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Folding of *Desulfovibrio desulfuricans* flavodoxin is accelerated by cofactor fly-casting

B.K. Muralidhara [a,*], Ramesh Rathinakumar [b], Pernilla Wittung-Stafshede [c,d,e,*]

[a] *Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77555, USA*
[b] *Biochemistry Department, Tulane University, New Orleans, LA 70112, USA*
[c] *Department of Biochemistry and Cell Biology, Rice University, USA*
[d] *Keck Center for Structural Computational Biology, Rice University, USA*
[e] *Department of Chemistry, Rice University, 6100 Main Street, Houston, TX 77251, USA*

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Abstract

Folding of cofactor-binding proteins involves ligand binding in addition to polypeptide folding. We here assess the kinetic folding/binding landscape for *Desulfovibrio desulfuricans* flavodoxin that coordinates an FMN cofactor. The apo-form folds in a two-step process involving a burst-phase intermediate. Studies on Tyr98Ala and Trp60Ala variants reveal that these aromatics—that stack with the FMN in the holo-form—are not participating in the apo-protein folding pathway. However, these residues are essential for FMN interactions with the unfolded protein during refolding of holo-flavodoxin. Unfolding of wild-type holo-flavodoxin is coupled to FMN dissociation whereas for Tyr98Ala and Trp60Ala holo-variants, FMN dissociates before polypeptide unfolding. Both variants refold as apo-proteins before FMN rebinds. In sharp contrast, refolding of unfolded wild-type holo-flavodoxin is over an order of magnitude faster than that of the apo-form, the pathway does not include a burst-phase intermediate, and the speed is independent of FMN excess ratio. These observations demonstrate that FMN binds rapidly to the unfolded polypeptide and guides folding straight to the native state. As this path to functional *D. desulfuricans* holo-flavodoxin is faster than if the cofactor binds to pre-folded apo-protein, this is one of few examples where molecular recognition via a "fly-casting" mechanism is kinetically favored.

Keywords: Protein folding; Cofactor; Fly-casting; Folding speed; Flavodoxin; FMN

The mechanism by which an unfolded polypeptide folds into its final native structure is still not fully understood. Most protein-folding studies focus on the behavior of isolated polypeptides in vitro; the general understanding of the folding processes of small, single-domain proteins has significantly improved in recent years. However, the environment inside cells is highly crowded and numerous inter-molecular interactions may take place during polypeptide folding in vivo. Some of these interactions may be short-lived while others may persist until the protein eventually undergoes degradation. For cofactor-binding proteins, a cofactor must associate with the polypeptide to adopt the active, functional state of the protein. Thus, in addition to conformational changes, folding of cofactor-binding proteins also includes a bi-molecular binding event; this step may occur before, after or during polypeptide folding. It has been found that many cofactor-binding proteins are capable of binding their cognate cofactors in the unfolded state in vitro [1–4]. This, together with the discovery of intrinsically disordered proteins in the cells, emphasizes the importance of assessing the interplay between protein folding and cofactor binding.

Proposed mechanisms for inter-molecular recognition, such as between a protein and a cofactor, include "induced-fit" (i.e., flexible recognition), "lock-and-key" (i.e., rigid-body docking), and "conformational-selection" (i.e., recognition of

* Corresponding authors. Fax: +1 409 772 9691 (B.K. Muralidhara), +1 713 348 5154 (P. Wittung-Stafshede).
E-mail addresses: bkmurali@utmb.edu (B.K. Muralidhara), pernilla@rice.edu (P. Wittung-Stafshede).

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pre-selected conformers) scenarios [5–9]. While these three mechanisms assume the presence of a folded or almost completely-folded protein before the inter-molecular recognition step, another possibility, the "fly-casting" mechanism that involves binding to an unstructured protein, was recently proposed [10]. In this scenario, the unfolded protein engages its cofactor at relatively large distances. Based on the greater capture radius of an unstructured polypeptide as compared to a folded protein, the fly-casting model predicts that the formation of a compact cofactor-protein complex (i.e., a folded, functional holo-protein) will occur more rapidly for polypeptides that are initially unfolded, either partially or fully. In addition, the greater flexibility of a non-native polypeptide might facilitate conformational sampling of the energy landscape en route towards the functional protein-cofactor complex.

Here, we test the fly-casting prediction by studying the interplay between polypeptide folding and cofactor binding in *Desulfovibrio desulfuricans* flavodoxin. Flavodoxins are small, bacterial flavo-proteins that participate in low-potential electron-transfer pathways [11–15]. There are two types of flavodoxins: long- and short-chain variants [11]. Long-chain flavodoxins (such as *Azotobacter vinelandii* and *Anabaena* PCC 7119) only differ from short-chain versions (such as *D. desulfuricans*, *Desulfovibrio vulgaris*, and *Clostridium beijerinckii*) in that they have a 20–25 residue loop that interrupts β-strand five. All known flavodoxins contain a single, non-covalently bound flavin mononucleotide (FMN)[1] cofactor [16,17]. The FMN interacts with three loops of the protein (Fig. 1): two aromatic residues (Trp60 and Tyr98 in *D. desulfuricans* flavodoxin), located on opposite loops, and flank either side of the FMN isoalloxazine ring allowing for considerable π-orbital overlap. The 5′-phosphate moiety of FMN is bound in an atypical phosphate-binding site, anchored by several hydrogen bonds in a loop near the N-terminus, but with no ion-pairing interactions [16–18]. Time-resolved folding studies of both long- and short-chain apo-flavodoxins demonstrate that the folding landscape involves burst-phase (on- and/or off-pathway) intermediates [19–23].

FMN-binding experiments to *D. desulfuricans* apo-flavodoxin at different denaturant concentrations have been used to propose that the fastest path to holo-flavodoxin will correspond to apo-protein folding before cofactor binding [24,25]. The experiments presented in this work challenge that scenario; at solvent conditions favoring refolding, we observe that FMN binds rapidly to unfolded *D. desulfuricans* flavodoxin and speeds up the folding process by over one order of magnitude. Since, this path to functional holo-flavodoxin is faster than is FMN binding to pre-folded apo-protein, *D. desulfuricans* flavodoxin is one of few characterized systems that follow the kinetic trend predicted by the fly-casting model.

[1] *Abbreviations used:* FMN, flavin mononucleotide; CD, circular dichroism.

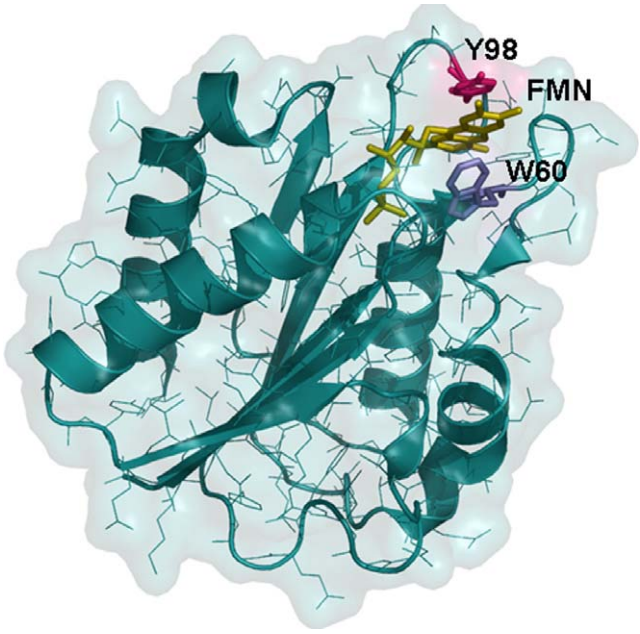

Fig. 1. Cartoon representation (generated in PyMOL) of the three-dimensional structure of holo-flavodoxin from *D. vulgaris* (pdb:1J8Q) revealing the typical flavodoxin-like fold of a five-stranded parallel β-sheet surrounded by 4 α-helices. The two aromatic residues (Tyr98: pink and Trp60: purple) that flank the isoalloxazine ring of FMN (yellow) are shown in stick representation.

Materials and methods

Protein preparation

Flavodoxin from *D. desulfuricans* (ATCC strain 29577) was expressed in *Escherichia coli* [19,26] and purified as described earlier. Construction and purification of the two mutants used (Trp60Ala and Tyr98Ala) have also been reported [24]. In short, apo-forms of wild-type, Trp60Ala, and Tyr98Ala flavodoxins were isolated on a Q-Sepharose column in phosphate buffer and further purified by gel permeation on a Superdex-75 using an FPLC system (Amersham-Pharmacia) [27] using phosphate buffer. HPLC-purified FMN (Sigma Chemicals) was used in binding/reconstitution studies. FMN was oxidized in all experiments and its concentration determined using a molar extinction coefficient of $\varepsilon_{445\ nm} = 12{,}500\ M^{-1}cm^{-1}$. Dialysis experiments to assess FMN dissociation from the holo-proteins in 3 M GuHCl were performed with 200 μM protein samples using 3 kDa cut-off membranes. Control experiments of membrane penetration by free FMN were performed with samples of 200 μM FMN. The concentrations of FMN outside the membrane at different time points were measured via FMN fluorescence (excitation at 445 nm; emission monitored at 525 nm).

Equilibrium protein unfolding

GuHCl-induced equilibrium-unfolding experiments with wild-type, Trp60Ala, and Tyr98Ala flavodoxins were probed in 50 mM phosphate buffer (pH 7, 25 °C). Fluorescence and