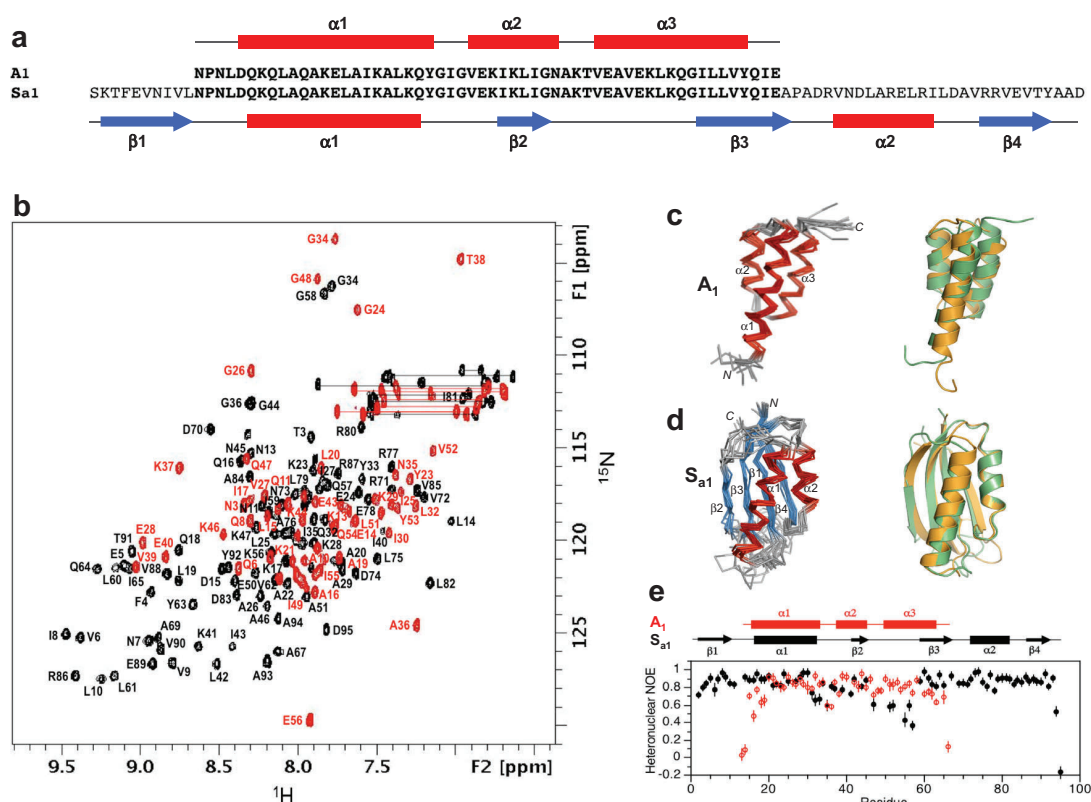


Fig. 3 | Structure and dynamics of A₁ and S_{a1}. **a** Sequence alignment of A₁ and S_{a1}, which are 100% identical over the 56 amino acid A-region. **b** Overlaid two-dimensional ¹H-¹⁵N HSQC spectra of S_{a1} (black) and A₁ (red) with backbone amide assignments. Spectra were recorded at 25 and 5 °C, respectively. **c** Ensemble of 10 lowest energy CS-Rosetta structures for A₁ (left panel). Superposition of the A₁ structure (green) with the parent G_A fold (orange) (right panel). **d** Ensemble of 10

lowest energy CS-Rosetta structures for S_{a1} (left panel). Superposition of S_{a1} (green) with the parent S₆ fold (orange) (right panel). **e** Backbone dynamics in designed proteins. Plot of ¹H-¹⁵N steady state heteronuclear NOE values at 600 MHz versus residue for A₁ (red) and for S_{a1} (black). Each set of heteronuclear NOEs was obtained from a single experiment. Errors were estimated based on the measured background noise level.

T_M of -100 °C and an estimated $\Delta G_{\text{folding}}$ of -5.3 kcal/mol at 25 °C (Fig. 5b, Supplementary Table 1)⁵⁸. The $\Delta G_{\text{folding}}$ of the parent S₆ is -8.5 kcal/mol⁴⁰. The Rosetta energy of the S_{a1} design is similar to that of the native sequence (Supplementary Fig. 3). A₁ has a T_M of 65 °C and a $\Delta G_{\text{folding}}$ = -4.0 kcal/mol at 25 °C⁵⁸ (Fig. 5a, Supplementary Table 1). The $\Delta G_{\text{folding}}$ of the parent G_A is -5.6 kcal/mol^{59,60}. The Rosetta energy of the A₁ design is slightly more favorable than for the native sequence (Supplementary Fig. 3).

HSA binding

Initial engineering of the fold switch was carried out without consideration of preserving function. As a result, A₁ does not have detectable HSA binding affinity because two amino acids in the binding interface were mutated. Significant HSA-binding is recovered, however, when the surface mutations, E28Y and K29Y, are made in A₁ (denoted A₂). These mutations do not appear to affect the structure of A₁ (Supplementary Fig. 5) but result in HSA binding of $K_D \leq 1 \mu\text{M}$ (Supplementary Table 1). This was determined by measuring binding to immobilized HSA as described in the “Methods” section.

Protease inhibition

S_{a1} does not bind protease because C-terminal amino acids were not preserved in its design. It can be converted into a protease inhibitor, however, by replacing its three C-terminal amino acids (AAD) with DKLYRAL (denoted S_{a1}I). A version of S_{a1}I was also made that contains the exact 56 amino acid A₂ sequence by making E38Y, K39Y mutations (denoted S_{a2}I). S_{a1}, S_{a1}I, and S_{a2}I are

similar in structure by CD analysis (Supplementary Fig. 5). The inhibition constant of S_{a2}I with the engineered subtilisin was determined to be 50 nM as described in the “Methods” section (Supplementary Table 1). Thus, a stable A-fold with HSA-binding function can be embedded within a 99 amino acid S-fold with protease inhibitor function (Fig. 2c, e). It should be noted that all HSA contact amino acids are preserved in both the A₂ and S_{a2}I sequences, but the three-dimensional topology necessary to form the HSA contact surface occurs only in the A-fold⁵⁰. Nevertheless, S_{a2}I was observed to bind weakly to HSA ($K_D \sim 100 \mu\text{M}$, Supplementary Table 1). This weak affinity suggests that some S_{a2}I molecules may populate the 3 α fold even though the α/β -plait fold strongly predominates.

Designing a switch from α/β -plait protease inhibitor to β -grasp IgG-binding protein

In designing an S- to B-fold switch, we used two topological alignments. The first was between SI- and B-folds, where the β 1 strands of each fold were aligned (Supplementary Figs. 2B and 6A). The second alignment was between S1- and B-folds, where the long loop between β 2 and β 3 in SI was shortened in S1 to be more consistent with natural protease inhibitors. In this scheme, the α 1 β 3 β 4 topology of the B-fold was aligned with the α 1 β 2 β 3 topology of the S1-fold (Fig. 6a, Supplementary Fig. 2C).

Design and characterization of B₁, S_{b1}, B₂, and S_{b2}

In the first approach, alignment of the β 1-strands of the B-fold and the S-fold and subsequent mutation to resolve catastrophic interactions

Table 1 | Structure statistics for A₁, B₁, and B₄

	A ₁	B ₁	B ₄
A. Experimental chemical shift inputs			
¹³ Cα	55	56	56
¹³ Cβ	51	50	53
¹³ CO	54	55	53
¹⁵ N	54	55	54
¹ H _N	54	55	54
¹ Hα	–	55	–
B. RMSDs to the mean structure (Å)			
Over all residues			
Backbone atoms	1.73 ± 0.47	0.86 ± 0.22	0.85 ± 0.34
Heavy atoms	2.27 ± 0.53	1.37 ± 0.29	1.43 ± 0.45
Secondary structures ^a			
Backbone atoms	0.90 ± 0.29	0.67 ± 0.22	0.62 ± 0.25
Heavy atoms	1.55 ± 0.42	1.12 ± 0.25	1.33 ± 0.41
C. Measures of structure quality (%)			
Ramachandran distribution			
Most favored	98.78 ± 1.64	92.31 ± 3.35	92.36 ± 2.71
Additionally allowed	1.22 ± 1.64	7.69 ± 3.36	7.64 ± 2.71
Generously allowed	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Disallowed	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
D. Backbone RMSDs to the parent structure^b (Å)			
Over all residues	2.48	0.62	0.64
Secondary structures	1.21	0.57	0.49
E. PDB/BMRB codes			
PDBDEV	00000083	00000084	00000085
BMRB	50,907	50,910	50,909

^aThe secondary elements used were as follows: A₁, residues 5–23, 27–35, 39–53; B₁, residues 2–8, 13–19, 23–37, 43–46, 51–55; B₄, residues: 2–8, 13–19, 23–37, 42–46, 51–55.

^bThe parent structure for A₁ is G_A (PDB 2FS1). The parent structure for B₁ and B₄ is G_B (PDB 1PGA). RMSDs were calculated by superimposing the mean structure from the NMR ensemble with either the mean structure (in the case of 2FS1) or the X-ray structure (in the case of 1PGA) for the parent.

produced low-energy switch candidates denoted B₁ and S_{b1}. The exact sequence of B₁ is embedded in S_{b1} at positions 4–59 (Supplementary Fig. 6A). The computational models of B₁ and S_{b1} show relatively small increases in energy compared to the corresponding relaxed native structures (Supplementary Fig. 3). The NMR structure of B₁ displayed a ββαββ topology identical to that of the parent B-fold, with a backbone RMSD of 0.6 Å (Supplementary Fig. 6B, C). The topology of S_{b1} is not the same as the parent S6 structure, however, and instead has a fold similar to that of B₁ (Supplementary Figs. 6B, D, and 7, PDB 7MQ4). Introducing 13 mutations into S_{b1} generated a protein denoted S_{b2} (Supplementary Fig. 8). S_{b2} contains four β-strands and two α-helices and has the general features of the parent S-fold (Supplementary Fig. 9, PDB 7MN2). The 56 amino acid version of S_{b2} (denoted B₂) has a significantly higher Rosetta energy than B₁, however, and is presumably unfolded (Supplementary Fig. 3). Thus, neither the B₁/S_{b1} nor B₂/S_{b2} protein pairs resulted in high identity sequences with different folds. Nonetheless, B₁ is 80% identical to the corresponding embedded region in the S-folded protein S_{b2} (Supplementary Fig. 9A). The structures of B₁, S_{b1}, and S_{b2} are described further in the Supplement and Tables 1 and 2.

Design of S_{b3} and B₃

To improve the design of the S-to-B switch we aligned the B-fold with the S' inhibitor fold and chose an alignment that creates a topological match between α1β3β4 in B and α1β2β3 in S' (Supplementary Fig. 2C). Mutation to resolve deleterious interactions

Table 2 | Structure statistics for S_{a1}, S_{b1}, S_{b2}, and S_{b3}

	S _{a1}	S _{b1}	S _{b2}	S _{b3}
A. Experimental restraint inputs				
NOE restraints				
Sequential (<i>i</i> – <i>j</i> = 1)	89	–	35	40
Medium range (1 < <i>i</i> – <i>j</i> ≤ 5)	35	–	31	10
Long range (<i>i</i> – <i>j</i> > 5)	92	–	67	66
Hydrogen bond restraints	88	–	82	83
TALOS dihedral angle restraints	–	–	–	91
Total NOE restraint inputs	304		215	290
PRE restraints		41		
B. Experimental chemical shift inputs				
¹³ Cα	88	79	83	–
¹³ Cβ	86	70	76	–
¹³ CO	69	67	72	–
¹⁵ N	88	69	76	–
¹ H _N	88	69	76	–
¹ Hα	76	45	61	–
C. RMSDs to the mean structure (Å)				
Over all residues ^a				
Backbone atoms	2.77 ± 0.82	5.47 ± 1.86	2.34 ± 0.60	2.46 ± 0.64
Heavy atoms	3.51 ± 0.85	6.32 ± 1.86	3.00 ± 0.62	3.29 ± 0.61
Secondary structures ^b				
Backbone atoms	1.07 ± 0.23	3.79 ± 1.50	1.08 ± 0.24	0.68 ± 0.14
		(0.71 ± 0.23) ^c		
Heavy atoms	1.84 ± 0.37	4.37 ± 1.42	1.78 ± 0.32	1.42 ± 0.25
		(1.24 ± 0.30) ^c		
D. Measures of structure quality (%)				
Ramachandran distribution				
Most favored	86.46 ± 4.07	92.35 ± 2.38	92.19 ± 1.83	86.30 ± 2.38
Additionally allowed	13.54 ± 4.07	7.54 ± 2.60	7.81 ± 1.83	10.52 ± 2.68
Generously allowed	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.47 ± 1.54
Disallowed	0.00 ± 0.00	0.12 ± 0.38	0.00 ± 0.00	1.96 ± 1.10
E. Backbone RMSDs to the parent structure (Å)^d				
Over all residues	2.37	0.49	6.16	11.67
Secondary structures	0.88	0.41	3.39	3.20
F. PDB/BMRB codes				
PDB	7MN1	7MQ4	7MN2	7MP7
BMRB	30,901	30,905	30,902	30,904

^aOver all residues used as follows: S_{a1}, 1–95; S_{b1}, 4–85; S_{b2}, 1–93; S_{b3}, 1–87.

^bThe secondary elements used are as follows: S_{a1}, residues 2–10, 16–32, 40–44, 59–67, 72–81, 86–92; S_{b1}, residues 5–12, 17–24, 27–41, 46–50, 55–59, 73–83; S_{b2}, residues 2–9, 23–32, 43–48, 59–65, 71–80, 85–91; S_{b3}, residues 4–10, 24–37, 40–46, 51–57, 62–71, 79–85.

^cRMSDs for S_{b1} minus the putative α2 region: residues 5–12, 17–24, 27–41, 46–50, 55–59.

^dThe parent structure for S_{a1}, S_{b2}, and S_{b3} is PDB 1RIS. The parent structure for S_{b1} is PDB 1PGA. In this case, the structure alignment is over the 56 amino acid B-region of S_{b1}.

in this alignment produced low-energy switch candidates denoted B₃ and S_{b3} (Supplementary Fig. 10). The exact sequence of B₃ is embedded in S_{b3} at positions 1–56. The energy of the computational model for S_{b3} is slightly more favorable than the relaxed native structure. The designed model of B₃ shows relatively small

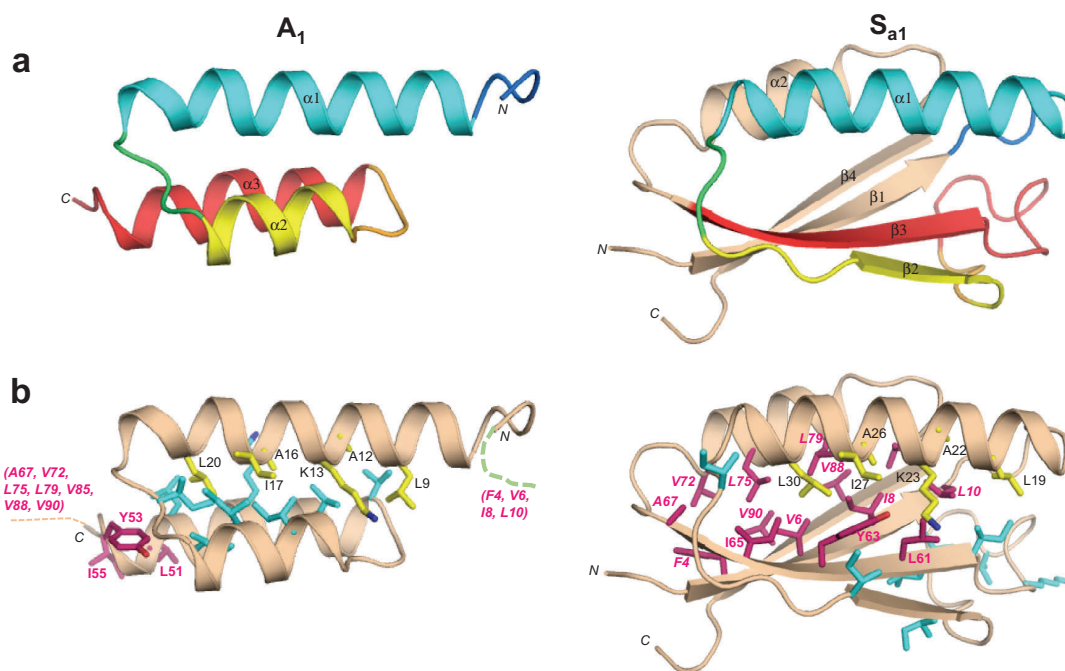


Fig. 4 | Structural differences between the 100% sequence identical regions of A_1 and S_{a1} . **a** Main chain comparisons. (Left panel) CS-Rosetta structure of A_1 with color coding for secondary structured elements. (Right panel) Corresponding color-coded regions mapped onto the CS-Rosetta structure of S_{a1} , illustrating changes in backbone conformation. Regions outside the 56 amino acid sequence of A_1 are shown in wheat. **b** Side chain comparisons. (Left panel) Residues contributing

to the core of A_1 from the $\alpha 1$ -helix (yellow), and from other regions (cyan). The non- $\alpha 1$ core residues from S_{a1} (pink) do not overlap with the A_1 core (see text for further details). (Right panel) Residues contributing to the core of S_{a1} from the $\alpha 1$ -helix (yellow), and most of the other participating core residues (pink). The non- $\alpha 1$ core residues from A_1 are also shown (cyan), highlighting the low degree of overlap.

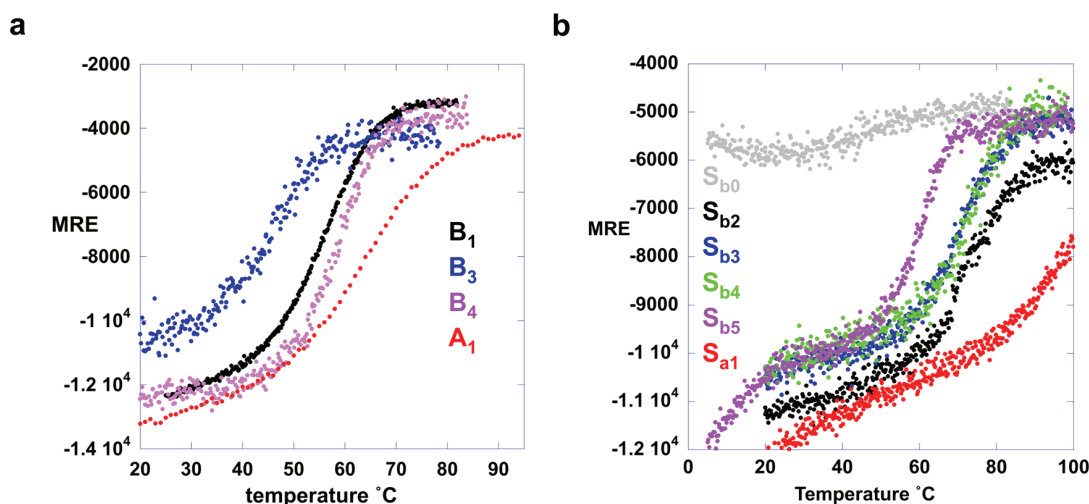


Fig. 5 | CD melting curves. **a** Ellipticity at 222 nm plot versus temperature for A- and B-variations. **b** Ellipticity at 222 nm plot versus temperature for S-variations. S_{b0} is a low-stability variant (F7V) of S_{b1} used to measure the temperature dependence of the unfolded state. Source data are provided as a Source Data file.

increases in energy compared to the relaxed native structure (Supplementary Fig. 3).

Structural analysis of S_{b3} and B_3

NMR-based structure determination indicated that S_{b3} has a $\beta\alpha\beta\beta\alpha\beta$ secondary structure and an S-fold topology (Fig. 6a, b, d, PDB 7MP7). Ordered regions correspond with residues 4–10 ($\beta 1$), 24–37 ($\alpha 1$), 42–46 ($\beta 2$), 51–56 ($\beta 3$), 62–70 ($\alpha 2$), and 79–85 ($\beta 4$). Comparison of S_{b3} with the parent S-fold indicates that the $\beta 1/\alpha 2/\beta 4$ portion of the fold is similar in both. In contrast, the $\beta 1$ – $\alpha 1$ loop is longer in S_{b3} (13 residues) than in the parent S-fold (5 residues), while $\alpha 1$, $\beta 2$, the $\beta 2$ – $\beta 3$ loop, and

$\beta 3$ are all shorter than in the parent (Fig. 6d). Consistent with the S_{b3} structure, the 13 amino acid $\beta 1$ – $\alpha 1$ loop is highly flexible (Fig. 6e). We also expressed and purified a truncated protein corresponding to the embedded B-fold, the 56 amino acid version of S_{b3} (denoted B_3). The 2D ^1H – ^{15}N HSQC spectrum of B_3 at 5 °C and low concentrations ($<20\ \mu\text{M}$) was consistent with a predominant, monomeric B-fold (Supplementary Fig. 11) but showed significant exchange broadening at 25 °C, indicative of low stability (see below). Presumably, the low stability is due to the less favorable packing of Y5 in the core of the B-fold compared with a smaller aliphatic leucine. However, additional, putatively oligomeric, species were also present for which relative

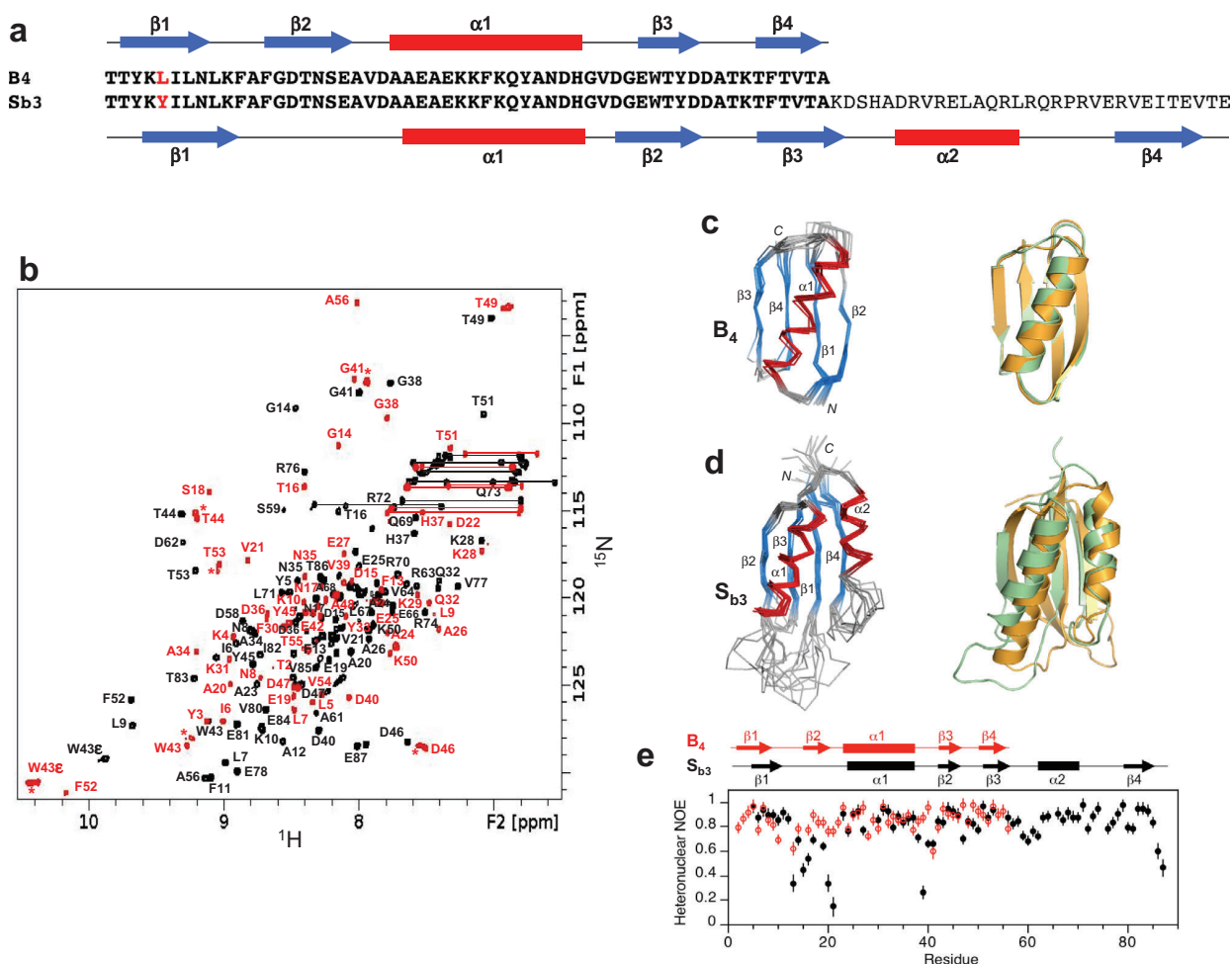


Fig. 6 | Structure and dynamics of S_{b3} and B₄. **a** Sequence alignment of B₄ and S_{b3}, differing by one residue (LSY) over the 56 amino acid B-region. **b** Overlaid two-dimensional ¹H-¹⁵N HSQC spectra of S_{b3} (black) and B₄ (red) with backbone amide assignments. Spectra were recorded at 25 °C. The A56 peak is an aliased signal. Peaks labeled with an asterisk decrease in relative intensity as the B₄ concentration is lowered, indicating the presence of a weakly associated putative dimer in addition to monomer. **c** Ensemble of 10 lowest energy CS-Rosetta structures for B₄ (left

panel). Superposition of the B₄ structure (green) with the parent G_B fold (orange) (right panel). **d** Ensemble of 10 lowest energy CS-Rosetta structures for S_{b3} (left panel). Superposition of S_{b3} (green) with the parent S₆ fold (orange) (right panel). **e** Plot of ¹H-¹⁵N steady-state heteronuclear NOE values at 600 MHz versus residue for B₄ (red) and S_{b3} (black). Each set of heteronuclear NOEs was obtained from a single experiment. Errors were estimated based on the measured background noise level.

peak intensities increased with increasing protein concentration. Due to its relatively low stability and sample heterogeneity, B₃ was not analyzed further structurally.

Design and analysis of point mutations that switch the fold of S_{b3}

We used the NMR structure of S_{b3} to design a point mutation, tyrosine 5 to leucine (Y5L), that would stabilize the embedded B-fold without compromising native contacts in the S-fold (Supplementary Figure 10). This mutant was therefore expected to shift the population to the B-fold. Two mutants were prepared, a Y5L mutant of S_{b3} (denoted S_{b4}) and a Y5L mutant of B₃ (denoted B₄). B₄ is indeed more stable than B₃ (Fig. 5a, Supplementary Table 1). Assignment and structure determination of B₄ showed its topology to be identical to the parent B-fold (Fig. 6b, c). At concentrations above 100 μM, B₄ displayed a tendency for weak self-association similar to that seen for B₃. For S_{b4}, the HSQC spectrum exhibited approximately twice the number of amide cross-peaks relative to S_{b3} (Fig. 7a), suggesting that S- and B-states were populated simultaneously. This was confirmed by the NMR assignment and also a comparison of the HSQC spectra for S_{b4}, B₄, and S_{b3}. A significant fraction of the S_{b4} backbone amide signals (~50 peaks) closely matched those of B₄, indicating the presence of a B-state (Supplementary Fig. 12A–C). The

close matching of these peaks is presumably because residues 1–56 in the B-state of S_{b4} are identical in sequence to B₄. The largest amide shift perturbations between the B-state of S_{b4} and B₄ occur for residues proximal to the C-terminus of the B-fold, such as G41, where S_{b4} has additional residues and B₄ does not. Many of the S_{b4} signals also matched well with S_{b3}, although the degree of similarity was not as extensive as with B₄ (Supplementary Fig. 12D–F). More significant amide chemical shift differences between the S-state of S_{b4} and S_{b3} are likely due to the Y5L mutation, which is a relatively large change located adjacent to the core. To resolve these ambiguities, backbone resonance assignments were made for the S-state of S_{b4} (Fig. 7a, <https://doi.org/10.13018/BMR51719> see the “Methods” section for details). Comparison of S_{b4} S-state assignments with S_{b3} indicated that most of the larger amide shift perturbations were in the β1 and β4 strands. Secondary structure analysis showed that the pattern of secondary structure elements for the S-state of S_{b4} is similar to that of S_{b3} (Fig. 7b). Inter-proton NOE analysis indicated that the arrangement of the β-strands is also similar (Fig. 7c). Together, these results show that S_{b4} populates both S- and B-folds approximately equally at 25 °C. Moreover, a ZZ-exchange spectrum demonstrated that the S- and B-states of S_{b4} are in slow conformational exchange on the NMR timescale (Fig. 7d).

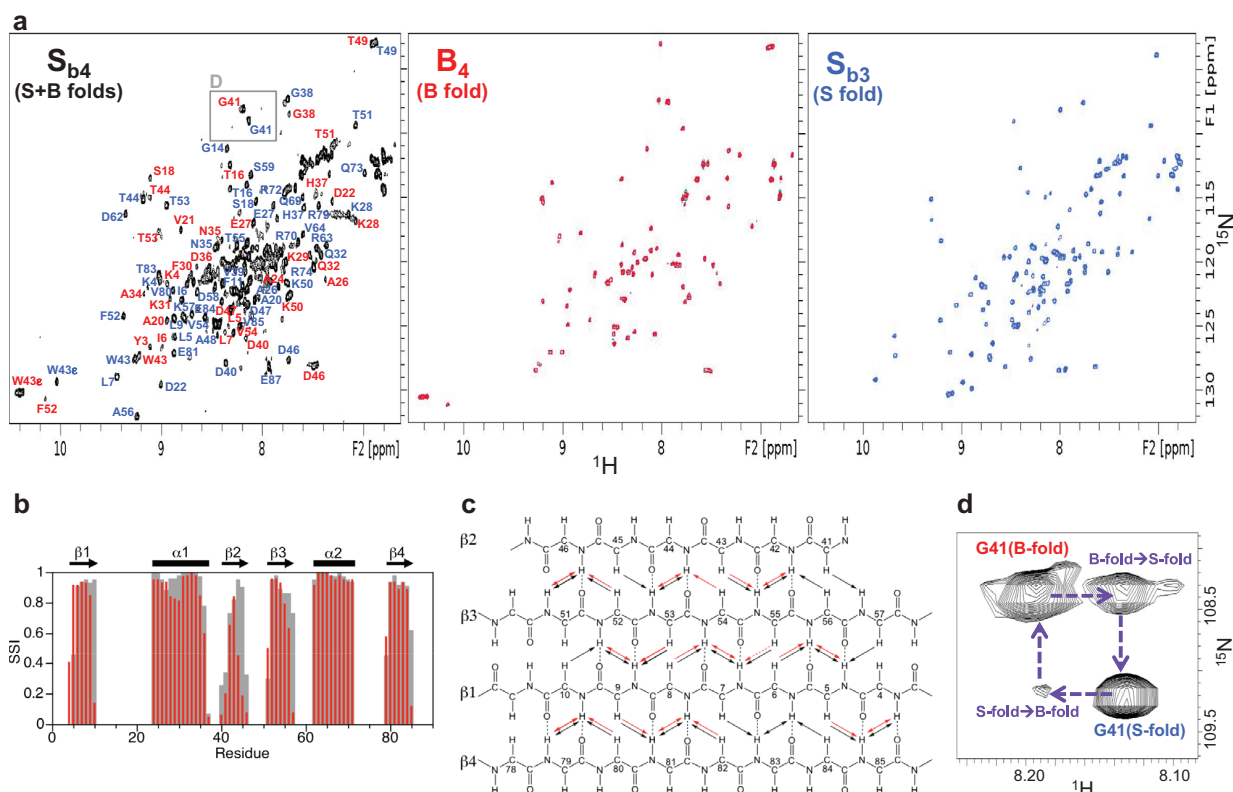


Fig. 7 | S_{b4} is an equilibrium mixture of S- and B-states. a Two-dimensional ¹H-¹⁵N HSQC spectra of S_{b4} (left), B₄ (center), and S_{b3} (right) at 25 °C. In the S_{b4} spectrum, backbone amide resonance assignments are shown for the S-state (blue) and the B-state (red). **b** Confidence levels of ordered secondary structure regions for the S-state of S_{b4} at 30 °C (red) and for S_{b3} at 25 °C (gray) from chemical shifts using

TALOS-N. The secondary structure elements of S_{b3}, determined from the three-dimensional structure, are shown above the plot. **c** Summary of long-range backbone NOEs from 3D ¹⁵N-NOESY data for S_{b3} at 25 °C (black) and the S-state of S_{b4} at 30 °C (red). **d** Gly41 region of the 300 ms ZZ-exchange spectrum for S_{b4} at 25 °C, showing exchange peaks between the S- and B-states.

Finally, we designed a mutation of leucine 67 to arginine (L67R) in S_{b4} to destabilize the S-fold without changing the sequence of the embedded B-fold. The mutant is denoted as S_{b5} (Supplementary Fig. 10). This was expected to shift the population to the B-fold. The 2D ¹H-¹⁵N HSQC spectrum of S_{b5} indicates that the L67R mutation does indeed destabilize the S-fold, with the loss of S-type amide cross-peaks and the concurrent appearance of a new set of signals indicating a switch to a B-fold. The superposition of the spectrum of S_{b5} with that of B₄ shows that the new signals in S_{b5} largely correspond with the spectrum of B₄ (Supplementary Fig. 13). Thus, the L67R mutation shifts the equilibrium from the S-fold to the B-fold. The additional signals (–25–30) in the central region of the HSQC spectrum that are not detected in B₄ are presumably due to the disordered C-terminal tail of S_{b5}. The C-terminal tail of S_{b5} does not appear to interact extensively with the B-fold, as evidenced by few changes in chemical shifts or peak intensities in the B-region of S_{b5} compared with B₄.

Structural comparison of S_{b3} and B₄

The aligned amino acids 1–56 of S_{b3} and B₄ have 98% sequence identity, the only difference being an L5Y mutation in S_{b3} (Fig. 6a). The global folds of S_{b3} and B₄ have large-scale differences, however (Fig. 8a, Supplementary Fig. 4). The β1-strands, while similar in length, are in opposite directions in S_{b3} and B₄. The β1-strand forms a parallel-stranded interaction with β4 in B₄, but an antiparallel interaction with the corresponding β3-strand in S_{b3}. Whereas residues 9–20 form the 6-residue β1–β2 turn and the 6-residue β2-strand of B₄, these same amino acids constitute the end of β1 and 10 residues of the largely disordered β1–α1 loop in S_{b3}. The remainder of the B-region is topologically similar, with the α1/β3/β4 structure in B₄ matching the α1/β2/β3 structure in S_{b3}.

Overall, however, the order of H-bonding in the 4-stranded β-sheets is quite different, with β2β3β1β4 in S_{b3} and β3β4β1β2 in B₄.

The main core residues of B₄ consist of Y3, L5, L7, and L9 from β1, A26, F30, and A34 from α1, and F52 and V54 from β4 (Fig. 8b). In S_{b3}, the topologically equivalent regions of the core are A26, F30, and A34 from α1, and F52 and V54 from β3. Residues Y5, L7, and L9 from the β1 strand of S_{b3} also form part of the core, but with different packing from B₄ due to the reverse orientation of β1. Residues A12 and A20, which contribute to the periphery of the core in B₄, are solvent accessible in the β1–α1 loop of S_{b3}. Most of the remaining core residues of S_{b3} come from outside of the B-region and include amino acids from β3 (A56), α2 (V64, L67, A68, L71), and β4 (V80 and I82).

Energetics of unfolding for B₃/S_{b3}, B₄/S_{b4}, and S_{b5}

Far-UV CD spectra were measured for B₃, B₄, S_{b3}, S_{b4}, and S_{b5} and their thermal unfolding profiles were determined by measuring ellipticity at 222 nm versus temperature (Fig. 5, Supplementary Fig. 10, Supplementary Table 1). As described above, the predominant form of S_{b3} is an S-fold. CD and NMR analyses show that B₃ is predominantly a B-fold with a ΔG_{folding} of –1.2 kcal/mol at 25 °C⁵⁸. From the NMR analysis, it appears that the B-fold is in equilibrium with putatively dimeric states. This creates a situation in which the B-fold is both temperature-dependent and concentration-dependent. The predominant form at 5 °C and ≤18 μM is the B-fold, however. The low stability and concentration-dependent behavior of B₃ may indicate that some propensity for S-type conformations could persist in the 56-residue protein.

S_{b4} has a temperature unfolding profile very similar to S_{b3} (Fig. 5) even though both S- and B- are approximately equally populated at 25 °C in S_{b4} (Fig. 7). This shows that the YSL mutation results in two folds that

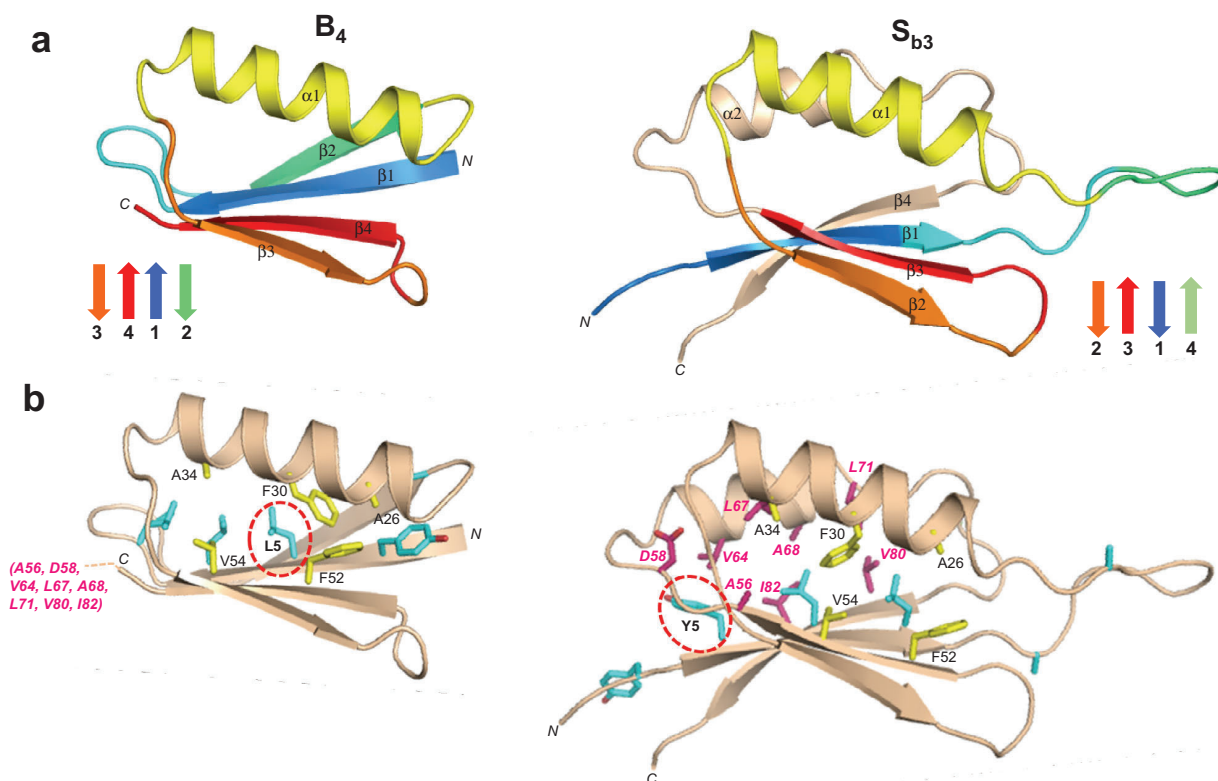


Fig. 8 | Structural differences in the high (~98%) sequence identity regions of B_4 and S_{b3} . **a** Main chain comparisons. (Left panel) CS-Rosetta structure of B_4 with secondary structure elements color coded. (Right panel) Corresponding color-coded regions mapped onto the CS-Rosetta structure of S_{b3} , showing changes in backbone conformation. Regions outside the 56 amino acid sequence of B_4 are shown in wheat. **b** Side chain comparisons. (Left panel) Residues contributing to

the core of B_4 from $\alpha 1/\beta 3/\beta 4$ (yellow), and from other regions (cyan). The non- $\alpha 1/\beta 2/\beta 3$ core residues from S_{b3} (pink) do not overlap with the B_4 core (see text for further details). (Right panel) Residues contributing to the core of S_{b3} from $\alpha 1/\beta 2/\beta 3$ (yellow), and the other participating core residues (pink). The non- $\alpha 1/\beta 2/\beta 3$ core residues from B_4 are also shown (cyan). The single L5Y amino acid difference between B_4 and S_{b3} is highlighted.

are almost isoenergetic and both thermodynamically stable relative to the unfolded state. Further, because S- and B-folds are in equilibrium and approximately equally populated, the free energy of switching to the B-fold from the S-fold ($\Delta G_{B\text{-fold}/S\text{-fold}}$) is ~ 0 kcal/mol at 25 °C. The switch equilibrium reflects the influence of the antagonistic B-fold on the S-fold population in S_{b4} , where the leucine at residue 5 helps stabilize the alternative B-state at the expense of the S-state. Thermal denaturation by CD shows that B_4 has a $\Delta G_{\text{folding}} = -4.1$ kcal/mol at 25 °C⁵⁸. The thermal unfolding profile of S_{b5} shows a low-temperature transition with a midpoint ~ 10 °C and a major transition with a midpoint of ~ 60 °C (Fig. 5b). The NMR analysis indicates that the major transition is unfolding of the B-fold. Thus, the arginine at 67 in S_{b5} makes the B-fold more favorable by making the S-fold unfavorable, consistent with the change in population from mixed to B-fold observed by NMR.

Protease inhibition

The S_{b3} protein is closely related to S'1 but lacks inhibitor function because C-terminal amino acids were changed in the design of the switch. It can be converted into a protease inhibitor, however, by altering C-terminal amino acids VTE to DKLYRAL. This mutant is denoted $S_{b3}I$. S_{b3} and $S_{b3}I$ appear similar in structure by CD analysis (Supplementary Fig. 10). The K_i for $S_{b3}I$ with the engineered subtilisin was determined to be 50 nM (Supplementary Table 1).

IgG binding

Binding to IgG was determined for B_3 and $S_{b3}I$ (Supplementary Table 1). B_3 and $S_{b3}I$ bound to IgG Sepharose with $K_D \leq 1 \mu\text{M}$ and 10 μM , respectively. Presumably, $S_{b3}I$ has significant IgG-binding

activity because the $\alpha 1\beta 3$ IgG binding surface of the B-fold is largely preserved in the S-fold. Thus, $S_{b3}I$ is a dual-function protein with both IgG-binding and protease inhibitor functions (Fig. 2f).

Discussion

The entire network of intersecting pathways between the S-, A-, and B-folds is summarized in Fig. 9. The first node on the pathway is a functional switch from RNA binding protein to protease inhibitor without a fold switch. The α/β plait is a common fold, and proteins with this basic topology include many different functions⁴². Engineering the SI and S'1 nodes illustrates how protease inhibitor function can arise in the α/β plait topology with a few mutations. Replacing only C-terminal amino acids in the S6 protein creates interaction with the substrate binding cleft of the protease (Fig. 2a, b). This C-terminal interaction plus adventitious contact between the β -sheet surface of the α/β plait and two α -helices in the protease result in protease inhibition in the 50 nM range. Based on the structure of S6 in the 30S complex, the C-terminal modification may not have major effects on binding interactions with ribosomal RNA and the S15 protein (Fig. 2a)⁴³. Thus, the transition from RNA binding protein to protease inhibitor likely is uninterrupted. An insertion in the $\beta 1$ - $\alpha 1$ loop and a deletion $\beta 2$ - $\beta 3$ loop in the SI-inhibitor creates a topology that more closely resembles natural prodomain-type inhibitors^{44,46,61} and creates an $\alpha 1\beta 2\beta 3$ motif in the S'-fold that is similar to the $\alpha 1\beta 3\beta 4$ motif of the B-fold. This topological similarity brings the S'I closer to an intersection with the B-fold. Thus, SI and S'1 nodes are both functional switches and branch points for switching the S-fold into the A- and B-folds, respectively.

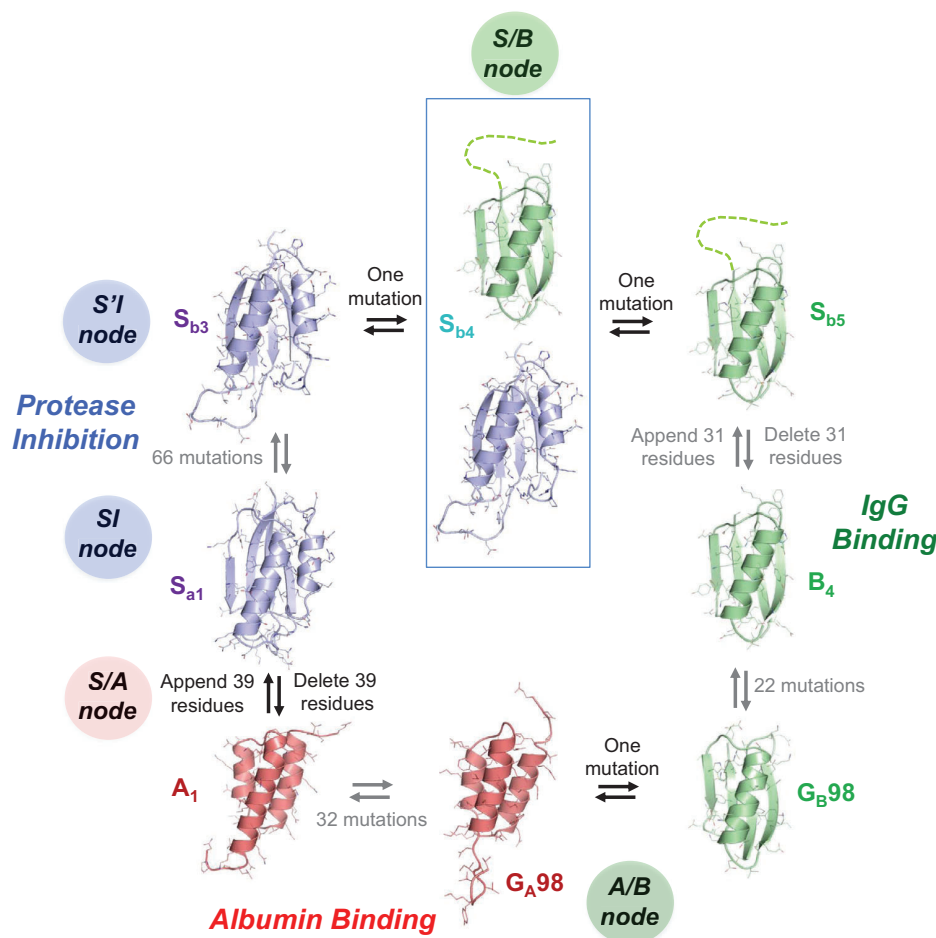


Fig. 9 | Sequence-fold relationships of engineered S/A, S/B, and A/B nodes.

Switches between stable folds can be induced by a single amino acid mutation or deleting/append terminal sequence that stabilizes the S-fold. Blue denotes an S-fold, green a B-fold, and red an A-fold. Gray arrows connect proteins that have been

reengineered without a fold switch. S_{b4} is observed with two folds simultaneously. The G_{A98} and G_{B98} structures are from PDB codes 2LHC and 2LHD (ref. ³²), respectively.

Engineering nodes at fold intersections required designing sequences that are compatible with native interactions in two different folds. We used simple rules to do this. The first rule was to align topologies rather than maximizing sequence similarities. Identifying a common topology can help determine a register that has fewer irreconcilable clashes. For example, topological alignment of the $\alpha 1$ helix of the SI fold and the $\alpha 1$ helix of the A-fold facilitated engineering the fold switch, because the regions flanking $\alpha 1$ of the SI-fold can encode two different fold motifs. When topological alignment is poor, as was the case with S- and B-folds, it was helpful to look for natural variations in the turns of the longer fold to create better alignment. Variation in loops and turns in a larger fold creates more freedom of design and a higher probability of switches. Once an alignment is chosen, the basic rule in resolving catastrophic clashes is to conserve original amino acids when possible. This reduces the uncertainties involved in computational design. The Rosetta energy function was not used to predict a favorable alignment but was important in evaluating mutations to resolve clashes once an alignment was chosen.

Selecting mutations compatible with two sets of native interactions required tradeoffs in the native state energetics of each individual fold^{5,11}. A node may be produced in cases in which both alternative folds are stable relative to the unfolded state. Stability relative to the unfolded state (i.e. a state with little secondary structure) was determined by CD melting (Fig. 5). It was informative to examine the stability of both short (56 residues) and longer forms of a putative node

sequence. The independent stability of the G-fold can be determined in the short form without the antagonism from the S-fold that is present in the longer sequence. The stabilities of the A_1 and A_2 proteins are about -4 kcal/mol at 25°C ⁵⁸ compared to -5.6 kcal/mol for the native G_A protein⁵⁶. The stabilities of B_3 and B_4 are -1.2 and -4.1 kcal/mol, respectively, at 25°C ⁵⁸ compared to -6.7 kcal/mol for the native G_B protein⁶². For the longer sequences, the $\Delta G_{\text{folding}}$ of S_{a1} and S_{b3} are -5.3 and -3.5 kcal/mol, respectively, at 25°C ⁵⁸ compared to -8.5 kcal/mol for the native S_6 protein⁴⁰.

In the case of the S-folds, however, the energetic effects of the stable, embedded G-fold must also be considered. Since the equilibria between both folded states and the unfolded state are thermodynamically linked, the free energy of a switch to a G-fold from an S-fold ($\Delta G_{\text{G-fold/S-fold}}$) is approximated by the difference in $\Delta G_{\text{folding}}$ ($\Delta\Delta G_{\text{folding}}$) between the short and long forms of a node protein. For example, based on $\Delta G_{\text{folding}}$ for A_1 and S_{a1} , the predicted $\Delta G_{\text{A-fold/S-fold}}$ of S_{a1} is 1.3 kcal/mol. This is consistent with the structure of the predominant S-fold determined by NMR but also with the small population of 3α fold suggested by weak HSA-binding. From the thermal denaturation profiles of B_3 and S_{b3} , the predicted $\Delta G_{\text{B-fold/S-fold}}$ of S_{b3} is 2.3 kcal/mol, a value consistent with the stable S-fold observed in NMR experiments. The S_{b3} sequence is also approaching a critical point, however. A substitution in S_{b3} that stabilizes the B-fold (YSL) shifts the equilibrium of S_{b4} to an approximately equal mixture of B- and S-folds.

That is, $\Delta G_{B\text{-fold}/S\text{-fold}}$ of S_{b4} is -0 kcal/mol at 25°C . One further substitution that destabilizes the S-fold (L67R) shifts the population of S_{b5} to a stable B-fold ($\Delta G_{B\text{-fold}/S\text{-fold}} \leq -5$ kcal/mol) (Fig. 9).

The existence of nodes between folds has implications for the evolution of new functions. In the case of the S/A node, all contact amino acids for HSA exist within the S-fold of the protease inhibitor $S_{a2}I$ albeit in a cryptic topology. Deletion of amino acids 67–99 (A_2) results in loss of inhibitor function and a fold switch from α/β plait to 3α . Acquisition of HSA binding activity ($K_D < 1\ \mu\text{M}$) results from unmasking the cryptic HSA binding amino acids via the fold switch (Fig. 2e). This level of binding affinity could be biologically relevant since the concentration of HSA in serum is $>500\ \mu\text{M}$ ⁶³. In the case of the S/B node, the $\alpha 1\beta 3$ motif contains all IgG contact amino acids and $S_{b3}I$ has some affinity for both IgG ($K_D = 10\ \mu\text{M}$) and protease ($K_I = 50\ \text{nM}$). In this case, the Y5L mutation (S_{b4}) or a deletion of 57–91 (B_4) causes a fold switch from α/β plait to the β -grasp and results in tighter IgG binding ($K_D \leq 1\ \mu\text{M}$) (Fig. 2f). This level of binding affinity could also be biologically relevant since the concentration of IgG in serum is $>50\ \mu\text{M}$ (or $>100\ \mu\text{M}$ Fc binding sites)⁶⁴. We have previously shown that an A-fold with HSA binding function can be switched to a B-fold with IgG-binding function via single amino acid substitutions that switch the folds and unmask cryptic contact amino acids for the two ligands^{29,32}.

In conclusion, it was possible to connect three common folds in a network of high-identity nodes that form critical points between two folds. As in other complex systems, a small change in a protein near a critical point can have a “butterfly effect” on how the folds are populated. This property of the protein folding code means that proteins with multiple folds and functions can exist in highly identical amino acid sequences. This suggests that the evolution of new folds and functions sometimes can follow uninterrupted mutational pathways.

Methods

Mutagenesis, protein expression and purification

Mutagenesis was carried out using Q5® Site-Directed Mutagenesis Kits (NEB). G_A and G_B variants were cloned into a vector (pH0720) encoding the sequence:

MEAVDANSLA QAKEAAIKEL KQYGIGDKYI KLINNAKTVE
GVESLKNEL KALPTEGSGN TIRVIVSVDK AKFNPHEVLG IGGHIVYQFK
LIPAVVDVP ANAVGKLKKM PGVEKVEFDH QYRGL

as an N-terminal fusion domain⁵⁶. Cell growth was carried out by auto-induction^{29,65}. Cells were harvested by centrifugation at $3750 \times g$ for 20 min and lysed by sonication on ice in 0.1 M KPi, pH 7.2. Cellular debris was pelleted by centrifugation at $10,000 \times g$ for 15 min. Supernatant was clarified by centrifugation at $45,000 \times g$ for 30 min. Proteins were purified using a second generation of the affinity-cleavage tag system employed previously to purify switch proteins^{29,66}. The second-generation tag results in high-level soluble expression of the switch proteins and also enables the capture of the fusion protein by binding tightly to an immobilized processing protease via the C-terminal EFDHQYRGL sequence. Loading and washing were at 5 mL/min for a 5 mL *Im-Prot* column using a running buffer of 20 mM KPi, pH 6.8. The amount of washing required for high purity depends on the stickiness of the target protein and how much of it is bound to the column. We typically wash with 10 column volumes (CV) of wash solution followed by 3 CV 0.5 M NaCl and then ~ 10 CV running buffer. This can be repeated as necessary. The 0.5 M NaCl shots are repeated until the amount of absorbance released with each high-salt shot becomes small and constant. All the high-salt solution is washed out before initiating the cleavage. The target protein was cleaved from the *Im-Prot* column by injecting 15 mL of imidazole solution (0.1 mM) at 1 mL/min, 22°C . The cleaved protein typically elutes as a sharp peak in 2–3 CV. The purified protein was then concentrated to 0.2–0.3 mM, as

required for NMR analysis. The columns were regenerated by injecting 15 mL of 0.1 N H_3PO_4 (0.227 mL concentrated phosphoric acid (85%) per 100 mL) at a flow rate of ~ 1 CV/min. The wash solution was neutralized immediately after stripping. The purification system is available from Potomac Affinity Proteins.

Protease inhibitor proteins were purified by binding to *Im-Prot* media and then stripping off the purified inhibitor with 0.1 N H_3PO_4 . Samples were then immediately neutralized by adding 1/10 volume 1 M K_2HPO_4 .

Rosetta calculations

Rosetta energies of all designed structures were generated using the Slow Relax routine⁵⁴. 1000 decoys were calculated for each design. PDB coordinates and energy parameters for the lowest energy decoy for each design are included as supplemental files.

Circular dichroism (CD)

CD measurements were performed in 100 mM KPi, pH 7.2 with a Jasco spectropolarimeter, model J-1100 with a Peltier temperature controller. Quartz cells with path lengths of 0.1 and 1 cm were used for protein concentrations of 3 and $30\ \mu\text{M}$, respectively. The ellipticity results were expressed as mean residue ellipticity, $[\theta]$, $\text{deg cm}^2 \text{dmol}^{-1}$. Ellipticities at 222 nm were continuously monitored at a scanning rate of $0.5^\circ/\text{min}$. Reversibility of denaturation was confirmed by comparing the CD spectra at 20°C before melting and after heating to 100°C and cooling to 20°C .

Measuring HSA and IgG binding affinity

Affinity of proteins to HSA and IgG was determined by their retention on the immobilized ligands. HSA and rabbit IgG were immobilized by reaction with NHS-activated Sepharose 4 Fast Flow (Cytiva) according to the manufacturer's instructions. The concentration of immobilized HSA was $100\ \mu\text{M}$. The concentration of immobilized IgG was $50\ \mu\text{M}$ (i.e. $100\ \mu\text{M}$ Fc binding sites). Generally, 0.2 mL of a $5\ \mu\text{M}$ solution of the test protein was injected into a 5 mL column at a flow rate of 0.5 mL/min. Determination of binding affinity assumes that binding is in rapid equilibrium such that the elution volume is proportional to the fraction of test protein bound to $100\ \mu\text{M}$ of binding sites. Proteins that are completely retained after 20 column volumes (CV) are assessed to have $K_D \leq 1\ \mu\text{M}$. Completely retained proteins are stripped from the column with 0.1 N H_3PO_4 at the end of the run.

Measuring protease inhibition

Competitive inhibition constants (K_i) were determined using the fluorogenic peptide substrate QEEYSAM-AMC (7-amino-4-methylcoumarin) purchased from AnaSpec Inc. and a highly specific, engineered protease known as RASProtease(I)⁴⁹. Competitive inhibition constants (K_i) were measured by determining the $K_{M(\text{apparent})}$ in the presence of 0, 50, and 100 nM of each inhibitor protein. The reactions were carried out in 100 mM KPi, 10 mM imidazole, 0.005% tween-20, pH 7.0 at 25°C with 1 nM RASProtease(I). The QEEYSAM-AMC concentrations used to determine K_M and $K_{M(\text{apparent})}$ were 0.1, 0.5, 1, 2, 5, and $10\ \mu\text{M}$. Initial rates were determined with a BioTek Synergy MT fluorescence microplate reader (Ex: 360/40, Em: 460/40) by measuring the release of the fluorescent AMC group via hydrolysis of the amide bond. Highly pure ($\geq 98\%$) protease and inhibitor proteins were used for all kinetic experiments.

NMR spectroscopy

Isotope-labeled samples were prepared at 0.2–0.3 mM concentrations in 100 mM potassium phosphate buffer (pH 7.0) containing 5% D_2O . NMR spectra were collected using Topspin3.6.1 software on Bruker AVANCE III 600 and 900 MHz spectrometers fitted with Z-gradient $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple resonance cryoprobes. Standard double and triple resonance experiments (HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, and

HNHA) were utilized to determine main chain NMR assignments. Inter-proton distances were obtained from 3D ^{15}N -edited NOESY and 3D ^{13}C -edited NOESY spectra with a mixing time of 150 ms. NmrPipe⁶⁷ was used for data processing and analysis was done with Sparky⁶⁸. Two-dimensional $\{^1\text{H}\}$ - ^{15}N steady-state heteronuclear NOE experiments were acquired with a 5 s relaxation delay between experiments. Errors in heteronuclear NOEs were estimated based on the background noise level. Chemical shift perturbations were calculated using $\Delta\delta_{\text{total}} = ((W_{\text{H}}\Delta\delta_{\text{H}})^2 + (W_{\text{N}}\Delta\delta_{\text{N}})^2)^{1/2}$, where W_{H} is 1, W_{N} is 0.2, and $\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$ represent ^1H and ^{15}N chemical shift changes, respectively. For PRE experiments on S_{b1} , single-site cysteine mutant samples were incubated with 10 equivalents of (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSL, Santa Cruz Biotechnology) at 25 °C for 1 h and completion of labeling was confirmed by MALDI mass spectrometry. Control samples were reduced with 10 equivalents of sodium ascorbate. Backbone amide peak intensities of the oxidized and reduced states were analyzed using Sparky. Three-dimensional structures were calculated with CS-Rosetta3.2 using experimental backbone ^{15}N , $^1\text{H}_{\text{N}}$, $^1\text{H}_{\alpha}$, $^{13}\text{C}_{\alpha}$, $^{13}\text{C}_{\beta}$, and ^{13}CO chemical shift restraints and were either validated by comparison with experimental backbone NOE patterns (A_1 , B_1 , B_4 , S_{b1}) or directly employed interproton NOEs (S_{a1} , S_{b2}) or PREs (S_{b1}) as additional restraints. One thousand CS-Rosetta structures were calculated from which the 10 lowest energy structures were chosen. For S_{b3} , CS-Rosetta failed to converge to a unique low-energy topology, producing an approximately even mixture of S- and B-type folds despite the chemical shifts and NOE pattern indicating an S-fold. In this case, CNS1.1⁶⁹ was employed to determine the structure⁵⁶, including backbone dihedral restraints from chemical shift data using TALOS-N⁷⁰. The backbone resonances for the S-state of S_{b4} were assigned using triple resonance methods as above, under conditions where the S-state is more favorably populated (30 °C, 100 mM KPi, 200 mM sodium chloride, pH 7.0). Amide assignments were then transferred to the two-dimensional ^1H - ^{15}N HSQC spectrum of S_{b4} at 25 °C in 100 mM KPi, pH 7.0. Inter-proton NOEs for the S-state of S_{b4} were obtained at the 30 °C/high salt condition, employing a 3D ^{15}N -edited NOESY spectrum with a 150 ms mixing time. A two-dimensional ZZ-exchange ^1H - ^{15}N HSQC spectrum was recorded on S_{b4} using a mixing time of 300 ms (25 °C, 100 mM KPi, pH 7.0)^{71,72}. Protein structures were displayed and analyzed utilizing PROCHECK-NMR⁷³, MOLMOL⁷⁴ and PyMol (Schrodinger)⁵⁵.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The NMR structures generated in this study have been deposited in the PDB: [<https://doi.org/10.2210/pdb7MN1/pdb>]; [<https://doi.org/10.2210/pdb7MQ4/pdb>]; [<https://doi.org/10.2210/pdb7MN2/pdb>]; [<https://doi.org/10.2210/pdb7MP7/pdb>]; [https://pdb-dev.wwpdb.org/entry.html?PDBDEV_00000083]; [https://pdb-dev.wwpdb.org/entry.html?PDBDEV_00000084]; [https://pdb-dev.wwpdb.org/entry.html?PDBDEV_00000085]. NMR Assignments have been deposited in the BMRB: [<https://doi.org/10.13018/BMR30901>]; [<https://doi.org/10.13018/BMR30902>]; [<https://doi.org/10.13018/BMR30904>]; [<https://doi.org/10.13018/BMR30905>]; [<https://doi.org/10.13018/BMR50907>]; [<https://doi.org/10.13018/BMR50909>]; [<https://doi.org/10.13018/BMR50910>]; [<https://doi.org/10.13018/BMR51719>]. The structures referenced in this paper are publicly available in the PDB: [<https://doi.org/10.2210/pdb1FKA/pdb>]; [<https://doi.org/10.2210/pdb2VDB/pdb>]; [<https://doi.org/10.2210/pdb1FCC/pdb>]; [<https://doi.org/10.2210/pdb6UAO/pdb>]; [<https://doi.org/10.2210/pdb2LHC/pdb>]; [<https://doi.org/10.2210/pdb1IRIS/pdb>]. Source data are provided with this paper. Design models are provided as files in the source data. Source data are provided with this paper.

References

- Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589 (2021).
- Baek, M. et al. Accurate prediction of protein structures and interactions using a three-track neural network. *Science* **373**, 871–876 (2021).
- Huang, P. S., Boyken, S. E. & Baker, D. The coming of age of de novo protein design. *Nature* **537**, 320–327 (2016).
- Ambroggio, X. I. & Kuhlman, B. Design of protein conformational switches. *Curr. Opin. Struct. Biol.* **16**, 525–530 (2006).
- Bryan, P. N. & Orban, J. Proteins that switch folds. *Curr. Opin. Struct. Biol.* **20**, 482–488 (2010).
- Dishman, A. F. et al. Evolution of fold switching in a metamorphic protein. *Science* **371**, 86–90 (2021).
- Wei, K. Y. et al. Computational design of closely related proteins that adopt two well-defined but structurally divergent folds. *Proc. Natl Acad. Sci. USA* **117**, 7208–7215 (2020).
- Anderson, W. J., Van Dorn, L. O., Ingram, W. M. & Cordes, M. H. Evolutionary bridges to new protein folds: design of C-terminal Cro protein chameleon sequences. *Protein Eng. Des. Sel.* **24**, 765–771 (2011).
- Burmann, B. M. et al. An α helix to β barrel domain switch transforms the transcription factor RfaH into a translation factor. *Cell* **150**, 291–303 (2012).
- Kulkarni, P. et al. Structural metamorphism and polymorphism in proteins on the brink of thermodynamic stability. *Protein Sci.* **27**, 1557–1567 (2018).
- Dishman, A. F. & Volkman, B. F. Design and discovery of metamorphic proteins. *Curr. Opin. Struct. Biol.* **74**, 102380 (2022).
- Alberstein, R. G., Guo, A. B. & Kortemme, T. Design principles of protein switches. *Curr. Opin. Struct. Biol.* **72**, 71–78 (2022).
- Rackovsky, S. Nonlinearities in protein space limit the utility of informatics in protein biophysics. *Proteins* **83**, 1923–1928 (2015).
- Chen, S. H., Meller, J. & Elber, R. Comprehensive analysis of sequences of a protein switch. *Protein Sci.* **25**, 135–146 (2016).
- Li, W., Kinch, L. N., Karplus, P. A. & Grishin, N. V. ChSeq: A database of chameleon sequences. *Protein Sci.* **24**, 1075–1086 (2015).
- Wolynes, P. G. Evolution, energy landscapes and the paradoxes of protein folding. *Biochimie* **119**, 218–230 (2015).
- Holzgräfe, C. & Wallin, S. Smooth functional transition along a mutational pathway with an abrupt protein fold switch. *Biophys. J.* **107**, 1217–1225 (2014).
- Scheraga, H. A. & Rackovsky, S. Homolog detection using global sequence properties suggests an alternate view of structural encoding in protein sequences. *Proc. Natl Acad. Sci. USA* **111**, 5225–5229 (2014).
- Ha, J. H. & Loh, S. N. Protein conformational switches: from nature to design. *Chemistry* **18**, 7984–7999 (2012).
- Yadid, I., Kirshenbaum, N., Sharon, M., Dym, O. & Tawfik, D. S. Metamorphic proteins mediate evolutionary transitions of structure. *Proc. Natl Acad. Sci. USA* **107**, 7287–7292 (2010).
- Lichtarge, O. & Wilkins, A. Evolution: a guide to perturb protein function and networks. *Curr. Opin. Struct. Biol.* **20**, 351–359 (2010).
- Rollins, N. J. et al. Inferring protein 3D structure from deep mutation scans. *Nat. Genet.* **51**, 1170–1176 (2019).
- Sikosek, T., Chan, H. S. & Bornberg-Bauer, E. Escape from Adaptive Conflict follows from weak functional trade-offs and mutational robustness. *Proc. Natl Acad. Sci. USA* **109**, 14888–14893 (2012).
- Chen, N., Das, M., LiWang, A. & Wang, L. P. Sequence-based prediction of metamorphic behavior in proteins. *Biophys. J.* **119**, 1380–1390 (2020).
- Porter, L. L. & Looger, L. L. Extant fold-switching proteins are widespread. *Proc. Natl Acad. Sci. USA* **115**, 5968–5973 (2018).
- Bedford, J. T., Poutsma, J., Diawara, N. & Greene, L. H. The nature of persistent interactions in two model β -grasp proteins reveals the

- advantage of symmetry in stability. *J. Comput. Chem.* **42**, 600–607 (2021).
27. Sykes, J., Holland, B. R. & Charleston, M. A. A review of visualisations of protein fold networks and their relationship with sequence and function. *Biol. Rev. Camb. Philos. Soc.* <https://doi.org/10.1111/brv.12905> (2022).
 28. Ambroggio, X. I. & Kuhlman, B. Computational design of a single amino acid sequence that can switch between two distinct protein folds. *J. Am. Chem. Soc.* **128**, 1154–1161 (2006).
 29. Alexander, P. A., He, Y., Chen, Y., Orban, J. & Bryan, P. N. A minimal sequence code for switching protein structure and function. *Proc. Natl Acad. Sci. USA* **106**, 21149–21154 (2009).
 30. Davey, J. A., Damry, A. M., Goto, N. K. & Chica, R. A. Rational design of proteins that exchange on functional timescales. *Nat. Chem. Biol.* **13**, 1280–1285 (2017).
 31. He, Y., Chen, Y., Alexander, P., Bryan, P. N. & Orban, J. NMR structures of two designed proteins with high sequence identity but different fold and function. *Proc. Natl Acad. Sci. USA* **105**, 14412–14417 (2008).
 32. He, Y., Chen, Y., Alexander, P. A., Bryan, P. N. & Orban, J. Mutational tipping points for switching protein folds and functions. *Structure* **20**, 283–291 (2012).
 33. Falkenberg, C., Bjorck, L. & Akerstrom, B. Localization of the binding site for streptococcal protein G on human serum albumin. Identification of a 5.5-kilodalton protein G binding albumin fragment. *Biochemistry* **31**, 1451–1457 (1992).
 34. Frick, I. M. et al. Convergent evolution among immunoglobulin G-binding bacterial proteins. *Proc. Natl Acad. Sci. USA* **89**, 8532–8536 (1992).
 35. Myhre, E. B. & Kronvall, G. Heterogeneity of nonimmune immunoglobulin Fc reactivity among gram-positive cocci: description of three major types of receptors for human immunoglobulin G. *Infect. Immun.* **17**, 475–482 (1977).
 36. Reis, K. J., Ayoub, E. M. & Boyle, M. D. P., Streptococcal Fc receptors. II. Comparison of the reactivity of a receptor from a group C streptococcus with staphylococcal protein A. *J. Immunol.* **132**, 3098–3102 (1984).
 37. Lindberg, M. O., Haglund, E., Hubner, I. A., Shakhnovich, E. I. & Oliveberg, M. Identification of the minimal protein-folding nucleus through loop-entropy perturbations. *Proc. Natl Acad. Sci. USA* **103**, 4083–4088 (2006).
 38. Haglund, E., Lindberg, M. O. & Oliveberg, M. Changes of protein folding pathways by circular permutation. Overlapping nuclei promote global cooperativity. *J. Biol. Chem.* **283**, 27904–27915 (2008).
 39. Haglund, E. et al. The HD-exchange motions of ribosomal protein S6 are insensitive to reversal of the protein-folding pathway. *Proc. Natl Acad. Sci. USA* **106**, 21619–21624 (2009).
 40. Haglund, E. et al. Trimming down a protein structure to its bare foldons: spatial organization of the cooperative unit. *J. Biol. Chem.* **287**, 2731–2738 (2012).
 41. Lindahl, M. et al. Crystal structure of the ribosomal protein S6 from *Thermus thermophilus*. *EMBO J.* **13**, 1249–1254 (1994).
 42. Day, R., Beck, D. A., Armen, R. S. & Daggett, V. A consensus view of fold space: combining SCOP, CATH, and the Dali Domain Dictionary. *Protein Sci.* **12**, 2150–2160 (2003).
 43. Schlutzen, F. et al. Structure of functionally activated small ribosomal subunit at 3.3 angstroms resolution. *Cell* **102**, 615–623 (2000).
 44. Gallagher, T. D., Gilliland, G., Wang, L. & Bryan, P. The prosegment-subtilisin BPN' complex: crystal structure of a specific foldase. *Structure* **3**, 907–914 (1995).
 45. Tangrea, M. A. et al. Stability and global fold of the mouse pro-hormone convertase 1 pro-domain. *Biochemistry* **40**, 5488–5495 (2001).
 46. Tangrea, M. A., Bryan, P. N., Sari, N. & Orban, J. Solution structure of the pro-hormone convertase 1 pro-domain from *Mus musculus*. *J. Mol. Biol.* **320**, 801–812 (2002).
 47. Sari, N. et al. Hydrogen-deuterium exchange in free and prodomain-complexed subtilisin. *Biochemistry* **46**, 652–658 (2007).
 48. Orengo, C. A. & Thornton, J. M. Alpha plus beta folds revisited: some favoured motifs. *Structure* **1**, 105–120 (1993).
 49. Chen, Y. et al. Engineering subtilisin proteases that specifically degrade active RAS. *Commun. Biol.* **4**, 299 (2021).
 50. Lejon, S., Frick, I. M., Bjorck, L., Wikstrom, M. & Svensson, S. Crystal structure and biological implications of a bacterial albumin binding module in complex with human serum albumin. *J. Biol. Chem.* **279**, 42924–42928 (2004).
 51. Sauer-Eriksson, A. E., Keywegt, G. J., Uhlen, M. & Jones, T. A. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. *Structure* **3**, 265–278 (1995).
 52. Alexander, P. A., Rozak, D. A., Orban, J. & Bryan, P. N. Directed evolution of highly homologous proteins with different folds by phage display: implications for the protein folding code. *Biochemistry* **44**, 14045–14054 (2005).
 53. Alexander, P. A., He, Y., Chen, Y., Orban, J. & Bryan, P. N. The design and characterization of two proteins with 88% sequence identity but different structure and function. *Proc. Natl Acad. Sci. USA* **104**, 11963–11968 (2007).
 54. Leaver-Fay, A. et al. ROSETTA3: an object-oriented software suite for the simulation and design of macromolecules. *Methods Enzymol.* **487**, 545–574 (2011).
 55. Delano, W. L. The PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, 2002).
 56. He, Y. et al. Structure, dynamics, and stability variation in bacterial albumin binding modules: implications for species specificity. *Biochemistry* **45**, 10102–10109 (2006).
 57. Shen, Y. et al. De novo structure generation using chemical shifts for proteins with high-sequence identity but different folds. *Protein Sci.* **19**, 349–356 (2010).
 58. Chen, Y. et al. Rules for designing protein fold switches and their implications for the folding code. Preprint at *bioRxiv* <https://doi.org/10.1101/2021.05.18.444643> (2021).
 59. Rozak, D. A., Orban, J. & Bryan, P. N. G148-GA3: a streptococcal virulence module with atypical thermodynamics of folding optimally binds human serum albumin at physiological temperatures. *Biochim. Biophys. Acta* **1753**, 226–233 (2005).
 60. He, Y., Chen, Y., Rozak, D. A., Bryan, P. N. & Orban, J. An artificially evolved albumin binding module facilitates chemical shift epitope mapping of GA domain interactions with phylogenetically diverse albumins. *Protein Sci.* **16**, 1490–1494 (2007).
 61. He, Y. et al. Solution NMR structure of a sheddase inhibitor prodomain from the malarial parasite *Plasmodium falciparum*. *Proteins* **80**, 2810–2817 (2012).
 62. Alexander, P., Fahnestock, S., Lee, T., Orban, J. & Bryan, P. Thermodynamic analysis of the folding of the Streptococcal protein G IgG-binding domains B1 and B2: why small proteins tend to have high denaturation temperatures. *Biochemistry* **31**, 3597–3603 (1992).
 63. Chien, S.-C., Chen, C.-Y., Lin, C.-F. & Yeh, H.-I. Critical appraisal of the role of serum albumin in cardiovascular disease. *Biomark. Res.* **5**, 31 (2017).
 64. Gonzalez-Quintela, A. et al. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin. Exp. Immunol.* **151**, 42–50 (2008).
 65. Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* **41**, 207–234 (2005).
 66. Ruan, B., Fisher, K. E., Alexander, P. A., Doroshko, V. & Bryan, P. N. Engineering subtilisin into a fluoride-triggered processing protease

- useful for one-step protein purification. *Biochemistry* **43**, 14539–14546 (2004).
67. Delaglio, F. et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293 (1995).
 68. Goddard, D. & Kneller, D. G. SPARKY 3 Vol. 3 (University of California, San Francisco, 2004).
 69. Brunger, A. T. et al. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D (Biol. Crystallogr.)* **54**, 905–921 (1998).
 70. Shen, Y. & Bax, A. Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *J. Biomol. NMR* **56**, 227–241 (2013).
 71. Farrow, N. A., Zhang, O., Forman-Kay, J. D. & Kay, L. E. A heteronuclear correlation experiment for simultaneous determination of ^{15}N longitudinal decay and chemical exchange rates of systems in slow equilibrium. *J. Biomol. NMR* **4**, 727–734 (1994).
 72. Montelione, G. T. & Wagner, G. 2D Chemical exchange NMR spectroscopy by proton-detected heteronuclear correlation. *J. Am. Chem. Soc.* **111**, 3096–3098 (1989).
 73. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R. & Thornton, J. M. AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**, 477–486 (1996).
 74. Koradi, R., Billeter, M. & Wuthrich, K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph. Model.* **14**, 51–55 (1996).

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Author contributions

Protein design: Yw.C., B.R., E.C., J.O., P.B.; Performed thermodynamic and binding analyses: B.R., Yw.C., D.M., R.S., P.B.; Performed dynamic light scattering experiments: T.G.; Performed NMR experiments/

structural analysis: Y.H., Yh.C., T.S., T.K., J.O.; Wrote the paper: J.O. (NMR and structural analysis), Yw.C., B.R., P.B. (remaining sections).

Competing interests

The authors declare no competing interests.

Additional information

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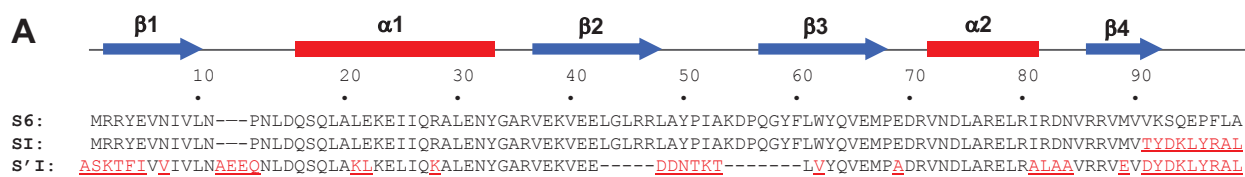
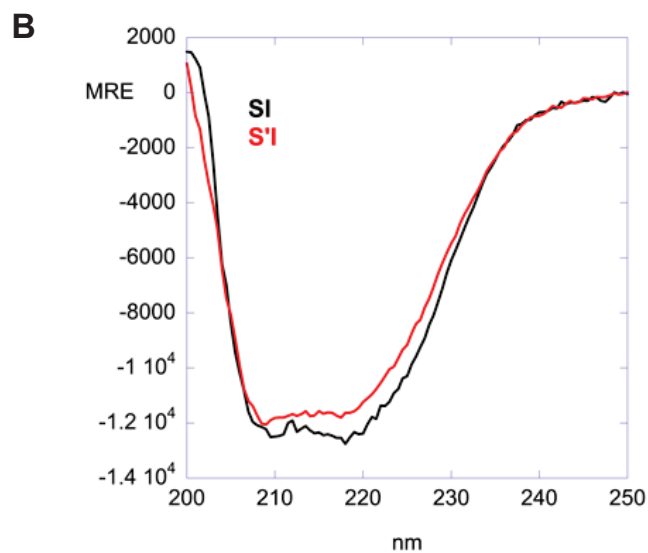
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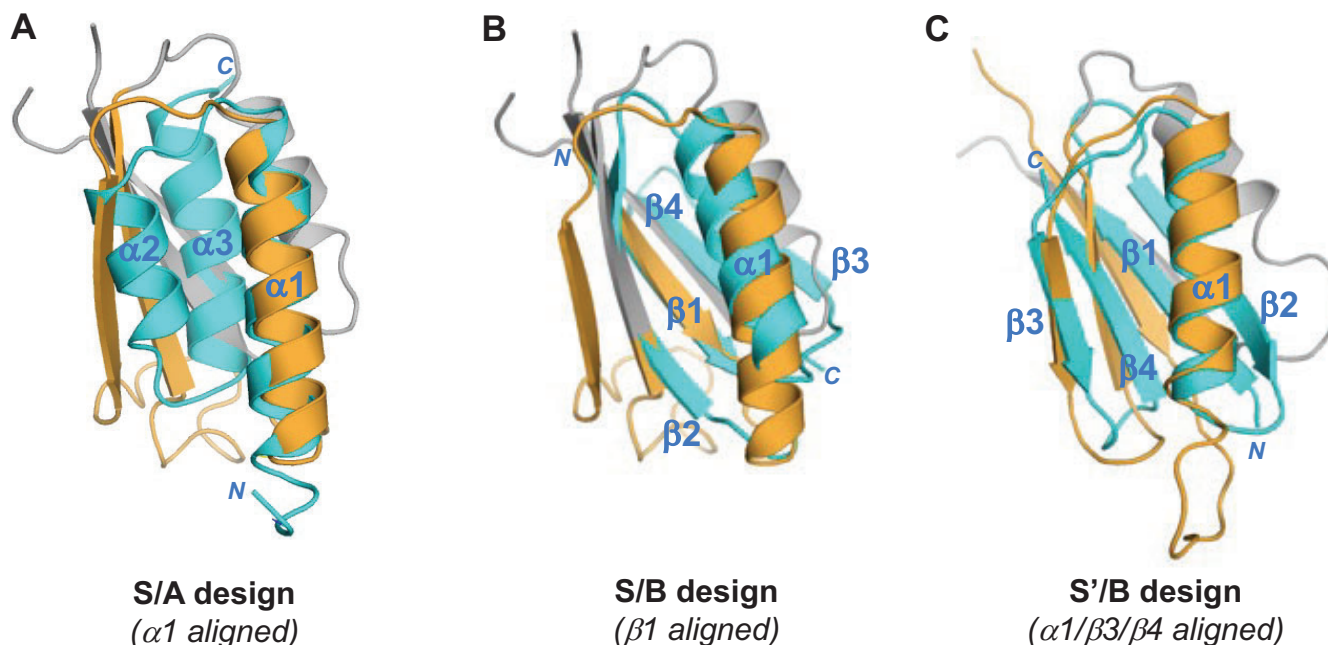
Supplemental Table 1. Fold, stability, and binding properties for each designed protein.

Mutant	Fold	$\Delta G_{\text{folding}}$ at 25°C	Binding ligand	K_i and or K_D
S _I	S	-8.5 kcal/mol	Protease	0.2μM
S _{a1I}	S	-5.3 kcal/mol	Protease	0.05μM
S _{a1}	S	-5.3 kcal/mol	---	
A ₁	A	-4 kcal/mol	---	
S _{a2I}	S	-5.3 kcal/mol	Protease/HSA	0.05μM/100μM
A ₂	A	-4 kcal/mol	HSA	≤ 1μM
S _{b1}	B	-1.1 kcal/mol	---	
B ₁	B	-4 kcal/mol	---	
S _{b2}	S	-4 kcal/mol	---	
B ₂	unfolded	>2 kcal/mol	---	
S' _I	S	-8.5 kcal/mol	Protease	0.06μM
S _{b3I}	S	-3.5 kcal/mol	Protease/IgG	0.05μM/10μM
S _{b3}	S	-3.5 kcal/mol	IgG	10μM
B ₃	B	-1.2 kcal/mol	IgG	≤ 1μM
S _{b4}	Mixed S/B	-4 kcal/mol ¹	IgG	≤ 1μM
B ₄	B	-4.1 kcal/mol	IgG	≤ 1μM
S _{b5}	B	-5 kcal/mol	IgG	10μM

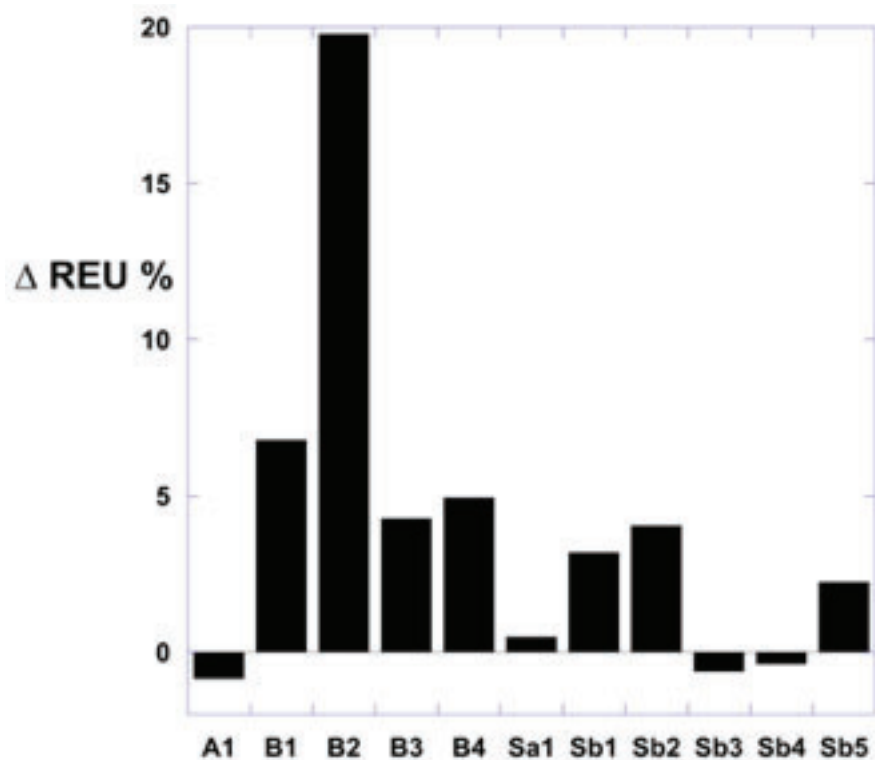
¹ S_{b4} populates both S- and B-folds in approximately equal mixture. Thus, $\Delta G_{\text{B-fold/S-fold}}$ is ~ 0 kcal/mol. This results from the fact that the stabilities for both B- and S-fold species relative to the unfolded state (determined by CD melting) are almost identical (i.e., $\Delta G_{\text{folding}}$ ~ -4 kcal/mol).



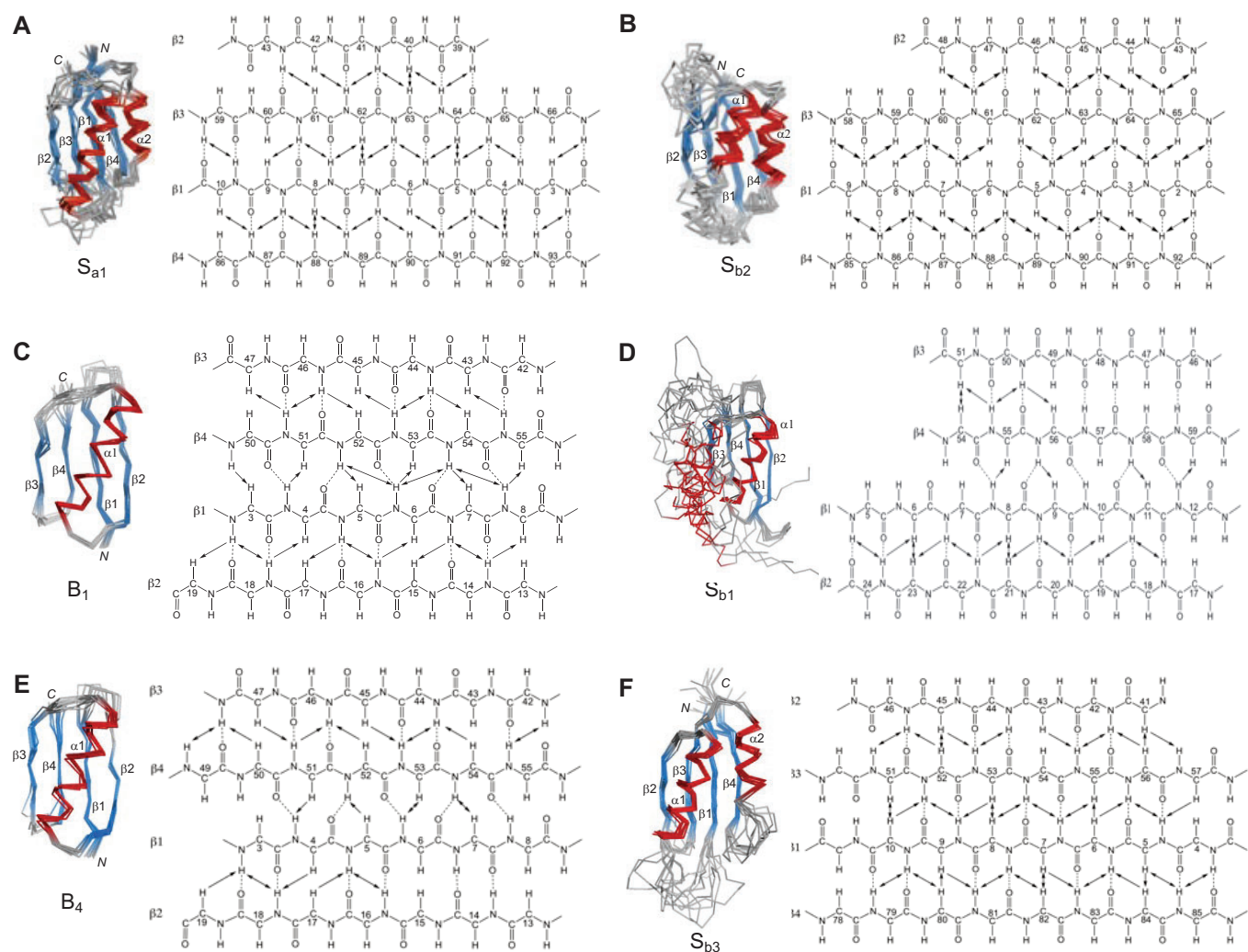
Supplemental Figure 1: Sequence comparison of S6, SI, and S'I. The secondary structure for the parent S6 fold is shown above the alignment. Mutations are shown by red text. CD spectra are compared in panel B. Source data are provided as a Source Data file.



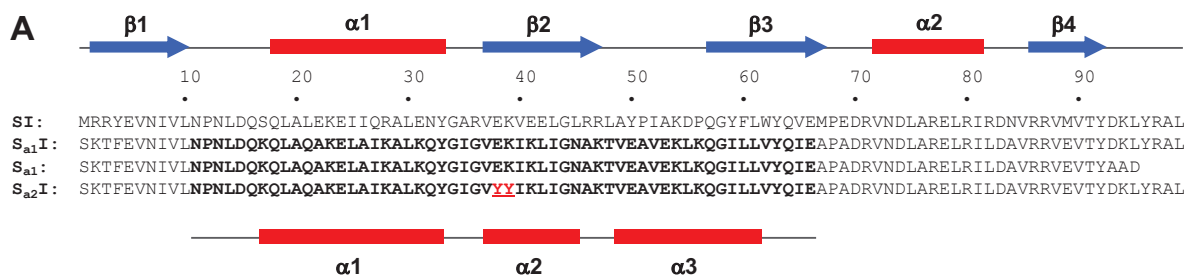
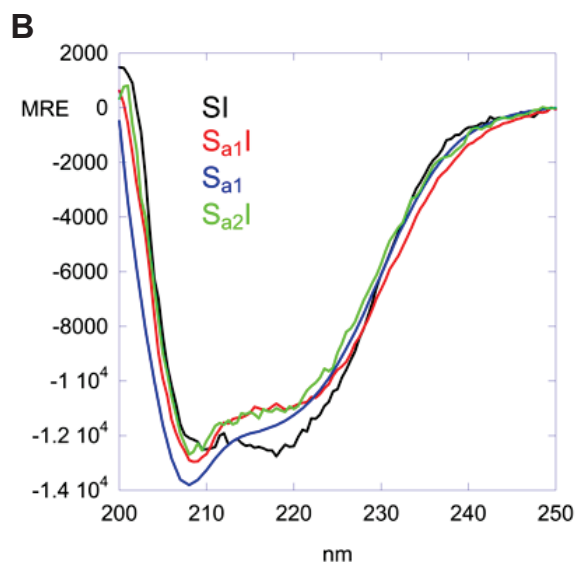
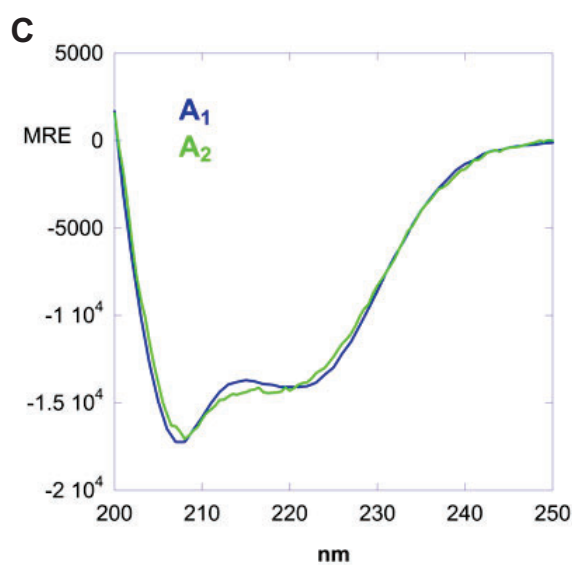
Supplemental Figure 2: Topological alignment of A and B-folds with S-folds. (A) The embedded A-fold occupies amino acid positions 11-66 with 46% identity in the aligned regions for the parent sequences. $\alpha 1(S)$ mostly coincides with $\alpha 1(A)$. $\beta 2(S)$ becomes $\alpha 2(A)$, and the long $\beta 2$ - $\beta 3$ turn and first half of $\beta 3(S)$ becomes $\alpha 3(A)$. $\beta 1$ and $\alpha 2$ - $\beta 4$ of the S-fold are outside of the overlap region. (B) The embedded B-fold occupies amino acid positions 4-59 with 9% identity in the aligned regions for the parent sequences. $\beta 1(S)$ mostly coincides with $\beta 1(B)$. The first half of $\alpha 1(S)$ becomes $\beta 2(B)$ and the second half of $\alpha 1(S)$ becomes the first half of $\alpha 1(B)$. The $\beta 2$ strand of S becomes the second half of $\alpha 1(B)$, a turn, and the first part of $\beta 3(B)$. The long $\beta 2$ - $\beta 3(S)$ turn and the first part of $\beta 3(S)$ become the second part of $\beta 3$ and $\beta 4$ of B. The second half of $\beta 3$ and $\alpha 2$ - $\beta 4$ of the S-fold are outside of the overlap region. (C) The embedded B-fold occupies amino acid positions 1-56 with 16% identity in the aligned regions for the parent sequences (excluding the 3 residue insertion and 12 residue deletion). The $\beta 1$ -strand is the same in both folds but changes orientation, the long turn between $\beta 1$ and $\alpha 1$ in S_{b3} becomes $\beta 2$ of the B-fold, and the $\alpha 1$ - $\beta 2$ - $\beta 3$ of S_{b3} maintains the same topology in both folds. The $\alpha 2$ -helix and the $\beta 4$ -strand of S_{b3} are outside the overlap region.



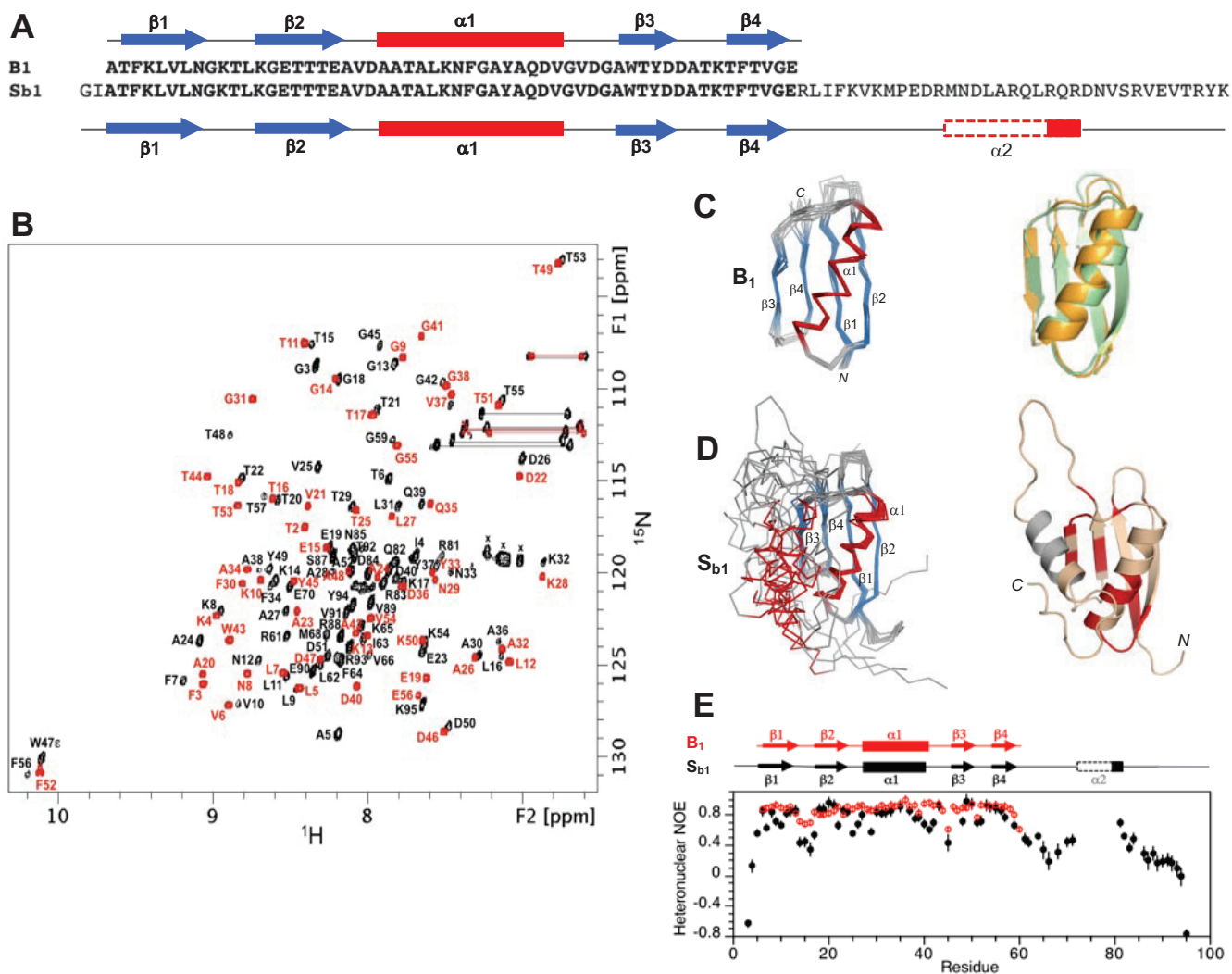
Supplemental Figure 3: The percent change in Rosetta Energy Units (REU) between the parent protein and the designed switch protein is plotted. The computational design of A₁ was compared to the relaxed structure of a highly stable A-fold². The designs of B₁, B₂, B₃, and B₄ were compared to a highly stable B-fold³. The designs of S_{a1}, S_{b1}, S_{b2}, S_{b3}, S_{b4}, and S_{b5} were compared to a highly stable S-fold⁴. All designed proteins have relatively small changes in %ΔREU except for B₂. The 20% increase in REU for B₂ is consistent with its low stability.



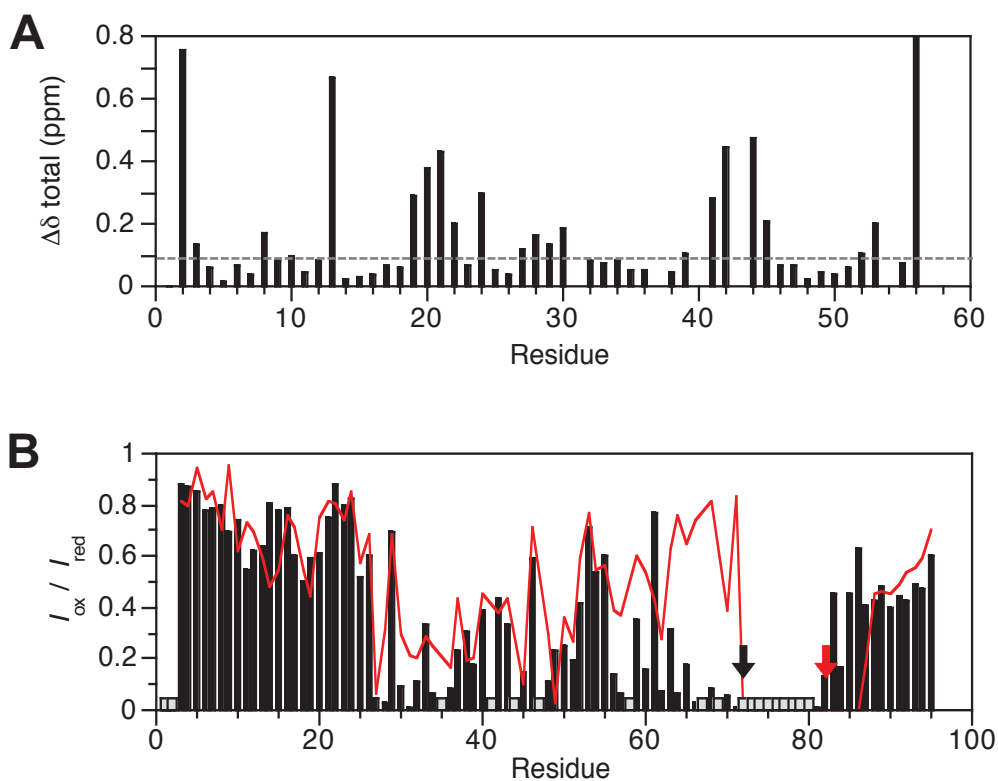
Supplemental Figure 4: Summary of long-range backbone NOEs observed for β -sheets in designed proteins.



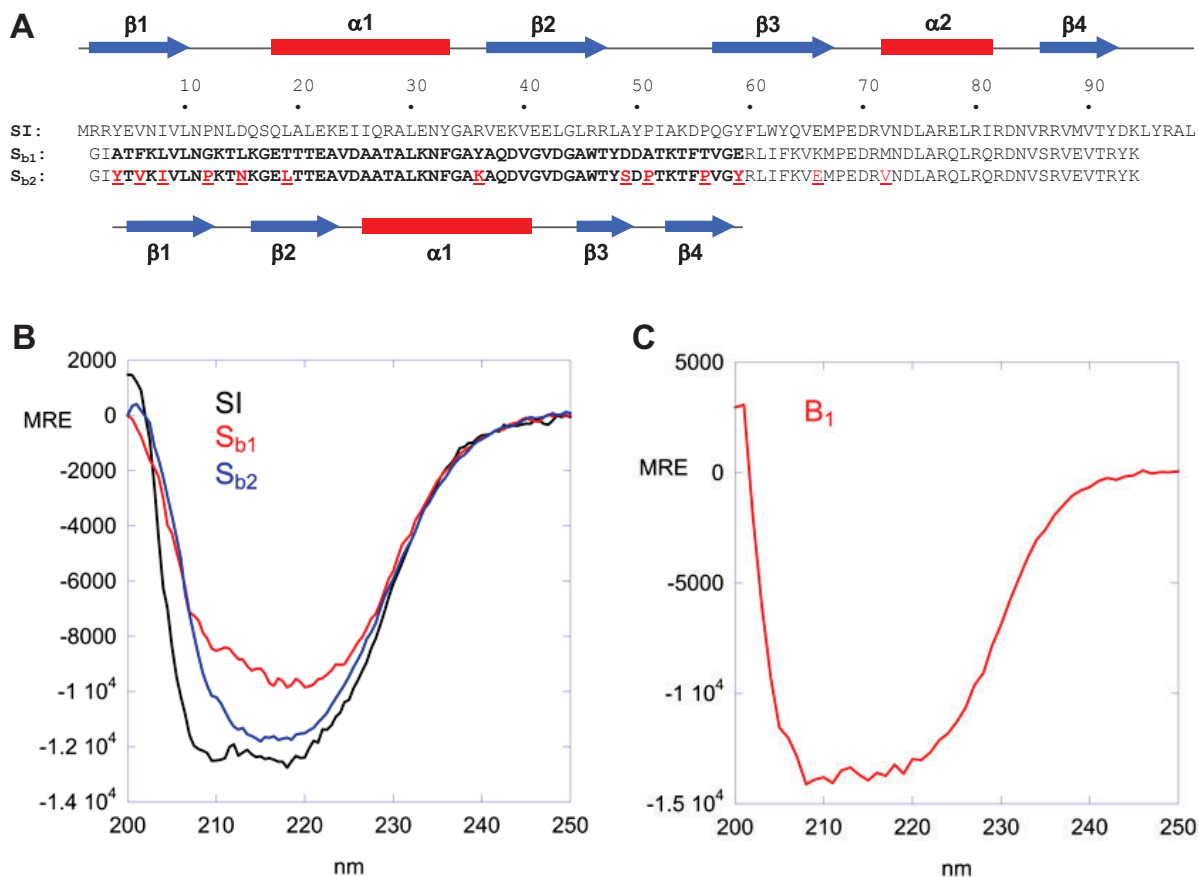
Supplemental Figure 5: Sequence comparison of SI, Sa₁I, Sa₂, and Sa₂I. The secondary structures for the parent S6 and A folds are shown above and below the alignment, respectively. Mutations are shown by red text. CD spectra are compared in panels B and C. Source data are provided as a Source Data file.



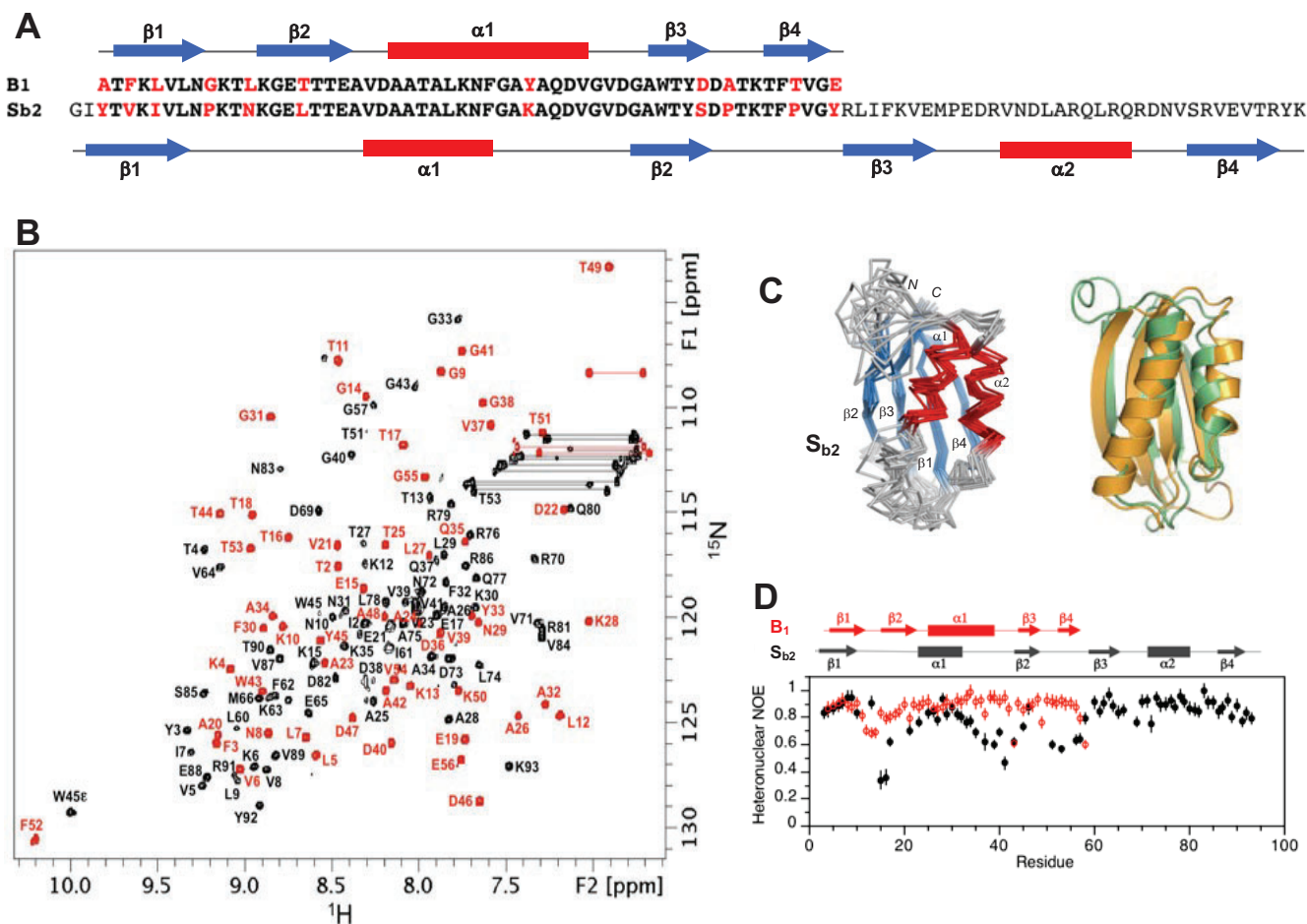
Supplemental Figure 6: Structure and dynamics of B₁ and S_{b1} - S_{b1} has a B-fold. (A) Sequence alignment of B₁ and S_{b1}, which are 100% identical over the B-region. (B) Overlaid two dimensional ¹H-¹⁵N HSQC spectra of S_{b1} (black) and B₁ (red) with backbone amide assignments. Spectra were recorded at 10°C. (C) Ensemble of 10 lowest energy CS-Rosetta structures for B₁ (left panel). Superposition of the B₁ structure (green) with the parent GB1 fold (orange) (right panel). (D) Ensemble of 10 lowest energy CS-Rosetta structures for S_{b1} using chemical shift, NOE, and PRE restraints (left panel). Cartoon representation of model 1 from the ensemble (right panel). Values of $\Delta\delta_{\text{total}} > 0.1$ ppm from Fig. S7A are mapped onto the structure (red). Unassigned residues in the putative $\alpha 2$ -helix are in gray. (E) Plot of $\{^1\text{H}\}$ -¹⁵N steady state heteronuclear NOE values at 600 MHz versus residue for B₁ (red) and for S_{b1} (black). Each set of heteronuclear NOEs was obtained from a single experiment. Errors were estimated based on the measured background noise level.



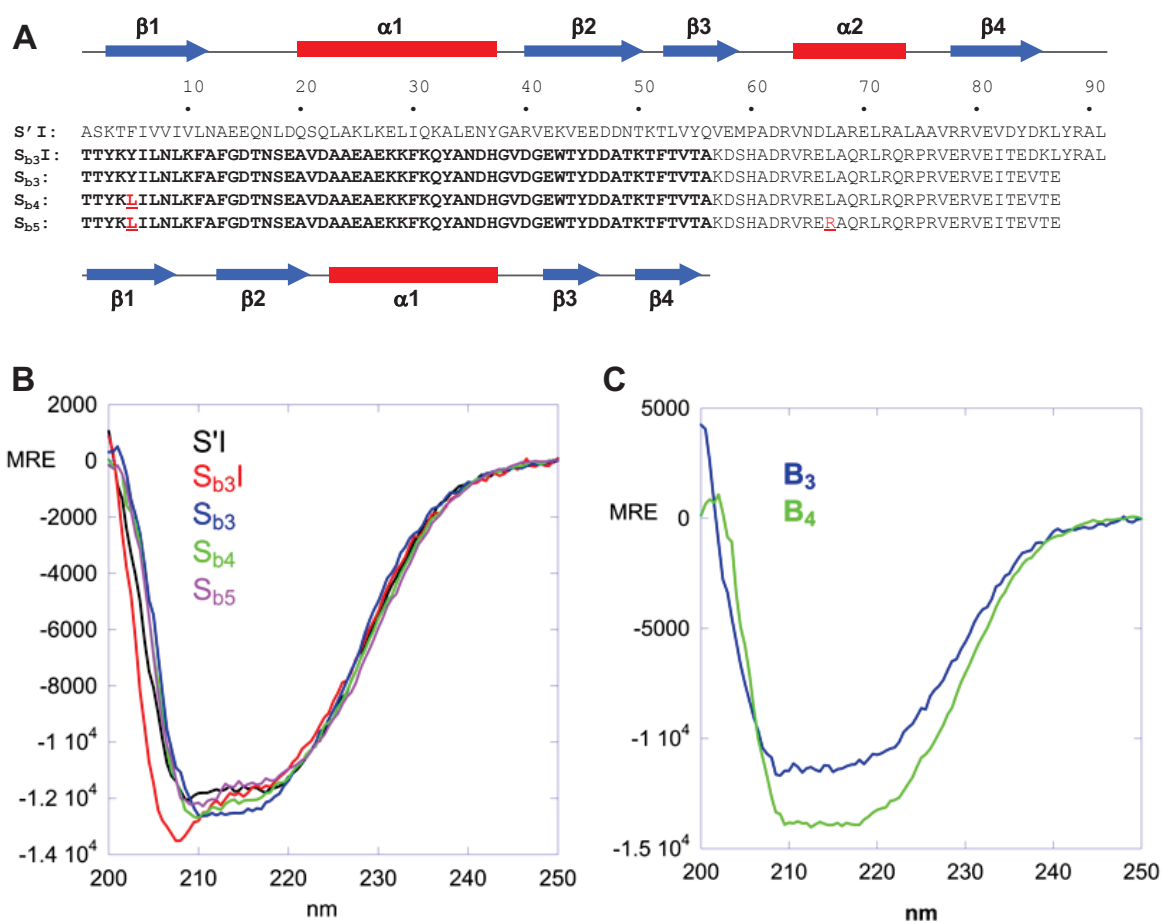
Supplemental Figure 7: Chemical shift perturbation (CSP) and PRE profiles for S_{b1} . (A) Plot of CSPs between S_{b1} and B_1 for backbone amides in the 56 amino acid identical region. Residue numbering is for B_1 . (B) Plot of I_{ox}/I_{red} versus residue for S_{b1} -R72C-MTSL (black) and S_{b1} -R83C-MTSL (red). Similar PRE profiles are observed in the B-folded part of the structure when the spin label is attached to the N- or C-terminus of the exchange-broadened region, which corresponds with $\alpha 2$ in S6. Gray columns indicate unassigned residues or prolines. The positions of the spin labels are indicated with arrows.



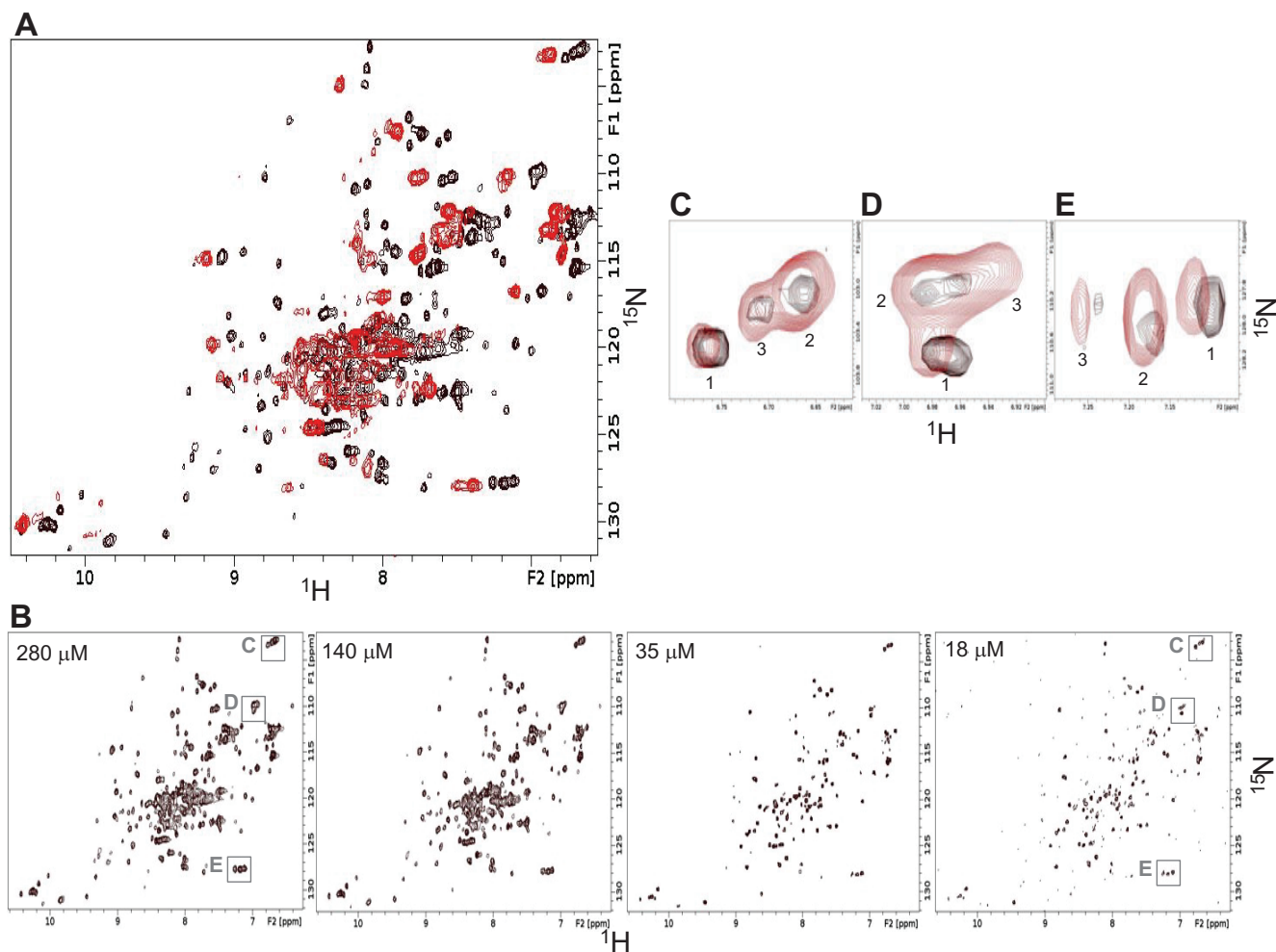
Supplemental Figure 8: Sequence comparison of SI, Sb₁, and Sb₂. The secondary structures for the parent S6 and B folds are shown above and below the alignment, respectively. Mutations are shown by red text. CD spectra are compared in panels B and C. Source data are provided as a Source Data file.



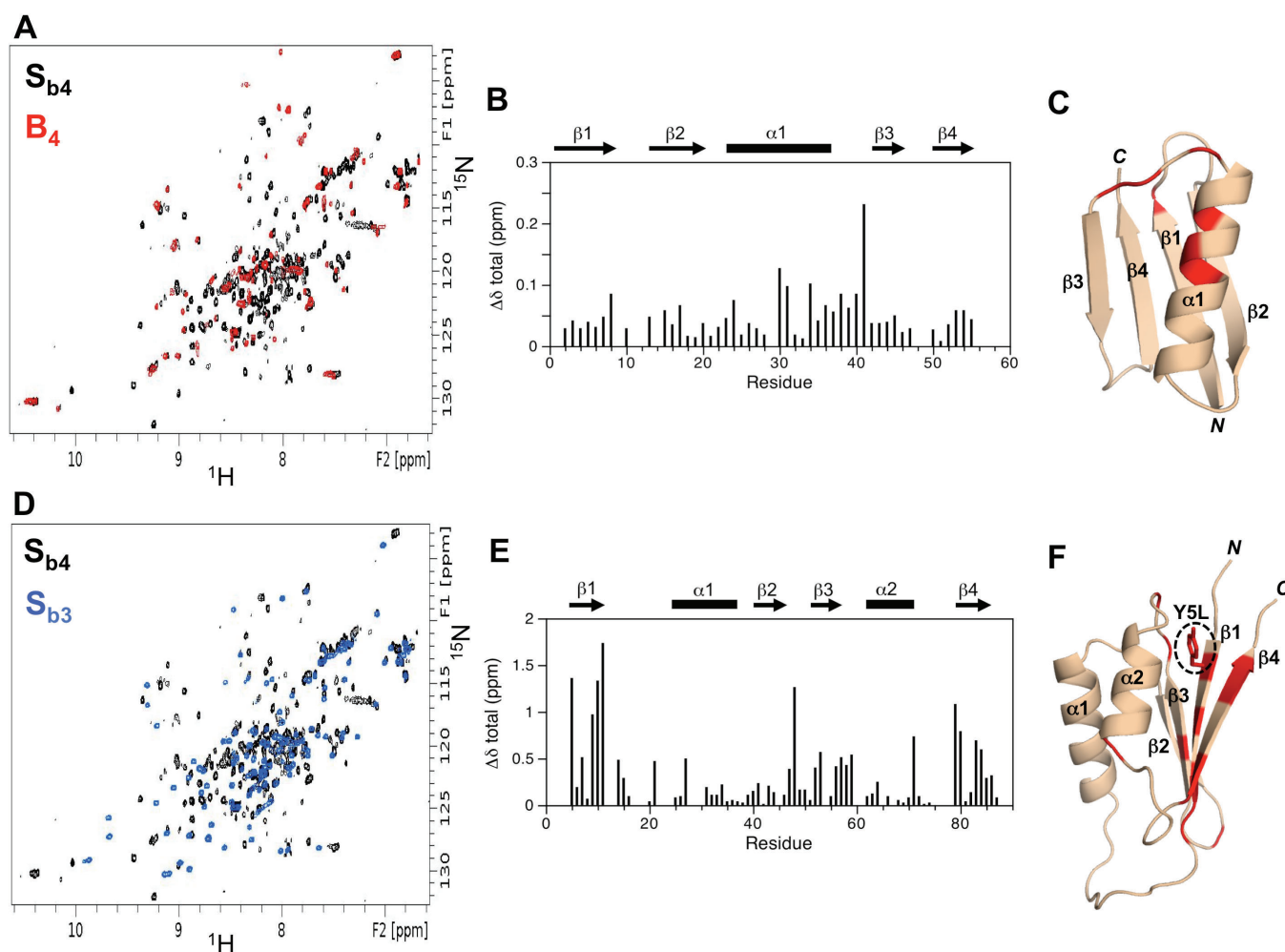
Supplemental Figure 9: Structure and dynamics of S_{b2} – S_{b2} has an S-fold. (A) Sequence alignment of S_{b2} and B_1 . The 11 amino acid differences are shown in red. (B) Overlaid two dimensional ^1H - ^{15}N HSQC spectra of S_{b2} (black) and B_1 (red) with backbone amide assignments. Spectra were recorded at 25°C. (C) Ensemble of 10 lowest energy CS-Rosetta structures for S_{b2} (left panel). Superposition of S_{b2} (green) with the parent S6 fold (orange) (right panel). (D) Plot of $\{^1\text{H}\}$ - ^{15}N steady state heteronuclear NOE values at 600 MHz versus residue for S_{b2} (black). Values are compared with B_1 , which has 80% sequence identity in the corresponding region. Each set of heteronuclear NOEs was obtained from a single experiment. Errors were estimated based on the measured background noise level.



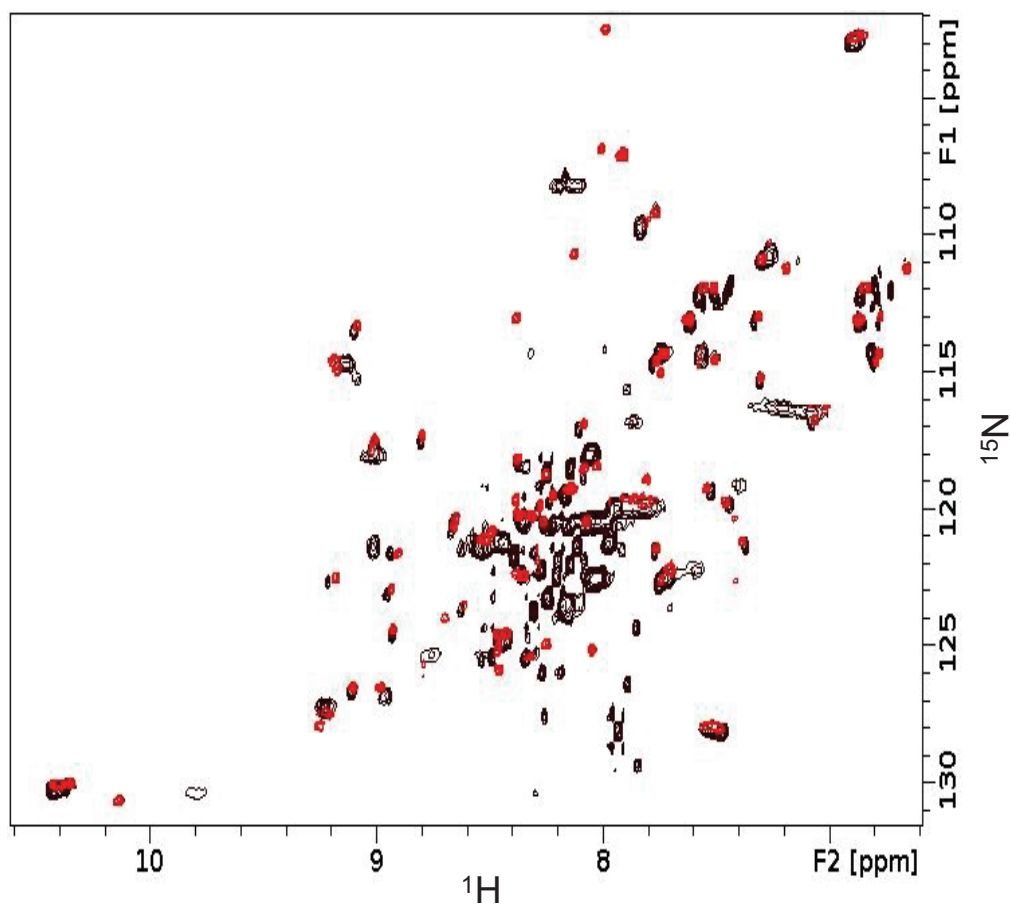
Supplemental Figure 10: Sequence comparison of S'I, S_{b3}, S_{b4}, and S_{b5}. The secondary structures for the parent S6 and B folds are shown above and below the alignment, respectively. Mutations are shown by red text. CD spectra are compared in panels B and C. Source data are provided as a Source Data file.



Supplemental Figure 11: Temperature and concentration dependence of B₃. (A) Overlaid two dimensional ^1H - ^{15}N HSQC spectra of B₃ at 25°C (red) and at 5°C (black). The protein concentration in both spectra is 280 μM . Broadened peaks at 25°C are consistent with low stability. (B) HSQC spectra of B₃ recorded at different concentrations as indicated. All spectra were acquired at 5°C. (C, D, E) Expanded regions from the overlaid concentration dependent spectra of B₃ at 18 μM (black) and 280 μM (red). Regions are as indicated in the leftmost and rightmost panels in (B). The peaks in (C, D, E) are labeled 1, 2 and 3, corresponding to three main concentration-dependent species. At 18 μM , the putatively monomeric species 1 is dominant, while at 280 μM the putatively dimeric species 2 is dominant.



Supplemental Figure 12: Chemical shift comparisons for S- and B-states of S_{b4} with S_{b3} and B₄, respectively. **(A)** Overlaid two-dimensional ¹H-¹⁵N HSQC spectra of S_{b4} (black) and B₄ (red). **(B)** Backbone amide chemical shift differences ($\Delta\delta_{\text{total}}$) between the B-state of S_{b4} and B₄ at 25°C versus residue. The secondary structure of B₄ is shown above the plot. **(C)** Residues with values of $\Delta\delta_{\text{total}} > 0.08$ ppm (red) are mapped onto the structure of B₄. **(D)** Overlaid two-dimensional ¹H-¹⁵N HSQC spectra of S_{b4} (black) and S_{b3} (blue). **(E)** Backbone amide chemical shift differences between the S-state of S_{b4} and S_{b3} at 25°C versus residue. The secondary structure of S_{b3} is shown above the plot. **(F)** Residues with values of $\Delta\delta_{\text{total}} > 0.5$ ppm (red) are mapped onto the structure of S_{b3}. The position of the single amino acid difference between S_{b3} and S_{b4}, Y5 in S_{b3} and L5 in S_{b4}, is highlighted.



Supplemental Figure 13: Two dimensional ^1H - ^{15}N HSQC spectrum of S_{b5} (black) compared with B_4 (red). Both spectra were acquired at 25°C . The significant overlap of folded signals in the two spectra indicates that S_{b5} has a B-fold with a disordered C-terminal tail.

Supplemental description of structures

Structure of B₁. The NMR structure consists of four β -strands defined by residues 2-9 (β 1), residues 13-20 (β 2), residues 42-46 (β 3), and residues 50-55 (β 4) and one α -helix from residues 23-37 (**Fig. S6B,C, Table 1**). The NOE pattern between main chain protons agrees well with a B-fold (**Fig. S4C**). Steady-state $\{^1\text{H}\}\text{-}^{15}\text{N}$ heteronuclear NOE values for B₁ are consistent with a well ordered structure, with the exception of the β 1- β 2, α 1- β 3, and β 3- β 4 internal loops (**Fig. S6E**).

Structure of S_{b1}. The topology of S_{b1} is not the same as the parent S6 structure. Instead, the 2D $^1\text{H}\text{-}^{15}\text{N}$ HSQC spectrum of S_{b1} has a pattern similar to that of B₁ (**Fig. S6B**). NMR assignment of the main chain resonances showed the presence of four β -strands and two α -helices, but the order of the secondary structure elements was $\beta\beta\alpha\beta\beta\alpha$ rather than the $\beta\alpha\beta\beta\alpha\beta$ arrangement expected for an S-type fold. Initial NMR structures of S_{b1} indicated a B-fold, which was supported by backbone NOE connectivities (**Fig. S4D**), with a mostly disordered C-terminal tail. CS-Rosetta modeled residues 73-83 near the C-terminus as an α 2-helix. Of these, amide signals due to residues 73-80 were not detectable in NMR spectra while residues 81-83 were helical based on assigned chemical shifts. Comparison of S_{b1} amide chemical shifts with those of B₁ indicated that most of the perturbations due to the C-terminal 35 amino acid tail were localized in α 1, β 3, and neighboring regions (**Fig. S7A**). This suggested that the putative α 2-helix interacts mostly with the B-fold in these contiguous regions. Mutations R72C and R83C were made at the N- and C-terminal ends of the α 2 region in separate samples of S_{b1} and these proteins were derivatized with the stable nitroxide spin label MTSL. Paramagnetic relaxation enhancement (PRE) measurements (**Fig. S7B**) showed significant decreases in amide peak intensity over the α 1 and β 3 regions for the B-core of S_{b1}, consistent with the chemical shift perturbation data. Furthermore, the PRE intensity profiles were similar regardless of which end of the α 2 region the spin label resided. This suggests that docking of the α 2 region against the B-folded core of S_{b1} is in exchange between multiple states, providing a plausible explanation for why most of the α 2 amide resonances are not detectable. Structures for S_{b1} were re-calculated using additional weak ($<20\text{\AA}$) PRE restraints, showing an ensemble with a well-defined B-core that has a putative α 2-helix packed against it loosely (**Fig. S6D, Table 2**).

Steady-state $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE data for S_{b1} were consistent with the structure (**Fig. S6E**). In particular, the C-terminal tail becomes more ordered around the $\alpha 2$ region, although these heteronuclear NOE values (0.4-0.7) are still below those of well-ordered regions (>0.8). Thus, the structure of S_{b1} may be viewed as a transitory state between the S- and B-folds. With two α -helices packed against a 4-stranded β -sheet, S_{b1} has the same overall two-layer α/β -sandwich architecture as the S-fold but differs in the topological arrangement of secondary structures.

Structure of S_{b2} . The three dimensional structure of S_{b2} contains four β -strands and two α -helices and has the general features of the parent S-fold (**Fig. S9B,C, Table 2**). The ordered regions in the structure are residues 1-9 ($\beta 1$), 23-32 ($\alpha 1$), 43-48 ($\beta 2$), 59-65 ($\beta 3$), 71-80 ($\alpha 2$), and 86-91 ($\beta 4$). While the parent S6 protein [<http://doi.org/10.2210/pdb1RIS/pdb>] and S_{a1} structures are very similar, the S_{b2} structure differs from both in a number of ways despite having the same overall topology. The $\alpha 1$ -helix in S_{b2} is shorter, comprising 10 amino acids compared with 17 amino acids in S_{a1} . Also, the $\beta 2$ -strand forms 4 amino acids later in the S_{b2} polypeptide chain than in S_{a1} . The first residue in the $\beta 2$ -strand of S_{b2} , G43, interacts with E65 in the $\beta 3$ -strand. This represents a two-residue shift in the register of hydrogen bonding between $\beta 2$ and $\beta 3$ in S_{b2} compared with S_{a1} (**Fig. S4B**). As a result of these differences, the loops connecting $\beta 1$ to $\alpha 1$ and $\alpha 1$ to $\beta 2$ are longer in S_{b2} (13 and 10 residues, respectively) than in S_{a1} (5 and 7 residues, respectively). The remainder of the S_{b2} structure encompassing $\beta 1$, $\beta 3$, $\alpha 2$, and $\beta 4$ is very similar to S_{a1} . Heteronuclear NOE dynamics data for S_{b2} were consistent with the NMR structure (**Fig. S9D**). In particular, the relatively long $\beta 1$ - $\alpha 1$, $\alpha 1$ - $\beta 2$, and $\beta 2$ - $\beta 3$ loops were found to be the most flexible on the ps-ns timescale.

- 1 Chen, Y. *et al.* Rules for designing protein fold switches and their implications for the folding code. *bioRxiv*, 2021.2005.2018.444643, doi:10.1101/2021.05.18.444643 (2021).
- 2 He, Y., Chen, Y., Rozak, D. A., Bryan, P. N. & Orban, J. An artificially evolved albumin binding module facilitates chemical shift epitope mapping of GA domain interactions with phylogenetically diverse albumins. *Protein Sci* **16**, 1490-1494 (2007).
- 3 Alexander, P., Fahnestock, S., Lee, T., Orban, J. & Bryan, P. Thermodynamic analysis of the folding of the Streptococcal Protein G IgG-binding domains B1 and B2: why small proteins tend to have high denaturation temperatures. *Biochemistry* **31**, 3597-3603 (1992).
- 4 Haglund, E. *et al.* Trimming down a protein structure to its bare foldons: spatial organization of the cooperative unit. *J Biol Chem* **287**, 2731-2738, doi:10.1074/jbc.M111.312447 (2012).