The flavodoxin-like fold is a protein architecture that can be traced back to the universal ancestor of the three kingdoms of life. Many proteins share this \(\alpha\)-\(\beta\) parallel topology and hence it is highly relevant to illuminate how they fold. Here, we review experiments and simulations concerning the folding of flavodoxins and CheY-like proteins, which share the flavodoxin-like fold. These polypeptides tend to temporarily misfold during unassisted folding to their functionally active forms. This susceptibility to frustration is caused by the more rapid formation of an \(\alpha\)-helix compared to a \(\beta\)-sheet, particularly when a parallel \(\beta\)-sheet is involved. As a result, flavodoxin-like proteins form intermediates that are off-pathway to native protein and several of these species are molten globules (MGs). Experiments suggest that the off-pathway species are of helical nature and that flavodoxin-like proteins have a nonconserved transition state that determines the rate of productive folding. Folding of flavodoxin from *Azotobacter vinelandii* has been investigated extensively, enabling a schematic construction of its folding energy landscape. It is the only flavodoxin-like protein of which cotranslational folding has been probed. New insights that emphasize differences between \textit{in vivo} and \textit{in vitro} folding energy landscapes are emerging: the ribosome modulates MG formation in nascent apoflavodoxin and forces this polypeptide toward the native state.
date. We also evaluate folding of the following CheY-related proteins: the bacterial response regulator CheY (Fig. 1C), the sporulation response regulator Spo0F, and the N-terminal receiver domain of nitrogen regulator protein NtrC (NT-NtrC).

**Flavodoxins: function, evolution, and categorization**

Flavodoxins are monomeric, single domain flavoproteins that are involved in electron transfer between various physiological redox partners. To shuttle electrons, flavodoxins contain a noncovalently bound flavin mononucleotide (FMN). Phylogenomic analysis shows that flavodoxin is evolutionary one of the earliest proteins, just as ferredoxin is. Both proteins are rather promiscuous in their choice of redox partners and evolved in the anaerobic environment, preceding the advent of oxygenic photosynthesis [11]. Before O$_2$ levels rose, ferredoxin was the obligatory redox protein. Once O$_2$ started to form, and bioavailability of iron became limiting, there was an intense selective pressure to replace oxidant-sensitive, iron-dependent proteins by oxidant-resistant, iron-free isofunctional counterparts. As flavodoxin satisfies both requirements, it temporarily took over ferrodoxin’s role [11,12].

The flavodoxin-like fold represents an architecture that can be traced back to the universal ancestor of all three kingdoms of life [11]. Despite its ancient origin and low susceptibility to O$_2$, flavodoxin itself has been lost in most eukaryotic lineages, including the entire plant and animal kingdom [12]. Cyanobacteria and algae thriving in iron-deficient oceanic environments usually contain the flavodoxin gene. It is assumed that plants evolved from macro-algae that already lacked flavodoxin, because they thrived in an iron-rich, freshwater habitat with no need to backup ferredoxin functions and therefore no selective pressure to keep the flavodoxin gene [11]. Flavodoxin is not found in mitochondria, although it is present in alphaproteobacteria, the suspected original endosymbiont. When both flavodoxin and ferredoxin are present in the same genome, like in many prokaryotes and algae, stress conditions such as iron starvation induce flavodoxin expression. Because electron transfer is essential for survival, flavodoxin largely replaces the roles of ferredoxin under these circumstances [11]. Although flavodoxin is not found in the higher kingdoms of life, it has become incorporated in important multidomain enzymes such as cytochrome P450 reductase [13] and nitric oxide synthase [14].

The flavodoxin-like fold (c.23) is the sixth fold that arose during evolution of protein architectures [15,16]. Interestingly, two thirds of the most ancient folds belong to the $\alpha/\beta$ class of proteins and more than half of the studied molecular fossils contain cofactors. Indeed, a flavodoxin made only of amino acid residues that were the most prevalent early during protein evolution is a functional protein with effective electron transfer capacity [17]. Homology exists between the flavodoxin-like fold and the ($\beta/\alpha$)$_8$-barrel fold (c.1 or TIM-barrel) [18], which is considered to be the second oldest protein fold [16]. The flavodoxin-like fold is one of the two preferred folds that bind FMN, and the other is the TIM-barrel fold [19].

Flavodoxins are categorized as short- or long-chain ones, depending on the presence of an insertion of about 20 amino acid residues in the last (i.e., fifth) $\beta$-strand (Figs 1A and 2). The function of this loop has not yet been determined, although it is suspected to be involved in binding of partner proteins [20]. The loop also seems to increase the binding affinity of FMN [20] and it is speculated that it is involved in the formation of folding intermediates [21]. Phylogenetic analyses suggests that short-chain flavodoxins derived from long-chain ones [22], although resolving this issue is complicated by several horizontal gene-transfer.

**Fig. 1.** Schematic representations of flavodoxin-like fold containing proteins. FMN is colored yellow. (A) Topology of the flavodoxin-like fold. $\alpha$-Helices are represented as circles and $\beta$-strands as squares. In long-chain flavodoxins the $\beta_5$-strand is split into $\beta_{5a}$ and $\beta_{5b}$. (B) Flavodoxin from A. vinelandii (PDB entry 1YOB [179]). (C) CheY from E. coli (PDB entry 3CHY) [180].
events between organisms [12]. Only short-chain flavodoxins are found in firmicutes (gram-positive bacteria), whereas cyanobacteria and algae exclusively synthesize long-chain flavodoxins [23]. Gram-negative bacteria can contain flavodoxins from either category and may even contain both short- and long-chain ones. In *Escherichia coli*, for example, there are four genes predicted to code for flavodoxins. Two of these proteins belong to the long-chain category (i.e., FldA and FldB) and the other to the short-chain one (i.e., MioC and YqcA) [24].

Because the flavodoxin-like fold is widespread in the protein universe, illumination of its folding is highly relevant. Folding of flavodoxins from *Anabaena*, *A. vinelandii*, *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris*, and *Helicobacter pylori* has been investigated with differing levels of depth. Of these proteins, the ones from *Desulfovibrio* are short-chain flavodoxins.

Energy landscapes of protein folding and role of intermediates

How are proteins able to form a wide variety of three-dimensional structures from differing amino acid sequences? How can similar sequences fold to disparate structures? Two principles are essential for solving these challenges [25–29]: Anfinsen’s thermodynamic hypothesis and Levinthal’s paradox. The thermodynamic hypothesis states that, at least for small globular proteins, the native structure is only determined by its amino acid sequence [25]. In the protein’s relevant physiological environment the native structure is a unique, stable conformation at the kinetically accessible minimum of free energy. Levinthal calculated the time it would take for a 100-residue protein to randomly search all possible conformations and found that this would last longer than the lifetime of the universe [30]. Paradoxically, most small proteins fold to their native structure within micro- to milliseconds, and even folding of large multidomain proteins rarely exceeds folding times of minutes [31]. This issue can be resolved if the conformational search is not random, but involves intermediates in which local interactions form that direct the subsequent folding of the polypeptide chain and thereby accelerate the folding reaction [30]. Both Anfinsen’s thermodynamic hypothesis and Levinthal’s paradox are integrated in the funnel-like energy landscape for protein folding [26].

All possible conformations of a single polypeptide can be visualized forming a folding funnel [26]. This funnel (Fig. 3A) depicts the free energy of folding as a function of conformational entropy. The vertical axis represents the free energy of a conformation, and the lateral area at a given depth (i.e., the width of the funnel) represents the number of conformations (or conformational entropy). The rim of the funnel is the starting area for folding (i.e., the unfolded state), which is not just one defined protein structure. Instead, the unfolded state comprises a conformational ensemble of rapidly interconverting unfolded structures, which is characterized by large entropy. Starting from the unfolded state, the peptide chain searches for the native state, which is the lowest point in the folding energy landscape [25]. A myriad of interactions is involved in folding an unfolded protein, such as

![Fig. 2. Three-dimensional structures of long- and short-chain flavodoxins. Shown are (A) a long-chain protein from *A. vinelandii* (blue, PDB entry 1YOB) and (B) a short-chain one from *Desulfovibrio vulgaris* (cyan, PDB entry 1J8Q), both without FMN. An overlay of both structures with FMN is shown in (C). The loop shown on the left divides the fifth β-strand of the long-chain flavodoxin into βsa and βsb. FMN is shown in stick representation.](image-url)
establishment of hydrophobic contacts, van der Waals interactions and formation of intramolecular electrostatic connections and hydrogen bonds. Lowering of the chain entropy also plays a role. Proteins appear to firstly develop secondary structures in the chain (such as turns and helices), which may interact transiently, followed by growth into more global structures [29].

Formation of intermediates gives rise to a rugged folding surface, reflecting the presence of local energetic minima (Fig. 3A). Most intermediates are transient and only few of them have sufficient stability to be detectable. These species can be either on- or off-pathway to the native structure. On-pathway ones predominantly contain native-like interactions in their structured parts. In contrast, off-pathway species have significant non-native, misfolded structure and are trapped in the corresponding energetic minima. To return to the productive folding ensemble, these trapped species have to overcome significant energy barriers, which can be achieved by their (partial) unfolding. Intermediates that are off-pathway to the productive folding route can be visualized as residing in a ‘trenched’ landscape (Fig. 3B). Folding of certain proteins involves both on- and off-pathway species, because the corresponding energy landscape is complex (Fig. 3C). It is rugged and contains a trench. Figure 3C suggests that both intermediates are on direct routes to the native state, as they reside within the same funnel. To better visualize the distinct character of both species, Fig. 3D positions the off-pathway intermediate on the bottom of a separate trough that is connected to an on-pathway funnel.

**Molten globular folding intermediates**

An important folding intermediate is the molten globule (MG), which was first described for the protein α-lactalbumin [32–34]. MGS are characterized by a substantial amount of secondary structure, yet without the tertiary side-chain packing of natively folded protein. Furthermore, MGS are relatively compact (typical radius increase compared to native protein of about 10–30%), possess a loosely packed hydrophobic core and expose hydrophobic surfaces to

![Fig. 3. Energy surfaces of protein folding. Free energies are shown vertically and reaction coordinates horizontally. Unfolded protein structures reside at the rim of each folding funnel. N indicates native protein and \( I_{\text{off}} \) signifies off-pathway folding intermediate. (A) In a rugged folding funnel a protein can form intermediates on its way to the native state. (B) In a trenched folding funnel a protein can become kinetically trapped in a trench, resulting in formation of an off-pathway intermediate. (C) Example of a folding funnel that is rugged and contains a trench, that is, it contains on- and off-pathway intermediates. (D) Simplified alternative energy landscape of a protein that can temporarily form an off-pathway intermediate.](image)
the solvent [34,35]. MGs are prone to aggregation and consequently are implicated in various diseases [36]. In vitro, several proteins form MGs under mildly alkaline or acidic conditions, including α-lactalbumin, apomyoglobin, and cytochrome c [32,33,37–42]. These MGs all contain native-like secondary structure and packing, causing them to be on-pathway to the native state. In contrast, off-pathway MGs contain non-native secondary structure and/or packing [43–46]. Nowadays, many proteins are thought to fold via MGs [1,47–49]. For example, MG species are necessary during insertion of proteins into membranes or during translocation [50–52]. Such insertions often happen cotranslationally, that is, while the ribosome synthesizes the polypeptide concerned. Proteins can fold cotranslationally and sample intermediate folding states [53–59], which might include MGs.

Exploration of the folding of flavodoxin from A. vinelandii

We first consider the folding of flavodoxin from A. vinelandii. This bacterium contains three flavodoxins, of which the long-chain flavodoxin II is most abundant under nitrogen-fixating conditions [60]. Folding of both the apo- and the holoform of this 179-residue protein has been probed by using guanidinium hydrochloride (GuHCl) as denaturant. A wide variety of techniques were used, including (time-resolved) fluorescence, far-UV circular dichroism (CD), nuclear magnetic resonance (NMR) spectroscopy, hydrogen/deuterium exchange, (single-molecule) Förster resonance energy transfer, stopped-flow, paramagnetic relaxation, and FMN-binding kinetics [5,43–46,61–72]. These experiments show that apoflavodoxin folds autonomously and spontaneously to its native state [5] and that the subsequent binding of FMN is the last step in folding of flavodoxin [66]. The three-dimensional structures of both native proteins are nearly identical, except for considerable flexibility in the flavin-binding region of apoflavodoxin [62]. This flexibility is probably required for apoflavodoxin to capture FMN out of solution. After binding FMN with nanomolar affinity, the protein relaxes to an energetically even more favorable state with picomolar-binding affinity [71]. As a result, many amino acid residues dispersed throughout the structure of flavodoxin become stabilized against unfolding [63,71]. The stability of flavodoxin is so high that FMN needs to be released first before global unfolding of the protein can occur [66].

Characterization of how flavodoxin kinetically folds in vitro shows that two parallel folding routes are accessible to apoflavodoxin molecules with all prolyl peptide bonds in their native transconfiguration [5,66]. These routes, of which one is on- and the other off-pathway, are visualized in the energy landscape of Fig. 4. This landscape depicts a funnel to the native state and a trough in which a misfolded intermediate temporarily resides. Folding in the funnel and trough are described by schemes 1 and 2, respectively:

![Scheme 1](image1)

![Scheme 2](image2)

Scheme 1 represents the approximately 10% of unfolded molecules (U; rim of the funnel toward native protein) that directly follow the productive folding route to native apoflavodoxin (Apo; bottom of funnel). On this path, intermediate I_{on} is an obligatory, high-energy on-pathway species that rapidly converts to native apoflavodoxin [5]. I_{on} resides in a trench in the funnel toward native protein. Due to its instability, I_{on} is not observed during denaturant-dependent equilibrium folding of the protein. This productive folding of 10% of unfolded molecules happens on the microsecond timescale. Subsequently, flavodoxin (Holo; not depicted in Fig. 4) forms upon binding of FMN to native apoflavodoxin. Around 90% of unfolded molecules (U*; the outer edge of the off-pathway trough) misfold within milliseconds and form the off-pathway intermediate M_{Goff} (scheme 2). This relatively stable species is also detected during denaturant-dependent equilibrium folding of the protein. This productive folding of 10% of unfolded molecules happens on the microsecond timescale. Subsequently, flavodoxin (Holo; not depicted in Fig. 4) forms upon binding of FMN to native apoflavodoxin. Around 90% of unfolded molecules (U*; the outer edge of the off-pathway trough) misfold within milliseconds and form the off-pathway intermediate M_{Goff} (scheme 2). This relatively stable species is also detected during denaturant-dependent equilibrium folding of the protein. This productive folding of 10% of unfolded molecules happens on the microsecond timescale. Subsequently, flavodoxin (Holo; not depicted in Fig. 4) forms upon binding of FMN to native apoflavodoxin. Around 90% of unfolded molecules (U*; the outer edge of the off-pathway trough) misfold within milliseconds and form the off-pathway intermediate M_{Goff} (scheme 2). This relatively stable species is also detected during denaturant-dependent equilibrium folding of the protein. This productive folding of 10% of unfolded molecules happens on the microsecond timescale.
nonproductive pathways, ultimately pass through \( I_{on} \) before reaching the native state.

Native apoflavodoxin occasionally forms partially unfolded forms (PUFs). Four PUFs have been identified, which all are off the productive folding route [67,70]. This demonstrates that the energy landscape of apoflavodoxin folding is even more rugged than depicted in Fig. 4.

Unfolded apoflavodoxin is not a featureless statistical coil, but instead contains four transiently ordered regions [43]. Three of these regions are \( \alpha \)-helices, whereas the fourth adopts non-native structure that is neither \( \alpha \)-helix nor \( \beta \)-strand (Fig. 5) [43]. During folding, these structured elements interact and subsequently form the ordered core of MG\(_{off} \) [43,44]. This propensity to interact is visualized in Fig. 4 and in scheme 2 by \( U^* \). Non-native docking of the \( \alpha \)-helices in MG\(_{off} \) prevents formation of the parallel \( \beta \)-sheet of native apoflavodoxin [43,44,69,70]. Thus, the source for MG\(_{off} \) formation is situated in the unfolded state.

The off-pathway intermediate has a drastically different architecture compared with native protein: it is largely \( \alpha \)-helical and contains no \( \beta \)-sheet [45] and is slightly expanded compared to native apoflavodoxin [61]. This \( \alpha \)-helical MG acts as a trap and needs to unfold significantly in order to embark on a route to native \( \alpha \)-\( \beta \) parallel protein. Kinetic folding data of apoflavodoxin suggest the presence of an activation barrier during conversion between unfolded protein and off-pathway MG [5]. This barrier is not shown in Fig. 4.

While formation of native apoflavodoxin is highly cooperative [5,46,64,65], conversion of unfolded protein into MG\(_{off} \) is a noncooperative, gradual process that simultaneously involves separate regions within apoflavodoxin [44,46]. This suggests an energy landscape of MG folding with many barriers (visualized by shallow trenches in the off-pathway trough of Fig. 4). In vitro, folding of the C-terminal part of MG\(_{off} \) precedes folding of the N-terminal part and this intermediate gradually compacts due to progressive extension of its ordered core [46,72]. This noncooperative folding happens because helices involve relatively short-range interactions and the folding of one helix does not need to affect the folding of other helices [44]. Indeed, thermodynamically derived phase diagrams indicate that

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Fig. 4. Schematic folding energy landscape of A. vinelandii apoflavodoxin. The left funnel shows the on-pathway route of unfolded molecules \( U \) (rim of the funnel) to native protein (N), on which an on-pathway intermediate (\( I_{on} \)) resides. The trough on the right displays unfolded molecules \( U^* \) (rim of the trough) forming apoflavodoxin’s off-pathway MG (MG\(_{off} \)). This molten globular species folds gradually, as exemplified by the presence of shallow trenches. For simplicity’s sake, the rugged character of MG\(_{off} \) folding, as evidenced by the detection of partially unfolded forms [67], is not depicted in the MG\(_{off} \) trough. Partial unfolding excursions that start from native protein are also omitted. Only native apoflavodoxin binds FMN, which leads to considerable stabilization of the protein and deepening of the corresponding funnel (not shown). In reality, the ratio of the circumferences of the rims of the on- and off-pathway funnels is about 1 : 9. This ratio reflects that 10% of unfolded molecules (i.e., \( U \)) directly follow the productive folding route to native apoflavodoxin, whereas 90% of unfolded molecules (i.e., \( U^* \)) misfold and temporarily form MG\(_{off} \) [5]. Apoflavodoxin is in 100 mM potassium pyrophosphate, pH 6.0, at 25 °C.

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Fig. 5. Model of the off-pathway MG of A. vinelandii flavodoxin, in which the four, transiently structured regions detected in unfolded protein are highlighted. Helical parts also present in native protein are colored green and regions of the unfolded protein that adopt \( \alpha \)-helical structure not found in native protein are colored blue; the orange element is structured, yet is neither \( \alpha \)-helix nor \( \beta \)-strand in unfolded protein. These regions dock non-natively and form the core of apoflavodoxin’s MG [43–45,70]. The cartoon shows the structural elements of the off-pathway MG, but their relative positioning is unknown.
the transition from an unfolded species to a MG is a gradual, second-order-like process [73,74]. Ultimately, after folding, the helical off-pathway MG of apoflavodoxin is almost entirely structured [44,46].

**Folding of other flavodoxins**

**Anabaena flavodoxin**

In native apoflavodoxin from *Anabaena* PCC 7119, the cofactor-binding site is flexible and FMN binds preferentially to native apoprotein [75–77]. Full release of cofactor happens upon unfolding of this 169-residue long-chain flavodoxin [78]. Both urea- and GuHCl-dependent equilibrium folding of apoflavodoxin are two-state [76,78,79]. During kinetic folding an intermediate transiently accumulates, whose unfolding is rate-limiting [3]. Whether this off-pathway intermediate is a MG is unknown. Phi-value analysis of mutations [80] revealed that the transition state that separates unfolded and native apoprotein is diffuse, and various interactions seem to be similarly important for rate-limiting barrier crossing [81].

In an apoflavodoxin variant whose stability has been reduced by excision of the C-terminal helix, the resulting protein fragment adopts a molten globular conformation at pH 7 [82,83]. The structure of this MG is homogeneously weakened compared to the one of full-length native protein [83]. The MG can be stabilized by increasing the helical propensity of helical regions of full-length native apoflavodoxin, suggesting preservation of helices in this MG [83].

Thermal unfolding of *Anabaena* apoflavodoxin at equilibrium follows a three-state mechanism in which an intermediate, with spectroscopic properties of a MG, populates [78,84,85]. A low-resolution structure of this species has been inferred from Phi-value analysis [85]. Large parts of the intermediate seem to have native-like topology, with somewhat weakened native interactions, but a 40-residue region is unfolded [78,84,85]. The thermal intermediate neither resembles the MG that accumulates at low pH, nor the transition state for productive folding, nor the MG fragment. Whether this species is similar to the transient intermediate observed during kinetic folding of apoflavodoxin is unknown [86].

At 25 °C, apoflavodoxin variant F98N also shows the spectroscopic properties of a MG, similar to those of the thermal intermediate of wild-type protein [86]. This MG is less compact than native protein [87] and comprises all 5 α-helices and β-strands 1–4 and 5α of native apoflavodoxin as well as their packing [86]. Whether the thermal intermediate is on- or off-pathway to native apoflavodoxin has not been probed.

**H. pylori flavodoxin**

This 164-residue protein is essential to *H. pylori*, the bacterium responsible for gastric ulcers. Hence, this long-chain flavodoxin is a target for drug discovery [88–90]. Again, FMN only binds to native apoflavodoxin, which strongly stabilizes the protein [91], thereby enabling it to be functional between pH 2 and 10 [92]. Apoflavodoxin [93] and flavodoxin [90] are structurally similar, but in apoprotein, the loops binding the isoalloxazine are flexible [93]. This flavodoxin strongly resembles *A. vinelandii* and *Anabaena* flavodoxins, but its FMN-binding site is more accessible [90,93]. As a result, inhibitors can bind specifically to *H. pylori* flavodoxin and thereby disrupt its electron transfer function [89,94].

The folding characteristics of *H. pylori* (apo)flavodoxin have been studied by thermal unfolding and by lowering pH [91,92,95]. Both methods result in formation of intermediates, yet with very different features. Thermal unfolding of (apo)flavodoxin involves two equilibrium intermediates [95]. One species is similar to the thermal intermediate observed for *Anabaena* apoflavodoxin. Compared to native protein, this species has a slight increase in solvent-exposure of tryptophans and preserves most of the secondary structure of the native protein (around 80% of native helix content). The other intermediate more closely resembles unfolded protein and has lost the tertiary contacts of native protein and a large part of its secondary structure, but at least one of its two tryptophans is partially buried [95].

Under strongly acidic conditions, *H. pylori* apoflavodoxin forms a MG that is slightly expanded compared to native protein. It has high helical content, displays exposed hydrophobic surfaces, and is devoid of, or maybe has very weakened, tertiary native interactions [91]. This MG is structurally different from the two thermal intermediates identified at neutral pH [92,95]. Whereas, under acidic conditions the MG of *H. pylori* apoflavodoxin is soluble and monomeric [91], the MG of *Anabaena* apoflavodoxin aggregates massively [79]. For *H. pylori* apoflavodoxin at neutral pH, the ensemble of molecules comprises native protein, the MG discovered by acid unfolding and the two intermediates revealed by thermal unfolding of the protein [92]. The latter three species are present at relatively low mole fractions, with the MG state hardly populated at all [92]. As no studies are reported of the
folding kinetics of H. pylori flavodoxin, the on- or off-pathway nature of its intermediates is unidentified.

**D. desulfuricans flavodoxin**

This 148-residue protein is a short-chain flavodoxin and is very sensitive to buffer and ionic strength conditions [96]. A single folding study of *D. desulfuricans* (apo)flavodoxin from ATCC strain 27774 has been reported, which suggests that during its GuHCl-dependent equilibrium folding an intermediate populates. This species has a more solvent-exposed tryptophan than native protein and judged by far-UV CD ellipticity at 220 nm it has native-like secondary structure [97]. Folding of apoflavodoxin from *D. desulfuricans* strain ATCC 29577 (75% sequence identity with ATCC 27774 protein) has been studied more extensively. This protein unfolds in a two-state fashion in ATTC 27774 protein) has been studied more extensively. This protein unfolds in a two-state fashion in strain ATCC 29577 (75% sequence identity with ATCC 27774 protein) has been studied more extensively. 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through a highly populated intermediate state, which is a MG dimer [106]. Urea-dependent equilibrium folding of CheY monitored by fluorescence and far-UV CD fits a two-state model. However, ANS binding suggests that a MG-like folding state populates [106]. Characterization of the folding of the F14N/V83T variant of CheY by NMR spectroscopy also shows involvement of a MG, which can associate to a dimeric MG [107]. It has been proposed that the monomeric MG can sequentially unfold into another intermediate \( I^* \), in which the first half of the polypeptide chain remains non-native and collapsed, and the second half is unfolded [107]. In contrast, native protein unfolds highly cooperatively [107]. Unfolded F14N/V83T protein in 5 M urea has residual structure [108]. \(^1\)HNMR chemical shift values indicate that two segments of unfolded CheY form helices. The 70 N-terminal residues of unfolded CheY have restricted mobility compared to the rest of the polypeptide. This collapsed region is stabilized by nonlocal interactions [108]. It is tempting to speculate that formation of structured sequence segments in unfolded CheY leads to establishment of \( I^* \).

Characterization of the transition state on the productive pathway identified two folding subdomains: an N-terminal one that is highly structured in the transition state and an unstructured C-terminal one [109]. Formation of the 70-residue N-terminal subdomain is rate limiting and serves as the nucleus for subsequent condensation of the C-terminal subdomain [109,110]. Intermediate \( I^* \) resembles this transition state [107].

Upon folding, CheY collapses to a burst-phase intermediate that possesses significant stability and secondary structure [110,111]. Analysis of the folding kinetics suggests that all five helices of CheY are formed in this submillisecond species [111]. The far-UV CD spectrum of this intermediate resembles that of the native state. However, the packing of aromatic side chains might not be identical [9]. Neither can it be excluded that this species lacks a \( \beta \)-sheet. CheY folds through two parallel pathways, which are defined by the state of isomerization of a proline in the active site, which is \( \text{cis} \) in native protein. Proline isomerization in the highly structured burst-phase species differs between both pathways. However, formation of this intermediate is not a consequence of proline isomerization [9]. The most likely folding model places the burst-phase species off-pathway to the native state. Each intermediate has to at least partially unfold before productive folding occurs. The extent of secondary structure formation and side-chain packing in these off-pathway intermediates are consistent with a MG-like state [9].

Coarse-grained, Gō-like simulations were used to further explore CheY folding. One simulation shows formation of a misfolded intermediate, which has all five helices present in native CheY, but lacks its central parallel \( \beta \)-sheet. This misfolded species has to at least partially unfold before the native state is reached [112]. More recent ‘flavored’ Gō-model simulations, in which the interaction energies of side-chain native contacts were scaled according to their abundance in the PDB, also show the existence of a frustrated state. This topologically frustrated species is a consequence of competition for native van der Waals contacts between the N- and C-terminal subdomains of CheY, leading to premature docking of these domains [113]. This intermediate unfolds before productive folding in the N-subdomain occurs, leading to native protein. An alternative source for frustration during protein

| Table 1. Folding of flavodoxin-like proteins. |
|---------------------------------------------|
| **Protein** | **Folding intermediate (s)** | **On-off-pathway species** | **MG observed** | **Transition state determined** | **Simulations of folding** |
| Flavodoxin | | | | |
| A. vinelandii | Yes | On and off | Yes | Yes | n.d. |
| Anabaena | Yes | Off | Yes | Yes | n.d. |
| H. pylori | Yes | n.d. | Yes | n.d. | n.d. |
| D. desulfuricans | Yes | Off | Yes | Yes | Yes |
| D. vulgaris | n.d. | n.d. | n.d. | n.d. | n.d. |
| CheY-like | | | | |
| CheY | Yes | Off | Yes | Yes | Yes |
| SpoOF | Yes | Off | n.d. | Yes\(^a\) | Yes |
| NT-NtrC | Yes | Off | n.d. | Yes\(^a\) | Yes |

n.d., not determined.

\(^a\)Derived from simulation.
Folding that leads to off-pathway species could be the formation of an unproductive local-in-sequence cluster of side chains of isoleucine, leucine, and valine (ILV) [9, 114–116]. A small ILV cluster seems crucial to the off-pathway reaction [9].

Aspects of the models for premature docking of subdomains and for formation of an unproductive ILV cluster appear to describe the folding of CheY. The subdomain model fulfills the requirement for an intact N-terminal domain to access the native conformation. The ILV cluster model captures best the initial formation of an off-pathway intermediate [115]. The events that lead to this frustrated species comprise accumulation of substructures favored by low-contact-order nonpolar interactions in the polypeptide. This leads to burial of surface areas, maximization of participation of aliphatic side chains in one of the two ILV clusters, and reduction in chain entropy penalty. Ultimately, CheY arrivers in the lowest free energy minimum of its energy landscape of folding, which is the native state [115].

**Spo0F and NT-NtrC**

A combined experimental and simulation analysis was done on the folding of Spo0F from *Salmonella typhimurium* and NT-NtrC from *Bacillus subtilis*, which are 124-residue response-regulator proteins that have low sequence similarity [116]. Both proteins are allosterically activated through phosphorylation of D54. ‘Flavored’ Gō-model-like simulations show that in the productive folding transition state for nonphosphorylated Spo0F and phosphorylated NT-NtrC the N-terminal subdomain is partially structured, whereas the C-terminal one is not, just as observed for CheY [116]. A similar result was obtained for nonphosphorylated NT-NtrC through use of two different simulation approaches, which shows that the N-terminal half of the protein appears to fold earlier than the C-terminal half [117, 118]. Remarkably, for phosphorylated NT-NtrC the latter two simulations suggest that the C-terminal half of the protein folds first [117, 118], contradicting the simulations of [116].

Kinetic folding experiments show that both Spo0F and NT-NtrC fold via parallel pathways through highly structured submillisecond intermediates before accessing their *cis* prolyl peptide bond-containing native conformations. Global analysis of the data favors an off-pathway folding mechanism for both proteins [116]. Sequence-sensitive Gō-model simulations suggest that frustration in the folding of Spo0F leads to the appearance of an off-pathway species, reflecting competition for intrasubdomain van der Waals contacts between its N- and C-terminal subdomains. Local-in-sequence clusters of side chains of ILV stabilize the intermediate. Comparable observations were made for CheY folding. In case of NT-NtrC, the simulations fail to detect an off-pathway species. Experimental kinetic folding data show that the free-energy landscapes for folding of NT-NtrC and Spo0F are in many ways similar to the one of CheY [9, 116].

Table 1 shows that off-pathway intermediate formation is characteristic for the folding of CheY-like proteins. Just as for flavodoxins, differences in the stability of these intermediates, and hence in their populations, may be explained by sequence-specific interactions within these species.

**Flavodoxin-like proteins tend to temporarily misfold during unassisted folding in vitro and have nonconserved transition states of folding**

Flavodoxin-like proteins are susceptible to frustration in the early stages of folding, because an α-helix forms much more quickly than a parallel β-sheet. α-Helix formation is rapid due to the highly local character of interactions that produce this secondary structure. In contrast, many intervening residues separate the amino acids that constitute the strands of a parallel β-sheet and thus its formation is relatively slow. Since in Gō-like simulations only residue pairs that are in contact in the native state experience attractive forces, application of this model to flavodoxin-like proteins should produce comparably structured off-pathway intermediates. However, we note that slightly differing simulations, including those in which heterogeneity of the native contact energies is added to incorporate sequence effects, produce markedly dissimilar intermediates. For *D. desulfuricans* apoflavodoxin the misfolded species contains β-sheet structure [102]. For CheY the off-pathway intermediate is reported to be helical, lacking the central parallel β-sheet of native protein [112]. However, other simulations show that secondary structure elements α₂, β₁, α₃, and β₄ of native CheY seem to be involved in formation of this species [9, 113]. For Spo0F, simulations demonstrate that formation of this intermediate engages secondary structure elements α₃, β₄, α₄, and β₃ of native protein. Apparently, variations in sequence seem to modulate formation of these folding intermediates [115, 116].

It is important to experimentally characterize the structural features of the discussed off-pathway intermediates in order to verify whether theory correctly predicts their conformations. In addition,
characterization of the unfolded states of flavodoxin-like proteins is essential to comprehend how these misfolded species form, as residual structure in unfolded protein facilitates formation of folding intermediates. For \textit{A. vinelandii} flavodoxin [43,44,69,70] and CheY [108] the unfolded protein has been experimentally probed at the residue level. Transiently ordered regions exist in both unfolded proteins and largely comprise $\alpha$-helical structures. Besides native, non-native $\alpha$-helical structure may also develop (Fig. 5). Gō-like simulations cannot predict such a non-native structure. In flavodoxin-like proteins, hydrophobic interactions of side chains pack $\alpha$-helices onto the parallel $\beta$-sheet. This typical feature probably contributes to the observed misfolding, as upon folding the $\alpha$-helices fold first and can subsequently dock onto one another through hydrophobic interactions. This docking prevents formation of the parallel $\beta$-sheet and the resulting misfolded intermediate needs to unfold considerably before folding to native protein can take place, explaining why this species is off-pathway, as experimentally shown for \textit{A. vinelandii} flavodoxin [43,44,46,69,70,72]. Indeed, this off-pathway species is largely $\alpha$-helical [45]. The off-pathway intermediate of CheY also appears to be helical, as all five native $\alpha$-helices are formed and as stabilization of any of its $\alpha$-helices slows refolding at low denaturant concentrations [111]. Experiments thus strongly suggest that the off-pathway intermediate of flavodoxin-like proteins is of helical nature. Future experimental efforts, using, for example, Fourier transform infrared spectroscopy, should reveal whether the misfolded species of \textit{D. desulfuricans} apoflavodoxin and Spo0F really contain $\beta$-sheet structure, as simulations seem to suggest.

Current knowledge about the kinetics and energetics of protein folding largely stems from \textit{in vitro} studies and from simulations. These studies revealed that the topology of a native protein influences the rate with which the native state is formed \textit{in vitro}. This implies that the transition state that determines the rate of folding resembles the native state of a protein. Interactions involving a few key residues force a folding protein to adopt a rudimentary native-like architecture. Once the correct topology has been achieved, the native structure will then almost invariably be generated during the final stages of folding [119–123]. Phi-value analysis shows that the productive folding transition state of CheY is polarized with a structured N-terminal subdomain and an unstructured C-terminal one [109]. Simulations reveal transition states for non-phosphorylated Spo0F and phosphorylated NT-NtrC similar to the one of CheY [116]. This indicates that the N-subdomain serves as the folding nucleus for these proteins. In contrast, the transition states of \textit{Ana-}

\textit{babaena} and \textit{D. desulfuricans} apoflavodoxins are diffuse with partial formation of many inter-residue interactions [81,102]. Thus, the productive folding transition state of a flavodoxin-like protein seems to differ from one protein to another. Apparently, both chain topology and amino acid sequence contribute to defining the structure of this transition state and the folding energy landscapes of flavodoxin-like proteins.

In summary, flavodoxin-like proteins tend to temporarily misfold during their unassisted folding \textit{in vitro} and have a nonconserved transition state that determines their rate of productive folding.

**Protein folding \textit{in vivo}**

While most protein folding experiments have been done \textit{in vitro}, the circumstances under which folding occurs in the cellular environment differ dramatically (Fig. 6).

Firstly, the conditions under which most folding landscapes are probed \textit{in vitro} are physiologically speaking irrelevant. Illustrative in this regard is the use...
of high concentrations of GuHCl or urea (although data can be extrapolated to zero molar denaturant). More gentle techniques to study protein folding are alteration of pH or temperature. Yet, even results obtained by these methods need to be carefully considered before they can be judged to be physiologically relevant. For example, how pertinent are folding intermediates found at pH 4, such as MGs, when the cytoplasmic environment is buffered at approximately pH 7 [124]? Concerning proteins that originate from endothermic organisms: how significant are folding intermediates found at 20 or 50 °C? Ultimately, existence of folding intermediates in vitro does not necessarily imply occurrence of the same or similar species and their consequences in vivo.

Secondly, compared to most in vitro folding experiments, the cellular environment is immensely crowded (Fig. 6), with estimations of the concentration of proteins and other macromolecules ranging up to 300–400 g L⁻¹ [125]. This crowding can lead to a significant excluded volume effect, which in turn can influence the type and amount of interactions a polypeptide can form [126,127]. However, folding in vitro is typically done using dilute buffers with micromolar protein concentrations. For A. vinelandii apoflavodoxin, conditions that mimic macromolecular crowding inside cells lead to compaction of unfolded protein and slight stabilization of native protein [61]. Crowding causes severe aggregation of its off-pathway MG [61]. In case of H. pylori apoflavodoxin, crowding slightly stabilizes both native and molten globular protein, but does not significantly stabilize the native state relative to its MG [92]. Remarkably, crowding increases the mid-point of thermal unfolding of apoflavodoxin from D. desulfuricans by up to 20 °C [96]. In contrast to other apoflavodoxins, the stability of this protein is very sensitive to buffer composition. Its thermal mid-point of unfolding can decrease as much as 25 °C upon changing from phosphate to HEPES buffer [96]. Crowding makes the ensemble of unfolded conformations of D. desulfuricans apoflavodoxin less expanded, resulting in a folding funnel that apparently is smoother and narrower [128]. Upon changing the crowding agent the folding mechanism of this protein seems to be modulated differently, and folding routes experiencing topological frustrations might be either enhanced or relieved [129]. The influence of crowding on the folding CheY-like proteins has not been reported. For various other proteins the effects of artificial crowders, such as Ficoll, glycerol, dextran and others, and occasionally of similar kinds of crowders put together, have also been probed (for an extensive review see [130]). However, these crowders differ from those in a cell, in which they span broad spectra of size, hydrophobicity, and charge, and are present simultaneously. Artificial crowders thus possess shortcomings and their usefulness in predicting effects of crowding in vivo is contested [131].

Thirdly, a major difference between folding in vitro and in vivo is the circumstance under which a protein is introduced in either environment. During in vitro folding a full-length polypeptide, or sometimes fragments of it, is studied. The protein is chemically or thermally unfolded and subsequently it can in principle sample all of its folding states while progressing to the native state. In vivo, however, a polypeptide can start to fold as soon as it becomes produced by the ribosome (Fig. 6).

Ribosomes play a crucial role as a central hub in coordinating protein quality control [132,133]. They sense the nature of the emerging polypeptide, recruit protein folding and translocation factors, and integrate mRNA and nascent chain quality control. Ribosomes can control the cellular abundance of proteins at the translational level and thus are a significant constituent of protein homeostasis [134–138]. In growing cells, most ribosomes are active in translation and contain an emerging polypeptide. Although ribosomal structures have been elucidated in atomic detail [139–143], relatively little is known about the conformational events polypeptide chains undergo while they are produced.

Upon addition of amino acid residues, the growing nascent chain gradually emerges from the ribosomal exit tunnel. Folding or misfolding of the nascent chain may already start in the exit tunnel [144–147] and in most cases commences while the nascent chain gradually emerges from it [53–55,57,148–156]. As synthesis of the nascent chain is orders of magnitude slower than it takes a protein to fold (i.e., seconds versus micro- to milliseconds), nascent chains can already sample parts of their conformational space before the next amino acid residue is added. This phenomenon, in combination with gradual emergence of the polypeptide from the exit tunnel, might impose conformational constraints on the folding energy landscape of the protein involved. Folding during synthesis on the ribosome leads to constant remodeling of the folding energy landscape as translation proceeds.

The ribosome itself can also modulate protein folding, for example, through interaction of the nascent chain with the exit tunnel or, upon its emergence from the tunnel, with the ribosomal surface [59,137,158]. The tunnel prevents proper tertiary folding of larger protein domains and precludes the C-terminal residues of the nascent chain from participating in long-range
interactions [159]. As a consequence, productive folding can occur only after a complete protein or a domain has come out of the ribosome [160]. Once a nascent chain emerges its folding can be influenced by transient electrostatic interactions with the ribosomal surface. As the exterior of the ribosome consists for a large part of rRNA, it is negatively charged in many areas. Negative charge also surrounds the exit tunnel, thereby providing an environment that is very different from the cytoplasm [161]. This charge may influence protein folding, as it attracts positively charged residues of the nascent chain and repels negatively charged ones [157]. It also restricts the dynamics of nascent chains according to their charge [161]. Due to these electrostatic interactions, nascent chains may be protected from misfolding or aggregation.

Methodologies like epitope recognition, enzymatic activity, cofactor binding, NMR, and fluorescence spectroscopy indicate structural ordering and acquisition of activity of polypeptides once they arrive outside the exit tunnel [53–55,57,148–156]. Cotranslational folding has been observed for all α [53], all β [55,57,152,155], and α/β proteins [56,162,163]. Various nascent chains form native- or non–native-like intermediates [53–55,57,152,164–166].

Lastly, upon emergence from the exit tunnel, cellular proteins can start to interact with the nascent chain and affect its folding (Fig. 6). Some of the first proteins that meet the nascent chain in bacteria, mitochondria and chloroplasts are processing proteins such as peptide deformylase, which removes the formyl moiety from the N-terminal formyl-methionine. Another processing protein is methionine aminopeptidase, which hydrolyses the N-terminal methionine for more than 50% of nascent chains [132]. Chaperone proteins that hover at the exit tunnel facilitate nascent chain folding just outside this tunnel [167,168]. They prevent protein aggregation and/or stimulate folding toward the native state. To date, no study of the influences of chaperones on the folding of flavodoxin-like proteins has been reported.

These differences between in vitro and in vivo protein folding show that a demand exists to obtain a molecular description of how flavodoxin-like proteins fold in a cell. Formation of off-pathway intermediates during the in vitro folding of flavodoxin-like proteins is an important observation. It raises the question whether these species also form in the cellular context. In the cell, MG-like species tend to aggregate with potential detrimental effects on organisms. To start with, it needs to be explored how a flavodoxin-like protein folds on the ribosome. Cotranslational folding is suspected to be critical for many proteins in order to prevent inter- or intramolecular misfolding and aggregation [155,167]. Increasing our knowledge of nascent chain folding is relevant for the biotechnological production of proteins with a flavodoxin-like fold and to comprehend the molecular basis of folding defects. These defects underlie protein malfunction and protein homeostasis failure [135] that can ultimately manifest itself as a wide variety of diseases [36,134]. Possibly, the ribosome and/or chaperones regulate off-pathway intermediate formation. In the following, as a first step toward clarification of the conformational events a flavodoxin-like protein undergoes while it folds in a cell, we discuss the cotranslational folding of flavodoxin.

The ribosome affects flavodoxin folding

Various strategies exist to study folding of a protein while it emerges from the ribosome. To characterize nascent chain folding of A. vinelandii flavodoxin, the approach we followed is to produce, purify, and characterize stably arrested ribosome-nascent chain complexes (RNCs). RNCs consist of the entire 70S ribosome with a polypeptide that is stalled through tight interaction with the exit tunnel. Production of these RNCs happens in E. coli [163] or by using an in vitro protein synthesis kit [169]. To achieve translational stalling, a sequence derived from E. coli SecM (Secretion Monitor protein) is attached to the C terminus of the translating nascent protein [170,171]. In addition, the construct used contains a linker that connects SecM and flavodoxin and spans the length of the exit tunnel, thereby entirely exposing the flavodoxin polypeptide outside the ribosome [163,169]. Use is made of constructs that lack 0, 5, or 10 amino acid residues at the C terminus of stalled flavodoxin. This procedure allows mimicking of late stages during protein translation and enables one to obtain ‘snapshots’ of cotranslational protein folding. Due to physical forces on the nascent chain [172,173], the flavodoxin construct is sometimes released from the RNC [163]. Comparison of released protein and RNCs allows assessment of the influence the ribosome has on apoapoflavodoxin folding.

Flavin mononucleotide only binds to the flavin-binding site of full-length, isolated A. vinelandii apoflavodoxin when the protein is natively folded [66]. We addressed cofactor binding to nascent flavodoxin and showed that FMN does not bind to apoflavodoxin during translation [163]. The cofactor can only bind to the protein once it is entirely synthesized and exposed outside the ribosome, because then apoflavodoxin...
becomes natively folded (Fig. 7A). Even when incomplete apoflavodoxin that lacks only its five C-terminal residues emerges from the ribosomal exit tunnel it cannot incorporate FMN, because it forms a non-native intermediate, the conformation of which is presently unknown. In contrast, the corresponding released, shortened protein product does bind the cofactor (Fig. 7B). These differences in FMN-binding capacities show that the ribosome affects nascent flavodoxin folding and cofactor binding. As a result, the binding of the cofactor to released full-length protein is the last step in the production of this flavoprotein in the cell (Fig. 7A,B) [163]. The longer the chain of nascent apoflavodoxin, the more stabilized the protein becomes and the less prone apoprotein is to intracellular proteolytic degradation. Upon incorporation of FMN the corresponding holoproteins become protected against degradation (Fig. 7A,B) [163].

To further the understanding of flavodoxin folding in vivo, we investigated whether the ribosome modulates formation of apoflavodoxin’s off-pathway MG [169]. To this end, the F44Y mutation was introduced into an RNC construct that exposes the entire nascent chain outside the exit tunnel. Full-length, isolated F44Y apoflavodoxin switches from natively folded to off-pathway MG upon decreasing ionic strength to physiological values [45], thereby avoiding the use of denaturant, which could adversely affect ribosomal integrity. The F44Y mutation introduces an extra oxygen atom into a hydrophobic pocket of native apoflavodoxin, causing considerable destabilization [45,70]. It was verified that the SecM sequence, the linker, and other components of the RNC construct do not influence formation of the off-pathway MG of F44Y apoflavodoxin or impair FMN binding. In addition, it has been shown that FMN does not associate with ribosomes [169]. As the off-pathway MG needs to unfold before native apoflavodoxin can form, the rate of cofactor binding is delayed compared to the situation where only natively folded apoprotein is present. Thus, ascertaining the rate of FMN binding as a function of ionic strength is a suitable tool to detect the presence of apoflavodoxin’s off-pathway MG on the ribosome [169]. Because F44Y apoflavodoxin RNCs bind FMN rapidly at both physiological and at high salt concentration, the fully exposed polypeptide must be natively folded under both conditions. Therefore, the ribosome modulates MG formation and forces nascent apoflavodoxin toward the native state (Fig. 7C) [169]. This confinement of MG formation is an important observation that emphasizes differences between folding energy landscapes in vivo and in vitro. Possibly, electrostatic repulsion of the nascent chain (apoflavodoxin has a net charge of $-13$ at neutral pH) by the negatively charged ribosomal surface restricts the conformational space of unfolded protein, leading to entropic stabilization of native protein at physiological ionic strength [169]. When release of the nascent chain occurs at physiological ionic strength, released F44Y apoflavodoxin construct predominantly forms the off-pathway MG, which is unstable and thus susceptible to intracellular proteolytic degradation (Fig. 7C) [169]. Because molten globular F44Y apoflavodoxin is in equilibrium with native protein, binding of FMN withdraws this latter state from the equilibrium and protects the protein against the action of proteases (Fig. 7C).

### Future perspectives

Our understanding of the folding of isolated flavodoxin-like proteins in a test tube, including the influence of macromolecular crowding, has increased considerably during the past decades. Also, the first facets of cotranslational folding of flavodoxin have been illuminated. Various aspects of the folding of flavodoxin-like proteins still require clarification, some of which are highlighted below.

Experimental determination of folding landscapes is a challenge and therefore relatively little experimental knowledge exists about them [29]. Developments in biophysical techniques, including single-molecule ones, need to be applied to determine, refine, and compare folding energy landscapes of various flavodoxin-like proteins and obtain more insight into off-pathway
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intermediate formation. For example, it is currently unknown when off-pathway intermediates of flavodoxin-like proteins unfold and embark on productive folding routes, which specific intramolecular interactions need to be disrupted. Mechanical unfolding and refolding by using optical tweezers may provide insight into this issue and can also be applied to further our understanding of the cotranslational folding of these proteins [157].

Separate domains of multidomain proteins are expected to fold independently of each other during translation [165]. It would be interesting to verify whether the ribosome also forces the folding of a flavodoxin-like domain within multidomain proteins, like cytochrome P450 reductase (PDB entry 1AMO) and nitric oxide synthase (PDB entry 1TLL), toward the native state. When a flavodoxin-like domain is non-C-terminally located, it is tethered to the ribosome during further translation of the multidomain protein involved. Thus, once this domain emerges from the exit tunnel, the ribosome has ample opportunity to influence its folding. If the ribosome pushes the flavodoxin-like domain toward the native state it should be able to bind FMN, as demonstrated for A. vinelandii apoflavodoxin [163]. Cotranslational incorporation of flavin would impede proteolytic degradation of nascent multidomain chains that contain a flavodoxin-like domain, due to increased stability conferred by cofactor binding. This could increase protein production efficiency [163]. For several flavoproteins involved in diseases, a beneficial effect is observed upon supplementing the respective flavin [174].

While at equilibrium the ribosome restrains off-pathway MG formation in stalled RNC complexes that expose the entire F44Y flavodoxin protein outside the exit tunnel, comparable confinement may not happen during translation. For example, nascent flavodoxin that lacks five C-terminal amino acid residues adopts a non-native structure [163]. It would be fascinating to elucidate how non-native interactions evolve during translation. Recently, by exploiting a reconstituted in vitro translation system combined with labeling of nascent chains at defined positions with bright fluorophores, nascent protein folding could be monitored in real-time by Förster resonance energy transfer [59]. Application of this methodology can shed light onto folding of flavodoxin-like protein during translation and reveal whether temporary misfolding and formation of MGs occurs.

Isolated, full-length flavodoxin-like proteins tend to form off-pathway intermediates during folding in vitro. While this observation does not signify that they also form misfolded species cotranslationally, once released from the restraining influence of the ribosome these proteins may temporarily adopt misfolded structures in the cellular environment. Nevertheless, many flavodoxin-like proteins can be overexpressed in their native form, suggesting involvement of chaperones. Hence, a strong demand exists to clarify the influences of chaperones, like Trigger factor, DnaK/DnaJ, and GroEL/GroES, have on the folding of flavodoxin-like proteins.

The ultimate challenge is to resolve the folding of flavodoxin-like proteins in vivo. Although progress has been made in characterizing protein conformations at the atomic level in the crowded milieu of live cells [175–178], structural resolution of how proteins attain their native architecture in such an environment is still a formidable challenge. Surprises are likely to emerge about the folding of flavodoxin-like proteins in a living cell.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

JAH and CPMvM wrote the manuscript. JAH generated the figures.

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