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Effects of protein size, thermodynamic stability, and net charge on cotranslational folding on the ribosome

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Abbreviations: AP = arrest peptide28531225

Abstract

During the last five decades, studies of protein folding in dilute buffer solutions have produced a rich picture of this complex process. In the cell, however, proteins can start to fold while still attached to the ribosome (cotranslational folding) and it is not yet clear how the ribosome affects the folding of protein domains of different sizes, thermodynamic stabilities, and net charges. Here, by using arrest peptides as force sensors and on-ribosome pulse proteolysis, we show a strong correlation between protein size and the distance from the peptidyl transferase center in the ribosome at which proteins fold. Moreover, an analysis of a large collection of mutants of the *E. coli* ribosomal protein S6 shows that the force exerted on the nascent chain by protein folding varies linearly with the thermodynamic stability of the folded state, and that the ribosome environment strongly disfavors folding of domains of high net-negatively charge.

Introduction

More than 40 years ago Anfinsen postulated that "the native structure is determined only by the protein's amino acid sequence" [1]. His work established the protein folding field, which up until recently has focused almost exclusively on biophysical studies of purified proteins that can be reversibly unfolded/refolded *in vitro*. However, it is becoming increasingly clear that many proteins start to fold cotranslationally, *i.e.*, while still being synthesized on the ribosome [2-4] ; small protein domains may even fold completely inside the ribosome exit tunnel [5,6]. In contrast to *in vitro* folding, cotranslational folding is still a poorly understood process [7], and we lack basic information such as how protein size and net charge relate to where in the exit tunnel a protein starts to fold, and how protein stability impacts cotranslational folding.

Here, using arrest peptides as force sensors [8,9] and on-ribosome pulse proteolysis [10], we have analyzed the cotranslational folding of eight protein domains that display cooperative folding *in vitro*. The domains are of different size and fold type, of different thermodynamic stability, and of different net charge. We find direct correlations between protein size and the location in the ribosome exit tunnel at which a protein folds, and between thermodynamic stability and the pulling force generated on the nascent chain during folding. Further, it appears that nascent chain segments with high net-negative charge are pushed out of the negatively charged ribosome exit tunnel before they fold. These findings establish some basic facts about cotranslational folding and confirm the role of the exit tunnel as an environment that can have a strong impact on protein folding.

Results

Proteins tested

As detailed in Supplementary Tables 1 and 2, we have studied eight protein domains to understand how protein size affects cotranslational folding: the *de novo* designed protein 1FSD (28 residues) [11], a WW domain (35 residues) [12], the *de novo* designed protein EHEE_rd2_0005 (40 residues) [13], protein G (56 residues) [14], a calmodulin fragment (68 residues) [15], ribosomal protein S6 (101 residues), superoxide dismutase 1 (SOD1, 110 residues) [16], and Ileal binding protein (ILBP, 128 residues) [17]. Second, in order to understand the relation between thermodynamic stability and the force generated on the nascent chain by cotranslational folding, we have analyzed sixteen single point mutants of protein S6. Finally, we have assayed four variants of S6 of widely varying net charge [18] to study how the negative electrostatic potential inside the exit tunnel [19,20] affects cotranslational folding.

Folding assays

We have used two assays to follow cotranslational folding of protein domains: an arrest peptide-based assay that makes it possible to detect the tension generated in the nascent chain when a domain folds [5], and an on-ribosome pulse-proteolysis assay where thermolysin resistance is used as an indicator of folding [10].

In the arrest-peptide assay, the domain to be studied is placed *L* residues upstream of the last residue in the arrest peptide (AP), Fig. 1a, and a set of constructs with varying *L* is generated. As illustrated in Fig. 1b, for small *L* there is not enough space in the ribosome exit tunnel to allow folding, and little force is generated on the AP.

At some medium value of L, the chain is just long enough to allow folding if stretched, and the force on the AP is increased. At large L, the domain is already folded when the ribosome reaches the codon at end of the AP, and again little force is generated. Since APs stall translation less efficiently when under tension [9,21], the fraction full-length protein (f_{FL}) produced for constructs with different L reflects the variation in force generated on the AP by the folding reaction, and a plot of f_{FL} vs. L can be used to infer where in the exit tunnel folding occurs during translation [8]. f_{FL} profiles were recorded for each protein by *in vitro* translation in the PURE^{TM} system [22], separation of arrested (A) and full-length (FL) forms of the protein by SDS-PAGE, and quantitation of the respective bands by phosphoimager analysis.

The pulse-proteolysis assay [10] is based on the premise that a short exposure of a stalled ribosome-nascent chain complex to thermolysin will lead to degradation of the protein if it is partly or wholly unfolded, but not if it is folded, Fig. 1c. Examples of SDS-PAGE gels for both assays are shown in Supplementary Fig. S1.

The onset of cotranslational folding correlates with domain size

In order to put the possible relation between protein size and the location in the ribosome exit tunnel where folding commences [4,6,9] on a firm quantitative footing, we selected eight protein domains of different folds and with sizes ranging from 28 to 128 residues for study, Supplementary Tables 1 and 2. We used the moderately strong 17-residue *Escherichia coli* SecM(*Ec*) AP for all eight proteins [23]; in addition we also used the stronger eight-residue SecM(*Ms*) AP from *Mannheimia succiniciproducens* [24] for protein S6. All f_{FL} profiles are shown in Fig. 2.

The f_{FL} profile for FSD1 (a designed 28-residue αββ zinc finger domain that does not require Zn^{2+} to fold) has a maximum already at L_{max} = 17 residues, Fig. 2a, significantly different from the f_{FL} profile previously obtained for the 29-residue zinc finger protein ADR1a that has $L_{max} \approx 25$ residues [5]. It has been suggested that the α-helix in FSD1 is more rigid than the β-hairpin, and there is low cooperativity between the α-helix and the β-hairpin during folding [25]. Circular dichroism and thermal denaturation suggest that the melting temperature of FSD1 mainly reflects the melting of the α-helix [26]. It has also been shown that stretches of α-helix can form deep in the exit tunnel [21,27,28], and it therefore seems likely that the high f_{FL} value at L = 17 residues reflects the formation of the α-helix rather than folding of the whole domain. To confirm that a folding event is responsible for the peak we introduced a point mutation that breaks the α-helix (F21P) in the L = 17 residues construct; this caused f_{FL} to drop to background (from 0.77 to 0.47, red data point).

The WW domain is one of the smallest known β−sheet proteins that can fold autonomously [29]. The f_{FL} profile for the 35-residue WW domain (an antiparallel three-stranded β-sheet) peaks at L_{max} = 23-25 residues and the protein starts to fold at $L_{onset} \approx 22$ residues, Fig. 2b, in line with previous findings [6]. Introduction of the mutation Y19A that reduces the thermodynamic stability of the domain [30] caused a reduction of f_{FL} at L = 25 residues from 0.60 to 0.39 (red data point), as expected if the peak reflects a folding event.

The 40-residue protein EHEE_rd2_0005 (βαββ fold) is an artificial mini-protein produced by two rounds of design and selection, and claimed to be "the most stable minimal protein ever found (lacking disulfides or metal coordination)" [13]. The peak of the force profile has the highest amplitude of all the tested proteins, with f_{FL} = 1 between L = 28-40 residues. Fig. 2c. Folding starts at L_{onset} ≈ 24 residues.

The f_{FL} profile for the 56-residue Protein G (ββαββ fold) [31] has a pronounced peak that starts at L_{onset} ≈ 28 residues and reaches a maximum at L_{max} ≈ 35 residues, Fig. 2d. Previous studies have shown that a tether length of L = 35 residues corresponds to a location at the distal end of the exit tunnel, near a prominent loop in the ribosomal protein uL24 [32] .

To confirm that the peak is due to folding we introduced the destabilizing mutation F52L [33] that decreased f_{FL} from 0.7 to 0.3 at L = 30 residues (red data point).

Wildtype calmodulin contains four Ca^{2+}-binding sites in the so-called EF-hands EF-1 to EF-4. Each EF-hand folds into a helix-loop-helix motif; the 69-residue version of the protein tested here contains only the EF-2 and EF-3 domains [15]. This particular version of calmodulin requires Ca^{2+} to fold and has a well-defined hydrophobic core composed mainly of aromatic residues [15]. When translated in the presence of 3 mM calcium chloride, calmodulin EF-2-EF-3 displays a clear peak in the f_{FL} profile with L_{onset} ≈ 35 residues and at L_{max} ≈ 40 residues, Fig. 2e. No comparable peak is seen when the translation reaction is supplemented with the Ca^{2+} chelator EGTA (0.3 mM), validating folding as the source of the peak.

Ribosomal protein S6 from *Thermus thermophylus* is a 101-residue βαββαβ protein with a ferredoxin-like fold [34]. It has been subjected to multiple *in vitro* folding studies [18,35-39]. The f_{FL} profile of S6 has a peak with $L_{onset} \approx 27$ residues and $L_{max} \approx 28$ residues, Fig. 2f. We also obtained an f_{FL} profile with the stronger SecM(*Ms*) AP and determined the f_{FL} profile at single-residue resolution around L_{max}. With the stronger AP, the peak in the f_{FL} profile is better defined, with $L_{onset} = 28$ residues and $L_{max} = 29$ residues.

Super oxide dismutase 1 (SOD1) plays an important role in neurodegenerative disease [40], and its misfolding and aggregation due to de-metalation has been linked to amyotrophic lateral sclerosis. The version of SOD1 tested in this study has been through a process of loop removal, leaving a model β-barrel that does not bind copper or zinc but still folds cooperatively into a globular domain [16].

The SOD1 f_{FL} profile has a major peak at $L_{max} \approx 45$ residues, Fig. 2g. We further studied the folding of SOD1 using pulse proteolysis [10]. The protein acquires full thermolysin resistance (as measured by the fraction protease-resistant arrested form of the protein, f_{TR}) at $L \approx 40$ residues, coincident with the onset of folding at $L_{onset} \approx 40$ residues seen in the f_{FL} profile.

The Ileal binding protein (ILBP) is the biggest protein analyzed in this study (128 residues). It shows a significant increase in fraction full length at $L_{onset} \approx 40$ residues and a maximum at $L_{max} = 45$-50 residues, Fig. 2h. As was observed with SOD1,

thermolysin resistance reaches its maximum coincident with the onset of folding seen in the f_{FL} profile.

We also tested a variant of ILBP that is unable to fold without ligand [17], in which the two helices that cap the β-barrel were removed and replaced by a small GSGS linker (depicted in Supplementary Fig. S1a). In the absence of ligand, the f_{FL} profile of this variant remains at baseline throughout the range L = 20-60 residues, Supplementary Fig. S1d (red curve).

In contrast, upon ligand binding, the mutant protein displays a similar f_{FL} profile as the wildtype protein, but with somewhat lower amplitude, Supplementary Fig. S1d (gray curve).

Notably, in the presence of ligand the mutant protein has a lower melting temperature (T_m = 41.2°C) than the apo-wildtype-ILBP (T_m = 60.5 °C) [41], supporting the idea that the amplitude of the f_{FL} peak correlates with the thermodynamic stability of the protein.

One notable feature of all the f_{FL} profiles in Fig. 2 is the rather high f_{FL} values for the longest linker lengths compared to previously reported f_{FL} profiles [5,8,32].

The most obvious difference between the constructs analyzed here and those analyzed before is that we use a flexible $(GS)_n$ linker (that is resistant to thermolysin cleavage) rather than a linker derived from the *E. coli* LepB protein with more variable amino acid composition. In contrast to the $(GS)_n$ linker, the LepB-derived linker presumably can make some specific interactions with the ribosomal tunnel that might add to the

overall arrest potency of the SecM AP. Indeed, with the previously used LepB-derived linker, f_{FL} for the 1FSD[L=61] construct was reduced to 0.03, much lower than the value of ~0.5 seen for large L values with the $(GS)_n$ linker, Fig. 2a (blue data point).

In summary, we find strong correlations between protein size and the distance from the peptidyl transferase center at which the protein folds, and between the onset of folding as determined from f_{FL} and f_{TR} profiles.

f_{FL} correlates with thermodynamic stability

Although previous studies suggest a correlation between f_{FL} and the thermodynamic driving force that generates tension in the nascent chain [9,42], this has not been carefully tested for cotranslational protein folding. The S6 protein provides a good model system in this regard, since both thermodynamic stability ($\Delta G_{D\text{-}N}$) and folding/unfolding rate constants (k_f, k_u) have been measured for a large number of point mutants [38].

We therefore measured f_{FL} at linker length L = 30 residues, *i.e.,* at the middle of the peak of the f_{FL} profile for wildtype S6 obtained with the strong SecM(*Ms*) AP, for the 16 S6 mutants listed in Supplementary Table 3. As seen in Fig. 3, there is a good correlation between f_{FL} and $\Delta G_{D\text{-}N}$ (f_{FL} = -0.41 + 0.15 $\Delta G_{D\text{-}N}$; R^2 = 0.70).

A multiple regression analysis including both $\Delta G_{D\text{-}N}$ and log k_f values did not yield a statistically significant improvement in the correlation (p = 0.063, H0 accepted at the

5% level). Folding kinetics thus does not play a significant role in determining f_{FL} values under our experimental conditions. However, since the translation rate is ~10-fold slower in PURE than *in vivo* [43], further studies will be required to fully address this last point.

Net charge and cotranslational folding

In order to determine how the charge of a folded protein domain affects cotranslational folding on the ribosome, we compared the f_{FL} profiles for wildtype S6 and four mutants of different net charge [18], Fig. 4 and Supplementary Fig. S2. Wildtype S6 contains 16 positively charged and 16 negatively charged residues (zero net charge). The $S6^{0}$ variant also has zero net charge but lacks all charged residues. The f_{FL} profile of $S6^{0}$ is broadly similar to that of wildtype S6, but the peak is shifted to somewhat larger L values, Fig. 4c. The f_{FL} profile of the positively charged variant $S6^{+16-9}$ is also similar to the profile for wildtype S6, Fig. 4d, except that the peak is sharper. The removal of half of the positive charges in variant $S6^{+9-16}$ causes the peak in the f_{FL} profile to shift from $L_{max} \approx 28$ to $L_{max} \approx 33$ residues, Fig. 4e, and leads to a general increase in f_{FL} throughout the profile.

Removal of the remaining positively charged residues yields the supercharged but not very stable variant $S6^{-16}$; in this case, $f_{FL} \geq 0.7$ for all L values despite the low stability of the protein, and the profile has a broad maximum at $L_{max} \approx 48$ residues, Fig. 4f.

We also recorded the f_{TR} profiles for wildtype S6 and the supercharged S6^{-16} variant, using the stronger SecM(*Ms*) arrest peptide [24]. The f_{TR} profile for wildtype S6 mirrors the f_{FL} profile, starting to increase at $L \approx 27$ residues and plateauing at $L \approx 32$ residues, Fig. 4g. In contrast, the S6^{-16} f_{TR} profile starts out moderately high at small L values, drops to a lower value at L = 30-45 residues, and finally increases again to reach a plateau at $L > 55$ residues, Fig. 5h.

The relatively high thermolysin resistance of S6^{-16} at small L values is surprising. To analyze this further, we introduced the destabilizing mutation L10A in both wildtype S6 and S6^{-16} at linker lengths L = 17 and L = 57 residues. This mutation reduces the folding free energy of wildtype S6 from 8.97 kcal/mol to 4.62 kcal/mol [38]. The destabilization of S6^{-16} caused by the mutation has not been measured, but the folding free energy of S6^{-16} itself is only 2.9 kcal/mol [18]. As seen in Fig. 4g-h, f_{TR} of wildtype S6 was only marginally affected by the mutation – as expected, since even the mutated protein is relatively stable – but was strongly reduced for S6^{-16}, at both tether lengths. Taken together, the f_{FL} profiles and the thermolysin-resistance data suggest that S6^{-16} is folded into a marginally stable structure at $L > 50$ residues. We cannot readily explain the relatively high thermolysin resistance seen at small L values, however; the reduction in f_{TR} caused by the L10A mutation in the S6^{-16}[L=17] construct would be consistent with some kind of early folding intermediate, but there is no hint of a folding intermediate at this linker length for wildtype S6.

We conclude that S6 versions that contain many positively charged residues (wildtype and $S6^{+17-9}$) start to fold inside the ribosome exit tunnel, while those with few or no positively charged residues ($S6^{0}$ and $S6^{+9-16}$) fold in the mouth of the tunnel. There is a hint from both the f_{FL} and f_{TR} profiles that the supercharged $S6^{-16}$ version folds outside the exit tunnel, around $L \approx 50$ residues. For the negatively charged versions $S6^{+9-16}$ and $S6^{-16}$ f_{FL} values are high for all linker lengths, likely reflecting a strong electrostatic repulsion between the negatively charged exit tunnel and the nascent chain [19,20,44]. Electrostatic repulsion could also explain why folding starts at progressively longer linker lengths as the net charge of S6 becomes more negative.

Discussion

It was long thought that the ribosomal exit tunnel is an inert conduit for the nascent chain in its passage towards the cytosol and is too tight to allow proteins to fold. In the last 5 to10 years this view has changed dramatically, and the exit tunnel is now recognized as an environment that can have strong effects on protein folding. In this study, we have focused on understanding how the ribosome modulates cotranslational folding depending on three central protein characteristics: size, thermodynamic stability, and net charge.

Our findings on the relation between domain size and the location in the ribosome exit tunnel where folding commences are summarized in Fig. 5, which includes not only data from the present study but also from previous studies on ADR1a [5], spectrin domains [8], a penta-repeat β-helix protein [45], DHFR [46], the I27 [32] and FNL5 [47] Ig-domain proteins, and the designed protein Top7 [48], see Supplementary Table 4.

Small domains (≤ 65 residues in size) invariably start to fold while still located inside the tunnel (L_{onset} < 35 residues), and the smaller they are the deeper in the tunnel they fold. Large domains tend to fold in the mouth of, or even outside, the tunnel (L_{onset} ≥ 35 residues), but, depending on the location of the first accessible folding nucleus in the structure, may also start to fold while still well inside the tunnel, as exemplified by the spectrin domains [8]. Fig. 5 provides the most complete picture to date of how the gradual increase in tunnel dimensions from the so-called constriction site (~25 Å from the peptidyl transferase site) to the tunnel exit allows protein domains of increasing size to fold.

For the largest proteins analyzed, SOD1 and ILBP, in addition to a main folding peak at L = 45-50 residues, we see an early increase in f_{FL} at L = 25-30 residues that might be indicative of a folding intermediate, Fig. 2g-h. A similar increase in f_{FL} at small L values was seen previously for the 189-residue protein DHFR [46]. *In vitro*, SOD1 does not seem to fold via an intermediate state, but displays two-state behavior [16]. Further studies will be necessary to characterize these putative folding intermediates.

Cotranslational folding of the exceptionally stable 40-residue protein EHEE_rd2_0005 generates sufficient force on the AP to yield f_{FL} = 1.0 over the whole range L = 28-40 residues, Fig. 2c, setting it apart from the other proteins studied here. This and previous observations [42,46] suggest that the amplitude of the peak in the f_{FL} profile reflects the thermodynamic stability of the folded protein. Indeed, there is a good correlation between f_{FL} and $\Delta G_{D\text{-}N}$ for a large set of S6 mutants, Fig. 3.

Since different proteins fold in different locations inside or outside the exit tunnel and presumably push against the ribosome in different ways during folding, we do not expect the precise quantitative relation between f_{FL} and $\Delta G_{D\text{-}N}$ found for S6 (see above) to hold for other proteins, but only that the general form of the correlation applies.

Our results for protein S6 variants of different charge content show that protein net charge has a dramatic effect on the f_{FL} profile, Fig. 4. In general, the higher the content of positively charged residues, the deeper in the exit tunnel the protein folds. We further note that the f_{FL} profile for the fully uncharged $S6^{0}$ variant is similar to that of wildtype S6, demonstrating that a protein that is devoid of charged residues can fold within the exit tunnel. To our knowledge, this is only the second example of efficient folding of a protein completely devoid of charged residues [49]. Strikingly, for the negatively charged variants $S6^{+9\text{-}16}$ and $S6^{-16}$, high values of f_{FL} persist to much larger L values than for the neutral and positively charged variants, and the peaks in the f_{FL} profiles are broad and indistinct. Thermolysin resistance at high L values is markedly lower for $S6^{-16}$ than for wildtype S6, Fig. 4, possibly reflecting the lower thermodynamic stability of $S6^{-16}$. Mutagenesis results suggest that $S6^{-16}$ is nevertheless folded at L = 57 residues.

Given the net-negative charge of the exit tunnel [19,20,50] we hypothesize that the high f_{FL} values seen for $S6^{+9\text{-}16}$ and $S6^{-16}$ are caused by negatively charged residues in the nascent chain being pushed out of the exit tunnel by electrostatic forces even before

the protein folds, consistent with ribosome-profiling data showing that stretches of nascent chain of high net-negative charge show reduced ribosome densities [51] .

More generally, the fact that domains of up to ~65 residues in size can fold inside the exit tunnel and therefore should be less likely to aggregate or require chaperones for folding might favor the evolution of proteins composed of small (sub)-domains that can initiate early folding inside the ribosome. In support of such a scenario, statistical analyses have identified evolutionarily conserved clusters of rare codons located some 20-50 codons downstream of independently folding sub-domains that may facilitate their cotranslational folding by slowing down translation [52,53].

Finally, the observation that cotranslational folding generates force on the nascent chain raises the possibility that new sensor systems composed of a ligand binding domain coupled to an arrest peptide may be found in nature, or be constructed for biosensing purposes. In principle, the calmodulin EF-2-EF-3 and ILBP constructs analyzed here could be used as Ca^{2+} and glycochenodeoxycholic acid sensors, respectively, if fused to, *e.g.*, GFP [6].

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Author contributions

JF and GvH conceived the project. JF and GvH designed the experiments, which were performed by JF, FRS, IM. JF and GvH wrote the manuscript.

Materials and Methods

Enzymes and chemicals

Enzymes were purchased from Thermo Scientific (Waltham, MA, USA) and New England Biolabs (Ipswich, MA, USA). Thermolysin from *Geobacillus stearothermophilus* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). DNA/RNA purification kits were obtained from Qiagen (Hilden, Germany). The New England Biolabs PURExpress® In Vitro Protein Synthesis Kit was purchased from BioNordika (Stockholm, Sweden). [^{35}S]-methionine was purchased from PerkinElmer (Waltham, MA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

DNA manipulations

The genes of the full sequence design (FSD-1), Pin1 WW domain (*Homo sapiens*), and EHEE design were assembled from oligonucleotides [54]. The gene for protein G (*Streptococcus sp*.) was purchased from Genewiz (South Plainfield, NJ). The wildtype calmodulin gene (*Homo sapiens*) was kindly donated by Sara Linse (Lund University) and modified by an adaptation of the original QuickChange site-directed mutagenesis protocol [55] to obtain the fragment tested in this study. Superoxide dismutase 1 (SOD1, *Homo sapiens*) gene [16] was kindly donated by Mikael Oliveberg (Stockholm University). The gene for the Ileal Binding protein (ILBP) from rabbit (*Oryctolagus cuniculus*) was purchased from Invitrogen (Carlsbad, California, United States) and was truncated by an adaptation of the original QuickChange site-directed mutagenesis protocol [55].

The gene for ribosomal protein S6 from *Thermus thermophiles*, along with the genes for the five of its mutants [18], were provided by Mikael Oliveberg.

The constructs were cloned in a pET19b vector under the control of a T7 promoter. As shown in Supplementary Table 2, the small domain (FSD-1, Pin1 WW domain and EHEE) constructs contained a sequence corresponding to an N-terminal unstructured fragment of 158 residues from *E. coli* LepB (Ismail et al., 2012) (to facilitate visualization by SDS/PAGE), a sequence corresponding to a $(GS)_n$ flexible linker of 43 or 52 residues (to reach a 60-residues long tether length together with the AP), the sequence corresponding to the SecM AP from *E. coli* (FSTPVWISQAQGIRAGP) [56] (referred here as SecM(*Ec*)) or *M. succiniciproducens* (HAPIRGSP) [57] (referred here as SecM(*Ms*)) and a sequence corresponding to a 23-residues long LepB-derived C-terminal fragment (to distinguish between full-length and arrested version of proteins after SDS/PAGE). The genes were inserted into the expression vector by Gibson assembly [58] . Constructs with increasingly shorter tether length starting from the N-terminal part of the flexible linker and control constructs for the full-length (SecM(*Ec*) FSTPVWISQAQGIRAGA, SecM(*Ms*) HAPIRGSA) and arrested (SecM(*Ec*): FSTPVWISQAQGIRAG*, SecM(*Ms*): HAPIRGS*; where * is a stop codon) forms of the proteins were generated by an adaptation of the original QuickChange site-directed mutagenesis protocol [55]. PCR amplified products were subjected to DpnI digestion and purified with GeneJET PCR purification Kit or GeneJET Gel Extraction Kit prior to Gibson assembly. *E. coli* DH5α competent cells were transformed and plated onto LB agar plate supplemented with carbenicillin. Single colonies were picked to inoculate LB supplemented with ampicillin and were incubated for 12-18 hours at 37°C with shaking. GeneJET Plasmid Miniprep Kit was

used for plasmid isolation and purification. All constructs were verified by sequencing (Eurofins Genomics).

In vitro transcription and translation

Linear constructs were obtained by PCR amplification using oligonucleotides overlapping the T7 promoter and terminator on purified plasmids. After DpnI digestion and purification (GeneJET PCR Purification Kit, Thermo Scientific), the linear constructs were used as template for protein expression. The NEB PURExpress In Vitro Protein Synthesis Kit was used for *in vitro* transcription and translation. The reaction mix was supplemented with [^{32}S]-Methionine and the synthesis of the radio-labelled proteins was performed at 37°C, for 15 minutes, under constant shaking at 700 rpm in an Eppendorf 5417R thermomixer. The reaction was stopped by addition of 1:1 volume of 10% ice-cold TCA and the samples were incubated on ice for at least 30 minutes. After centrifugation at 14,000 rpm for 5 minutes at 4°C, the supernatant was discarded and the pellet was re-suspended in sample buffer at 37°C for 15 minutes, under constant shaking at 900 rpm. The samples were treated with RNase I (670 μg/mL) at 37°C for 30 minutes, and were subsequently resolved by SDS/PAGE. All expressions were performed in triplicates.

On-ribosome pulse-proteolysis

After synthesis of radio-labelled proteins as described in the previous section, the reactions were stopped by the addition of chloramphenicol (instead of TCA) at a final concentration of 3.3 mM. Each sample was divided in two aliquots of equal volume, one for pulse-proteolysis and the other serving as a non-treated control. Pulse-proteolysis was performed by treatment with thermolysin (buffered in 50mM Tris,

500μM $ZnCl_2$, pH7.0) at a final concentration of 0.75 mg/m for 1 min. at 37°C, under constant shaking at 700 rpm. The same conditions were applied for the non-treated controls with the difference that thermolysin was not included in the buffer solution. The reaction was stopped by addition of 3μl 500mM EDTA (pH 8.4), and protein precipitation was performed by addition of 1:1 volume of ice-cold 10% TCA and incubation on ice for 30 minutes. As described in the previous section, after centrifugation at 14,000 rpm for 5 minutes, at 4°C, the supernatant was discarded and the pellet was re-suspended in sample buffer at 37°C for 15 minutes, under constant shaking at 900 rpm. The samples were supplemented with RNase I (670 μg/mL) at 37°C, for 30 minutes, and were subsequently resolved by SDS/PAGE. All pulse-proteolysis assays were performed in triplicates.

Gel electrophoresis and quantitation

Proteins were resolved by SDS-PAGE and visualized on a Fuji FLA-3000 phosphoimager. For f_{FL} measurements, the bands were quantified to estimate the fraction full-length protein $f_{FL} = I_{FL}/(I_{FL} + I_A)$, where I_{FL} is the intensity of the band corresponding to the full-length protein and I_A is the intensity of the band corresponding to the arrested form of the protein.

For pulse proteolysis measurements the thermolysin-resistant fraction resistant was calculated as: $f_{TR} = I_{ATR} / I_{ABuff}$, where I_{ATR} is the intensity of the arrested band after pulse proteolysis and I_{ABuff} is the intensity of the non-treated control band. Bands were quantitated using ImageJ (http://rsb.info.nih.gov/ij/) to obtain an intensity cross

section, which was subsequently fit to a Gaussian distribution using in-house software.

Homology modelling of ILBP mutant

The structure (PBD; 2lba) of chicken Ileal bile acid-binding (ILBP) protein in complex with glycochenodeoxycholic acid (GDCG) was used to produce a homology model [59] of the truncated mutant (ILBP-tm) protein used in this study (59.6 % Seq. Id with 2lba PDB template). We show the estimated position of the ligand in stick representation GDCG in Supplementary Fig. 1a.

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Figure legends

Figure 1

(a) Schematic representation of *in vitro* translated constructs. The protein under study is placed L residues upstream of the last proline in the arrest peptide (AP), and a C-terminal 23-residue extension is added downstream of the arrest peptide to ensure that the arrested and full-length forms of the construct can be cleanly separated by SDS-PAGE. (b) The arrest-peptide based assay: at linker length L_1, the protein is too deep in the exit tunnel to be able to fold, hence no force is generated on the AP and mostly arrested nascent chains are produced ($f_{FL} \approx 0$). At L_2, if the linker is stretched beyond its equilibrium length the protein can just reach a location in the exit tunnel where there is sufficient space for it to fold. Some of the folding free energy is therefore stored as elastic energy in the linker, increasing the force on the AP. More full-length proton is produced ($f_{FL} > 0$). At L_3, finally, the protein is already folded when the ribosome reaches the C-terminal end of the AP, and little force is exerted on the AP ($f_{FL} \approx 0$). (c) The on-ribosome pulse proteolysis assay: at L_1, the protein is located too deep in the exit tunnel to be able to fold and the nascent chain is hence degraded by a brief thermolysin pulse. At L_2, the protein is folded and hence resistant to proteolysis. Note that the protein is attached to the ribosome via a $(GS)_n$ linker that in itself is insensitive to thermolysin.

Figure 2

Fraction full-length protein (f_{FL}) and thermolysin resistant protein (f_{TL}) plotted as function of L for the eight proteins discussed in the text. Each data point is an average

of three independent experiments (error bars indicate SEM values). (a) FSD1. The red data point at L = 17 residues is for the destabilizing mutation F21P. The blue data point at L = 61 residues is for a construct where the $(GS)_n$ linker was replaced by a linker derived from the LepB protein (see Methods). (b) WW domain. The red data point at L = 25 residues is for the destabilizing mutation Y19A. (c) Designed protein EHEE_rd2_0005. (d) Protein G, the red data point at L = 30 residues is for the destabilizing mutation F52L. (e) Calmodulin EF-2-EF-3. The black profile was obtained in 3 mM $CaCl_2$, and the red profile in 0.3 mM EGTA. (f) Ribosomal protein S6. The black profile was obtained with the SecM(*Ec*) AP, and the gray profile with the SecM(*Ms*) AP. (g) SOD1 (lacking the metal-loading loops IV and VII). The full curve (squares) is the f_{FL} profile and the dashed curve (triangles) is the thermolysin-resistance profile (f_{TR}). (h) ILBP. The full curve (squares) is the f_{FL} profile and the dashed curve (triangles) is the thermolysin-resistance profile (f_{TR}).

Figure 3

Correlation between f_{FL} and $\Delta G_{D\text{-}N}$ values for wildtype S6 and 16-point mutations listed in Supplementary Table 3. The best-fit linear regression line is shown. Statistical analysis was performed using the StatPlus:mac Pro software.

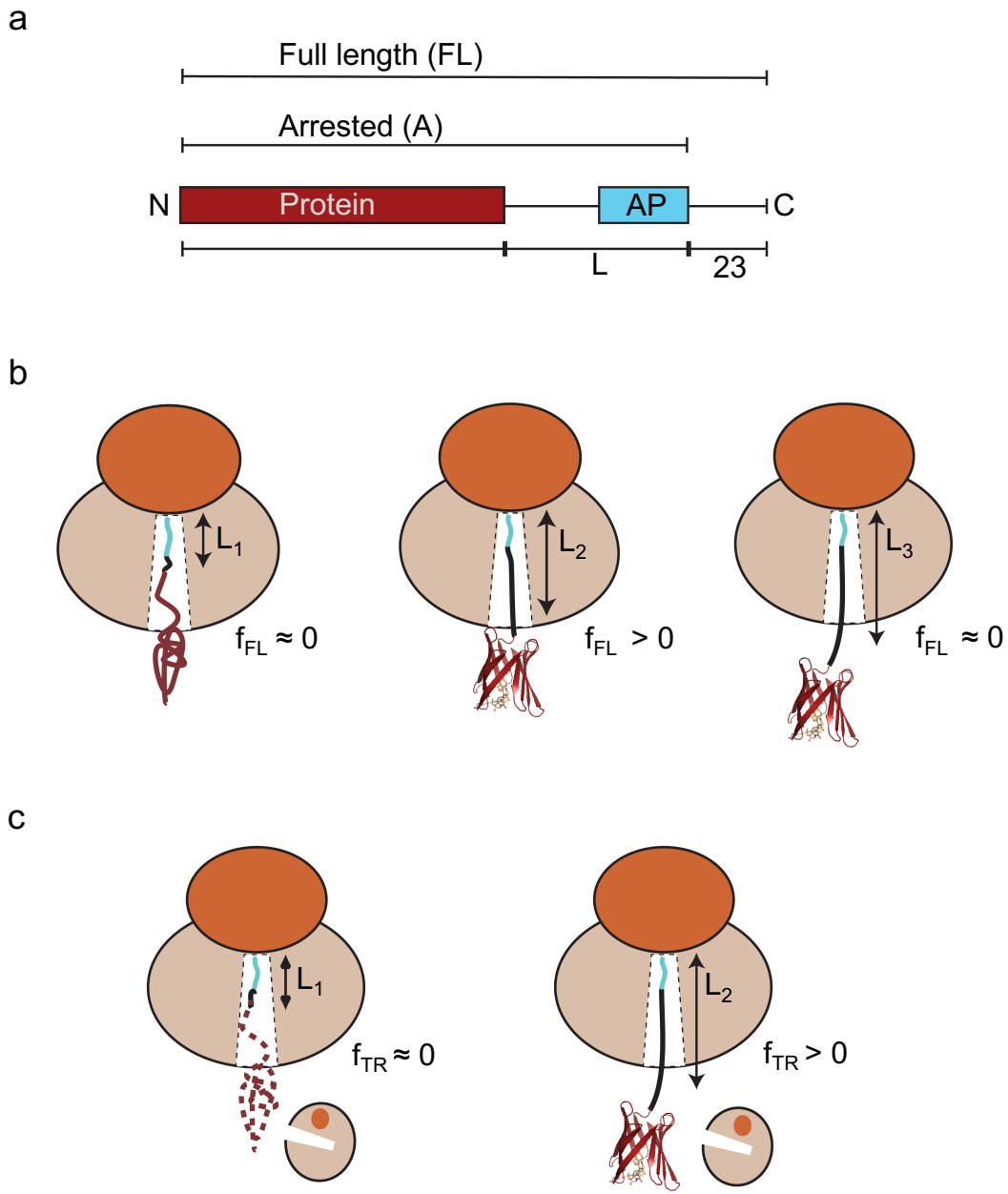
Figure 4

(a) NMR structure of wildtype S6 from *Thermus thermophilus* (PDB 2kjv). Positively charged residues (Arg+Lys) are colored in blue and negatively charged residues (Asp+Glu) in red. (b) f_{FL} profile of wildtype $S6^{+16\text{-}16}$ (for sequences, see Supplementary Figure S2). (c) f_{FL} profile of $S6^{0}$. (d) f_{FL} profile of $S6^{+16\text{-}9}$. (e) f_{FL}

profile of $S6^{+9-16}$. (f) f_{FL} profile of $S6^{-16}$. g) f_{FL} (squares, solid curve) and f_{TR} (triangles, dashed curve) profiles for wildtype S6. Red data points at L = 17 and 57 residues are for the destabilizing mutation L10A. (h) f_{FL} (squares, solid curve) and f_{TR} (triangles, dashed curve) profiles for $S6^{-16}$. Red data points at L = 17 and 57 residues are for the destabilizing mutation L10A. The SecM(Ec) AP was used in panels *a-f* and the SecM(*Ms*) AP in panels *g* and *h*.

Figure 5

Relation between protein size (in residues) and L_{onset} (*i.e.*, the L value corresponding to the half-maximal peak height on the up-side of the peak). The shaded gray indicates the approximate location of the mouth of the exit tunnel, and numbers on the right indicate the approximate distance (in Å) from the peptidyl transferase center. Error bars of ±2.5 residues have been added to indicate that the f_{FL} profiles in Fig. 2 were recorded with a 5-residue resolution, hence introducing an uncertainty in the L_{onset} values. Proteins are indicated as follows: 1 - FSD1, 2 - ADR1a1, 3 – WW domain, 4 - EHEE, 5 - Protein G, 6 - Calmodulin EF-2-EF-3, 7 - Penta-repeat protein, $8^{¶}$ - $S6^{+16-9}$, 8^{ω} - S6 wildtype, 8^{*}- $S6^{+16-9}$, $8^{\&}$- $S6^{0}$, $8^{\#}$ - $S6^{-16}$, 9^{ω} - spectrin β16, 9^{*} - spectrin R16, $9^{\#}$ - spectrin R15, 10 - I27, 11 - SOD1, 12 - ILBP, 13 - DHFR. Top7 and FLN5 are indicated by a green and a red X (for these two proteins L_{onset} was determined using, respectively, GFP fusions and NMR, while for all other proteins f_{FL} profiles were used.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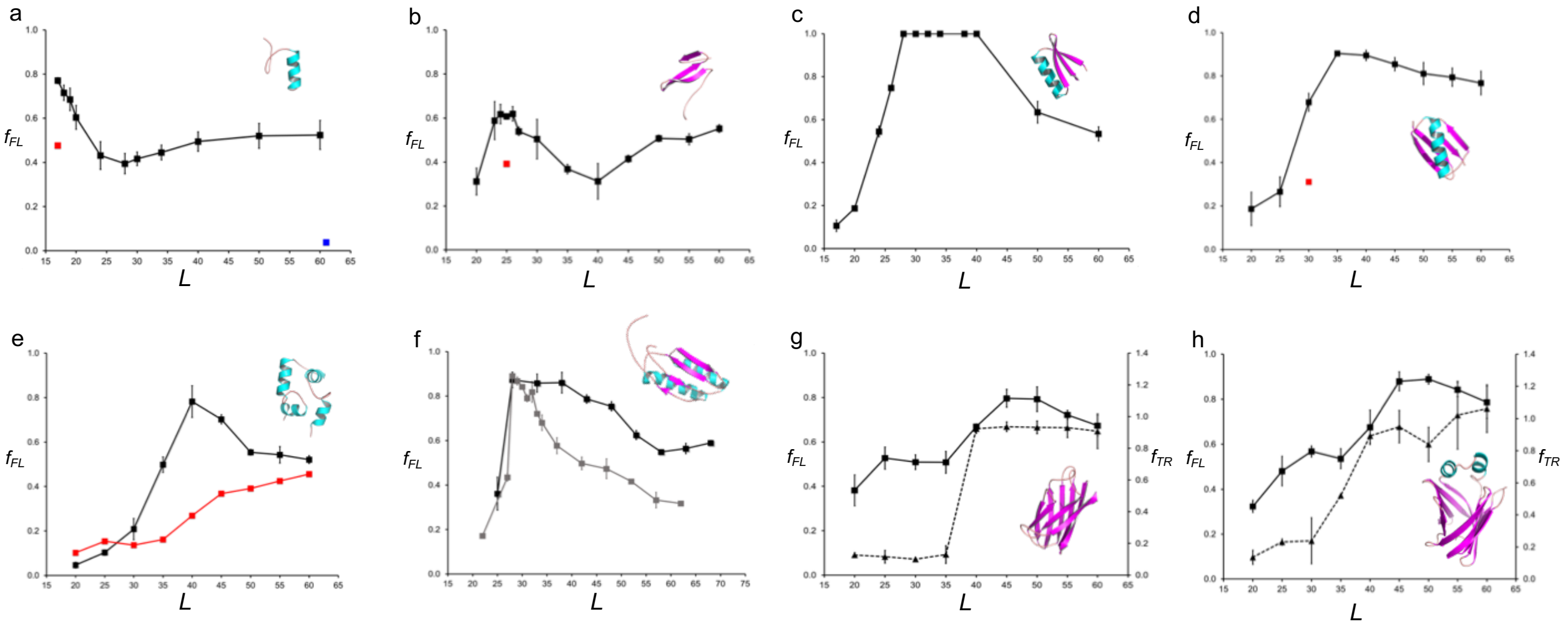
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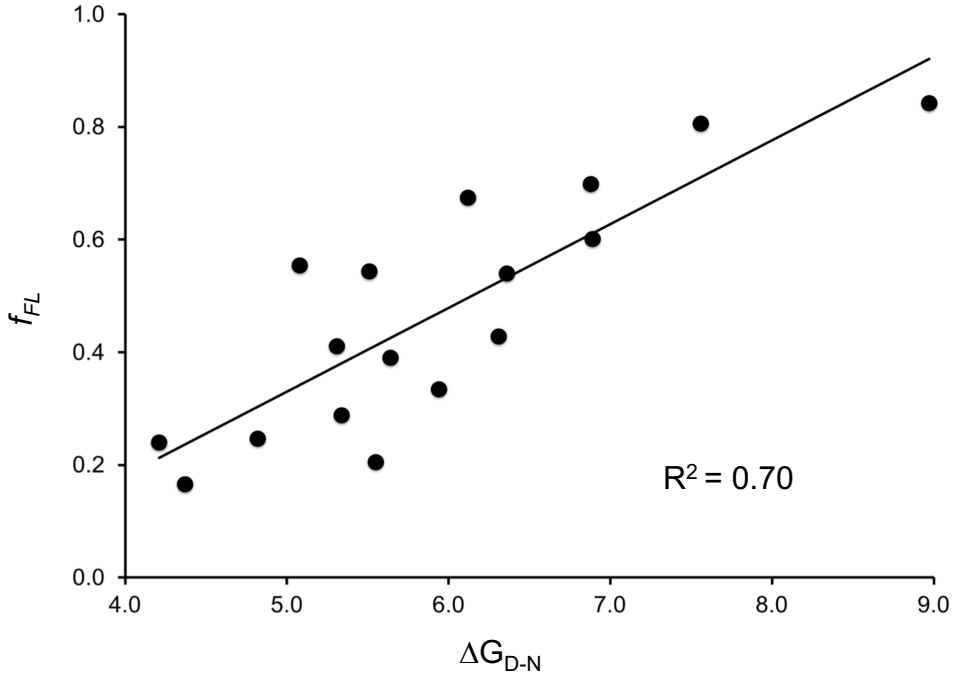
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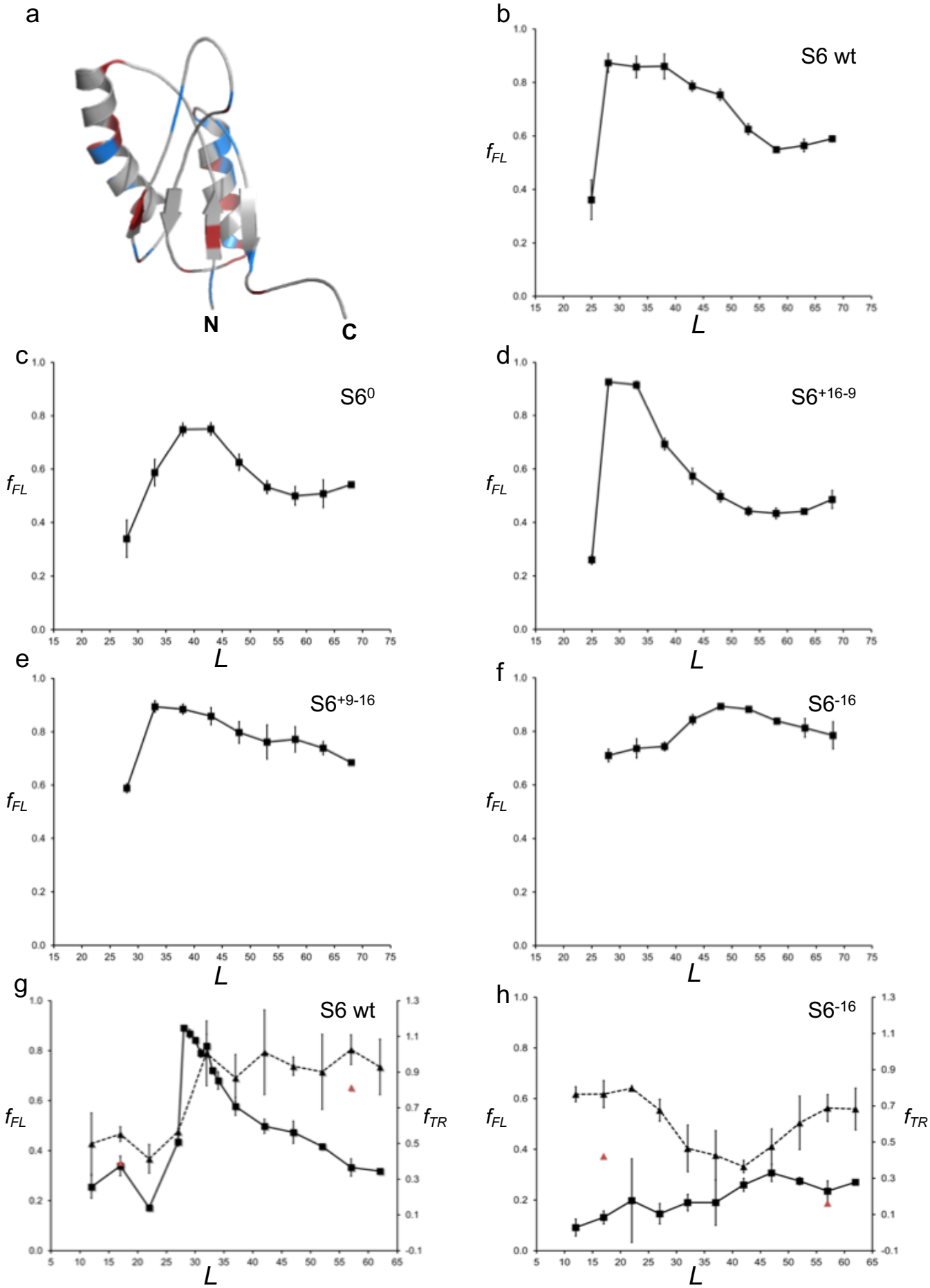
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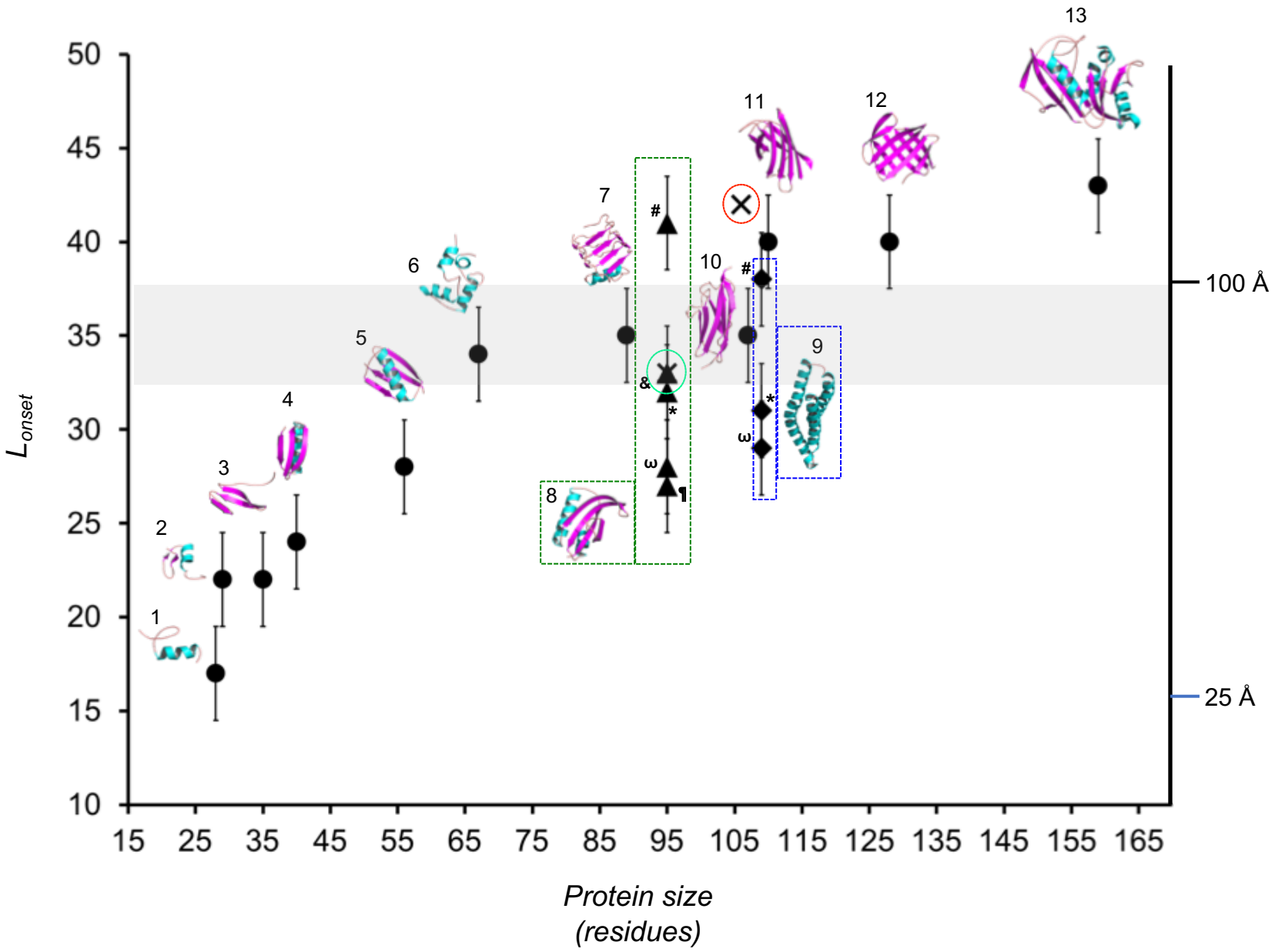
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Supplementary Tables 1-4

Supplementary Table 1. Proteins tested in the study. (#) EHEE is an artificial protein for which only a theoretical model is available. ($) Highlighted in green are the two Ileal bile acid-binding proteins used in this study, we prepared homology models based on PDB: 2LBA. Highlighted in gray are S6 and four of its variants, there are not determined structures for the differentially charged mutants.

Protein	**Reference**	**PDB ID**	**Length (residues)**	**Molecular weight (kDa)**	**Net charge**
FSD1	1	1fsd	28	3.48	+5
WW domain	2	2m8i	35	4.08	+4
EHEE	3	model#	40	4.94	+1
Protein G	4	3gb1	56	6.1	-4
Cam Frag	5	2hf5	69	7.83	-9
SOD1	6	4bcz	110	11.10	-3
ILBP	7	model$	128	14.40	+1
ILBP-tm	7	model$	105	11.51	+2
S6-wt-(+16/-16)	8,9	2kjv	101	11.97	0
S6-(+16/-9)	8,9	----	101	11.96	+7
S6-(0)	8,9	----	101	10.96	0
S6-(+9/-16)	8,9	----	101	11.54	-7
S6-(-16)	8,9	----	101	10.97	-16

Supplementary Table 2. Sequences of the longest construct tested for each protein domain (orange). Shorter constructs were obtained by shortening of the $(GS)_n$ linker (purple) For small domains, an unstructured segment from the *E. coli* LepB protein (light blue) was appended to the N terminus in order to make the constructs easy to identify by SDS-PAGE. The arrest peptide (green) is followed by a 23-residue C-terminal tail (light blue), also derived from the LepB protein.

```
>Lep-154-1fsd-SecM-L60 (Not listed in Uniprot, artificial protein,
PDB;1fsd)
MANRSFIYEPFQIPSGSMMPTLNSTDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYPEDP
KLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTYSNVEPSDFVQTFSRRNGGEAT
SGFFEVPKQETKENGIRLSETSGSGSQQYTAKIKGRTFRNEKELRDFIEKFKGRGSGSGSGSGSGS
GSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSR
IGGIH*

Number of amino acids: 28
Molecular weight: 3.48 kDa
Arg+Lys=4+5=9
Glu+Asp=3+1=4
Net charge at pH 7:+5


>WW domain UniProtKB - Q13526 (PIN1_HUMAN)
MANRSFIYEPFQIPSGSMMPTLNSTDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYPEDP
KLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTYSNVEPSDFVQTFSRRNGGEAT
SGFFEVPKQETKENGIRLSETSGSGSKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGGSGSGS
GSGSGSGSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPT
GLRLSRIGGIH*

Number of amino acids: 35
Molecular weight: 4.08 kDa
Arg+Lys=4+2=6
Glu+Asp=2+0=2
Net charge at pH 7:+4


>EHEE (Not listed in Uniprot, artificial protein,[3])
MANRSFIYEPFQIPSGSMMPTLNSTDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYPEDP
KLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTYSNVEPSDFVQTFSRRNGGEAT
SGFFEVPKQETKENGIRLSETSGSGSTTRYRFTDEEEARRAAKEWARRGYQVHVTQNGTYWEVEVR
GSGSGSGSGSGSGSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQ
EGEWPTGLRLSRIGGIH*

Number of amino acids: 40
Molecular weight: 4.94 kDa
Arg+Lys=7+1=8
Glu+Asp=6+1=7
Net charge at pH 7:+1
```

>Protein G UniProtKB - P06654 (SPG1_STRSG)
MTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEGSGS
GSGSGSGSGSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSD
KQEGEWPTGLRLSRIGGIH*

Number of amino acids: 56
Molecular weight: 6.1 kDa
Arg+Lys=0+6
Glu+Asp=5+5
Net charge at pH 7:-4

>CamFrag UniProtKB - G3V361 (G3V361_HUMAN)
MAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAE
LRHVMTNLGSGSGSGSGSGSGSGSGSGSGSGSGGSGSGSGSGSGSGSGSGSGSGFSTPVWIS
QAQGIRAGPGSSDKQEGEWPTGLRLSRIGGIH*

Number of amino acids: 69
Molecular weight: 7.83 kDa
Arg+Lys=4+3=7
Glu+Asp=8+8=16
Net charge at pH 7:-9

>SOD1 UniProtKB - P00441 (SODC_HUMAN)
MATKAVAVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHGAGGDLGNVTA
DKDGVADVSIEDSVISLSGDHSIIGRTLVVHEKAGAGAGSRLASGVIGIAQMMGSGSGSG
SGSGSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGE
WPTGLRLSRIGGIH*

Number of amino acids: 110
Molecular weight: 11.10 kDa
Arg+Lys=2+7=9
Glu+Asp=5+7=12
Net charge at pH 7:-3

>ILBP UniProtKB - P50119 (FABP6_RABIT)
MAFTGKFEMESEKNYDEFMKLLGLPSDVVEKSRNIKIVTEIKQDGQDFTWSHHYSGGQIM
TNKFTIGKESEIQTFGGKKFKAVVNMEGGKVVANFPNYQHTSEIKGDKLVEVSSIGGVTY
ERVSKRLAGSGSGSGSGSGSGSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQ
AQGIRAGPGSSDKQEGEWPTGLRLSRIGGIH*

Number of amino acids: 128
Molecular weight: 14.40 kDa
Arg+Lys=3+15=18
Glu+Asp=12+5=17
Net charge at pH 7:+1

>ILBP-tm UniProtKB - P50119 (FABP6_RABIT) mutant
MAFTGKFEMGGSGIVTEIKQDGQDFTWSHHYSGGQIMTNKFTIGKESEIQTFGGKKFKAV
VNMEGGKVVANFPNYQHTSEIKGDKLVEVSSIGGVTYERVSKRLAGSGSGSGSGSGSGSG
GSGSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLR
LSRIGGIH*

Number of amino acids: 105
Molecular weight: 11.51 kDa
Arg+Lys=2+11=13
Glu+Asp=8+3=11
Net charge at pH 7:+2

>S6-SecM(*Ec*) AP UniProtKB - P23370 (RS6_THETH)
MRRYEVNIVLNPNLDQSQLALEKEIIQRALENYGARVEKVEELGLRRLAYPIAKDPQGYF
LWYQVEMPEDRVNDLARELRIRDNVRRVMVVKSQEPFLANAGSGSGSGSGSGSGSGGSGS
GSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSRI
GGIH*

Number of amino acids: 101
Molecular weight: 11972.77
Arg+Lys=12+4=16
Glu+Asp=11+5=16
Net charge at pH 7:0

>S6^{+16-9}-SecM(*Ec*) AP UniProtKB - P23370 (RS6_THETH) mutant
MRRYEVNIVLNPNLDQSQLALEKQIIQRALENYGARVQKVQELGLRRLAYPIAKDPQGYF
LWYQVEMPQNRVNDLARQLRIRNNVRRVMVVKSQEPFLANAGSGSGSGSGSGSGSGGSGS
GSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSRI
GGIH*

Number of amino acids: 101
Molecular weight: 11965.88
Arg+Lys=12+4=16
Glu+Asp=6+3=9
Net charge at pH 7:+7

>S6^{0}-SecM(*Ec*) AP UniProtKB - P23370 (RS6_THETH) mutant
MSSYQVNIVLNPNLNQSQLALQSQIIQSALQNYGASVQSVQQLGLSSLAYPIASNPQGYF
LWYQVQMPQNSVNNLASQLSISNNVSSVMVVSSQQPFLANAGSGSGSGSGSGSGSGGSGS
GSGSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSRI
GGIH*

Number of amino acids: 101
Molecular weight: 10963.32
Arg+Lys=0+0=0
Glu+Asp=0+0=0
Net charge at pH 7:0

> S6^{+9-16}-SecM(*Ec*) AP UniProtKB - P23370 (RS6_THETH) mutant
MSRYEVNIVLNPNLDQSQLALESEIIQRALENYGARVESVEELGLSRLAYPIAKDPQGYF
LWYQVEMPEDRVNDLASELRISDNVRSVMVVKSQEPFLANAGSGSGSGSGSGSGSGGSGS
GSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSRI
GGIH*

Number of amino acids: 101
Molecular weight: 11545.03
Arg+Lys=7+2=0
Glu+Asp=11+5=16
Net charge at pH 7: -7

>S6^{-16}-SecM(*Ec*) AP UniProtKB - P23370 (RS6_THETH) mutant
MSSYEVNIVLNPNLDQSQLALESEIIQSALENYGASVESVEELGLSSLAYPIASDPQGYF
LWYQVEMPEDSVNDLASELSISDNVSSVMVVSSQEPFLANAGSGSGSGSGSGSGSGGSGS
GSGSGSGSGSGSGSGSGSGSGSGSGSFSTPVWISQAQGIRAGPGSSDKQEGEWPTGLRLSRI
GGIH*

Number of amino acids: 101
Molecular weight: 10979.08
Arg+Lys=0+0=0
Glu+Asp=11+5=0
Net charge at pH 7: -16

>S6-SecM(*Ms*) AP UniProtKB - P23370 (RS6_THETH) mutant
MRRYEVNIVLNPNLDQSQLALEKEIIQRALENYGARVEKVEELGLRRLAYPIAKDPQGYF
LWYQVEMPEDRVNDLARELRIRDNVRRVMVVKSQESGSGSGSGSGSGSGSGSGSGSGSGG
SGSGSGSGSGSGSGSGSGSGSGSGSGSGSHAPIRGSPGSSDKQEGEWPTGLRLSRIGGIH*

Number of amino acids: 95
Molecular weight: 11359.06
Arg+Lys=12+4=16
Glu+Asp=11+5=16
Net charge at pH 7:0

>S6^{-16}-SecM(*Ms*) AP UniProtKB - P23370 (RS6_THETH)
MSSYEVNIVLNPNLDQSQLALESEIIQSALENYGASVESVEELGLSSLAYPIASDPQGYF
LWYQVEMPEDSVNDLASELSISDNVSSVMVVSSQESGSGSGSGSGSGSGSGSGSGSGSGG
SGSGSGSGSGSGSGSGSGSGSGSGSGSGSHAPIRGSPGSSDKQEGEWPTGLRLSRIGGIH*

Number of amino acids: 95
Molecular weight: 10365.36
Arg+Lys=0+0=0
Glu+Asp=11+5=16
Net charge at pH 7:-16

Supplementary Table 3. Thermodynamic stabilities ($\Delta G_{D\text{-}N}$) in kcal/mol and $log\ k_f$ values for 16 single-point mutations in protein S6 [10], and fraction full length (f_{FL}) for the corresponding nascent chain mutants obtained with the SecM(*Ms*) AP at at L = 30 residues. The f_{FL} measurements are averages of 3 independent replicates and their corresponding standard deviations are shown.

	$\Delta G_{D\text{-}N}$	***log kf***	***fFL***	**ST dev.**
WT	8.97	2.64	0.84	0.005
V6A	5.34	1.86	0.28	0.012
I8A	4.82	1.61	0.24	0.011
L10A	4.37	1.83	0.16	0.011
L19A	6.36	2.42	0.53	0.031
I26A	5.94	1.86	0.33	0.038
L30A	5.31	2.03	0.41	0.026
V37A	6.31	2.39	0.42	0.034
L61A	5.55	2.4	0.20	0.012
Y63A	5.08	2.3	0.55	0.024
V65A	5.64	2.16	0.39	0.051
V72A	7.56	2.59	0.80	0.008
L75A	6.89	2.3	0.60	0.011
L79A	4.21	2.27	0.23	0.011
V85A	5.51	2.51	0.54	0.007
V88A	6.88	2.39	0.69	0.009
V90A	6.12	2.42	0.67	0.013

Supplementary Table 4. Proteins tested in previous studies

Protein	Reference	PDB ID	Length (residues)	Molecular weight (kDa)	Net charge
DHFR	11	8dfr	189	21.64	+1
PENT	12	6fls	105	12.03	-10
Titin I27	13	1tit	89	9.78	-6
R15 R16 β16	14	1u5p 1aj3 3lbx	109 109 109	12.99 12.73 13.43	+1 +1 0
ADR1a	15	2adr	29	3.47	+5
FLN5	16	1qfh	106	11.22	-9
TOP7	17,18	1qys	95	10.75	-4

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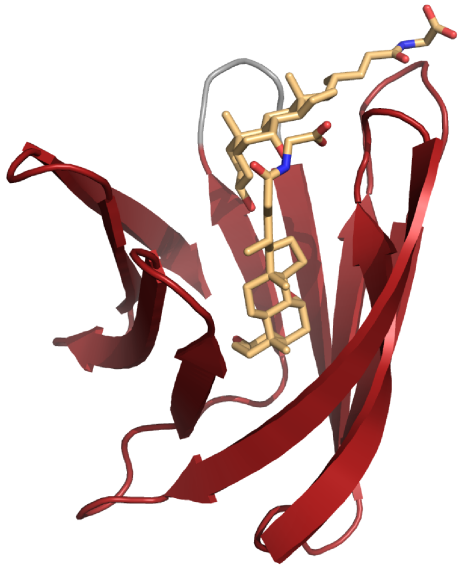

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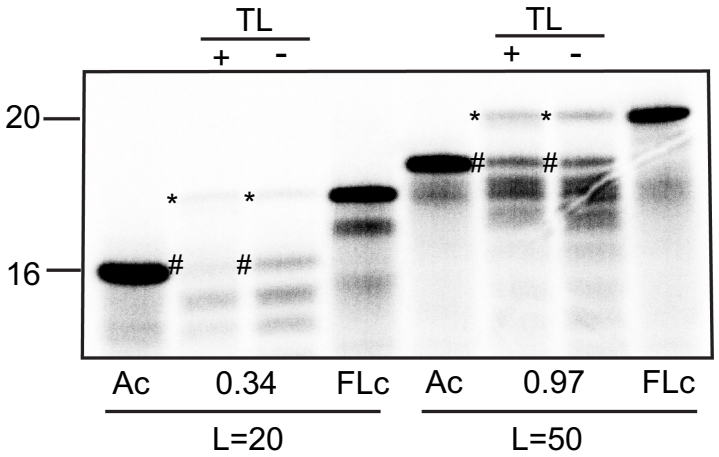
TL
+
-
TL
+
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20
16
*
#
Ac
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FLc
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0.97
FLc
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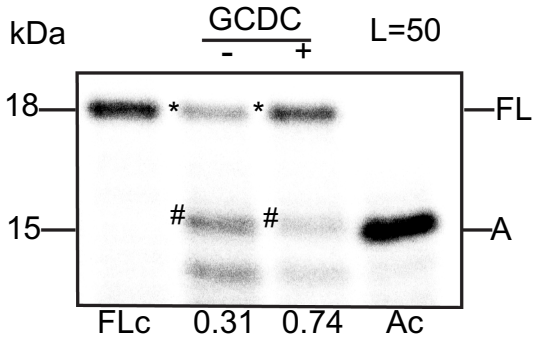
b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GCDC
-
+
L=50
18
15
FL
A
*
#
FLc
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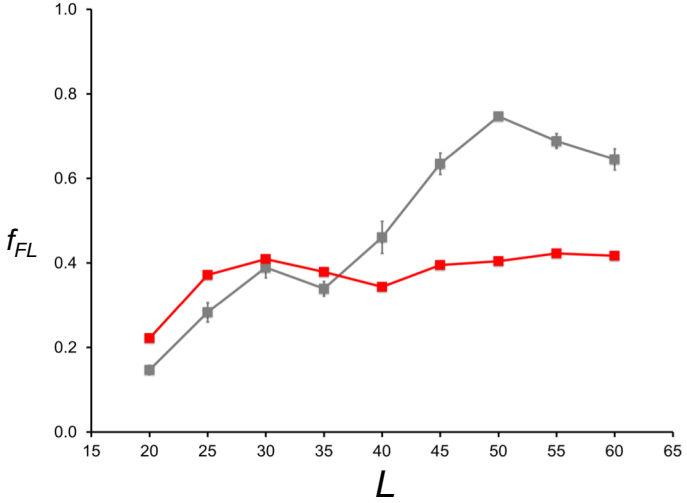
0.74
Ac

d


1.0
0.8
0.6
0.4
0.2
0.0
f_{FL}
15
20
25
30
35
40
45
50
55
60
65
L

S*upplementary Figure S1*. (a) Model for a loop-truncated ILBP mutant used to test the effects of ligand binding on the f_{FL} profile. The model is based on PDB 2lba, the modeling template showed 60 % sequence identity with the modeled targets and the position of the ligand reflects an estimation of the position in the modeled using as reference the position in the NMR structure. Biochemical data supports the binding of this ligand in all versions of the protein used for this study [17]. (b) *In vitro* translation of the loop-truncated ILBP[L=50] construct with the SecM(*Ms*) AP, showing that the protein exerts a stronger pulling force on the nascent chain in the presence (+) than in the absence (-) of the ligand GCDC (f_{FL} = 0.74 *vs*. 0.31; $f_{FL} = I_{FL}/(I_{FL}+I_A)$ where I_{FL} (or I_A) is the intensity of the band marked * (or #). Full-length (FLc) and arrested (Ac) controls are indicated; the FLc construct has a P-to-A mutation the critical Pro at the end of the AP and does not give any arrested product, the Ac construct has a stop codon inserted directly after the AP. (c) *In vitro* translation and pulse proteolysis of wildtype ILBP at L = 20 and L = 50 residues. The arrested ILBP nascent chain is susceptible to thermolysin at L = 20 residues (f_{TR} = 0.34) but not at L = 50 residues (f_{TR} = 0.97), compare bands marked # in the ±TL lanes. Full length (FLc) and arrested (Ac) controls are indicated. (d) f_{FL} profiles for loop-truncated ILBP (red curve) and loop-truncated ILBP translated in the presence of 400 μM of the ligand glycochenodeoxycholic acid (gray curve).

```
   WT MRRYEVNIVLNPNLDQSQLALEKEIIQRALENYGARVEKVEELGLRRLAYP
+16-9 MRRYEVNIVLNPNLDQSQLALEKQIIQRALENYGARVQKVQELGLRRLAYP
    0 MSSYQVNIVLNPNLNQSQLALQSQIIQSALQNYGASVQSVQQLGLSSLAYP
-9-16 MSRYEVNIVLNPNLDQSQLALESEIIQRALENYGARVESVEELGLSRLAYP
  -16 MSSYEVNIVLNPNLDQSQLALESEIIQSALENYGASVESVEELGLSSLAYP

   WT IAKDPQGYFLWYQVEMPEDRVNDLARELRIRDNVRRVMVVKSQEPFLANA
+16-9 IAKDPQGYFLWYQVEMPQNRVNDLARQLRIRNNVRRVMVVKSQEPFLANA
    0 IASNPQGYFLWYQVQMPQNSVNNLASQLSISNNVSSVMVVSSQQPFLANA
-9-16 IAKDPQGYFLWYQVEMPEDRVNDLASELRISDNVRSVMVVKSQEPFLANA
  -16 IASDPQGYFLWYQVEMPEDSVNDLASELSISDNVSSVMVVSSQEPFLANA
```

Supplementary Figure S2. Sequences of the protein S6 charge variants analyzed in Figure 4.