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Topological Switching between an α−β Parallel Protein and a Remarkably Helical Molten Globule

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Abstract: Partially folded protein species transiently exist during folding of most proteins. Often these species are molten globules, which may be on- or off-pathway to native protein. Molten globules have a substantial amount of secondary structure but lack virtually all the tertiary side-chain packing characteristic of natively folded proteins. These ensembles of interconverting conformers are prone to aggregation and potentially play a role in numerous devastating pathologies, and thus attract considerable attention. The molten globule that is observed during folding of apoflavodoxin from *Azotobacter vinelandii* is off-pathway, as it has to unfold before native protein can be formed. Here we report that this species can be trapped under nativelike conditions by substituting amino acid residue F44 by Y44, allowing spectroscopic characterization of its conformation. Whereas native apoflavodoxin contains a parallel β-sheet surrounded by α-helices (i.e., the flavodoxin-like or α−β parallel topology), it is shown that the molten globule has a totally different topology: it is helical and contains no β-sheet. The presence of this remarkably nonnative species shows that single polypeptide sequences can code for distinct folds that swap upon changing conditions. Topological switching between unrelated protein structures is likely a general phenomenon in the protein structure universe.

Introduction

Four decades ago, Levinthal[1] predicted that folding of proteins to their native states involves folding pathways on which folding intermediates reside. Today, the considerable advances made in experimental methods have shown that hardly any protein exhibits true two-state folding behavior.[2] Ultrarapid mixing experiments led to the detection of short-lived kinetic intermediates,[3] and techniques such as Förster resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), and NMR revealed partially folded states that are difficult to detect.[4,5] Occasionally proteins can be changed by mutagenesis to create a polypeptide that forms a relatively stable folding intermediate.[6−8] In general, experimental data on folding intermediates argue for near-native topology of these species, which have incompletely folded or partially misfolded structural elements.[8−10]

Most folding proteins encounter folding energy landscapes[11] that are rough. As a result, partially folded intermediates, which may be on- or off-pathway to the native state, are populated. When the intermediate is on-pathway, as is observed for the majority of proteins studied to date, it has nativelike topology and is productive for folding. In contrast, when the intermediate is off-pathway it is trapped in such a manner that the native state cannot be reached without substantial reorganizational events.[2] Several kinetic studies have revealed involvement of off-pathway intermediates during protein folding.[12−18]

Structural characterization of folding intermediates is challenging due to the transient nature of their existence. In addition, if an intermediate is observed at equilibrium, it is in most cases sparsely populated relative to the corresponding native and unfolded states. However, characterization of folding intermediates is crucial for the understanding of protein folding. Kinetic intermediates that appear early during folding have been shown to resemble the relatively stable molten globule[19−22] intermediates formed by several proteins under mildly denaturing

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conditions.[19] This resemblance has been demonstrated for α-lactalbumin,[23] apomyoglobin,[24] RNase H,[25] T4 lysozyme,[26] and Im7[27] and suggests that these molten globules can be considered as models of transient intermediates.[2] Due to exposed hydrophobic groups, molten globules are prone to aggregation. Particularly, a decrease of the folding rate due to the presence of an off-pathway molten globule, which is kinetically trapped and partially folded, increases the likelihood of protein aggregation. This aggregation phenomenon can have detrimental effects on organisms.[28] Understanding the formation and conformation of molten globules offers potential insights into factors responsible for protein misfolding, aggregation, and, potentially, for numerous devastating pathologies.[29]

Here, we report the trapping under nativelike conditions of the molten globule folding intermediate of a 179-residue flavodoxin from *Azotobacter vinelandii*, enabling characterization of its conformation. Flavodoxins are monomeric proteins involved in electron transport and contain a noncovalently bound flavin mononucleotide (FMN) cofactor. These proteins consist of a single structural domain and adopt the flavodoxin-like or $\alpha-\beta$ parallel topology, which is widely prevalent in nature. Both denaturant-induced equilibrium and kinetic (un)folding of flavodoxin and apoflavodoxin (i.e., flavodoxin without FMN) have been characterized by use of guanidine hydrochloride (GuHCl) as denaturant.[13,30−34] The folding data show that apoflavodoxin autonomously folds to its native state, which is structurally identical to flavodoxin with the exception of residues in the flavin-binding region of the apoprotein. These residues have considerable dynamics in apoflavodoxin.[35,36] The last step in flavodoxin folding is binding of the cofactor to native apoflavodoxin.[30]

Kinetic folding of apoflavodoxin involves an energy landscape with two intermediates and is described by $I_{off} \leftrightarrow$ unfolded apoflavodoxin $\leftrightarrow I_{on} \leftrightarrow$ native apoflavodoxin.[13] Intermediate I_{on} lies on the productive route from unfolded to native protein, is highly unstable and is therefore not observed during denaturant-induced equilibrium unfolding. Approximately 90% of folding molecules fold via off-pathway intermediate I_{off}, which is a relatively stable species that needs to unfold to produce native protein and thus acts as a trap.[13] The formation of an off-pathway species is typical for proteins with a flavodoxin-like topology.[32] An off-pathway intermediate is experimentally observed for all other $\alpha-\beta$ parallel proteins of which the kinetic folding has been investigated: apoflavodoxin from *Anabaena*,[12] CheY,[16] cutinase,[17] and UMP/CMP kinase.[18]

Equilibrium unfolding of apoflavodoxin is described by the three-state model $I_{off} \leftrightarrow$ unfolded apoflavodoxin $\leftrightarrow$ native apoflavodoxin (see Figure S1 in Supporting Information).[13] Unfolded apoflavodoxin contains several transiently structured regions that dock, causing formation of I_{off}.[37] The intermediate folds in a noncooperative manner[38] and populates significantly in the concentration range of 1−3 M GuHCl. Its maximal population is at 1.76 M GuHCl with a mole fraction of 0.63 (Figure S1d in Supporting Information). The off-pathway species is molten globule-like: it is compact, its three tryptophans are solvent-exposed, and it has severely broadened NMR resonances due to exchange between different conformers on the micro- to millisecond time scale.[13,33,39] Although the conformation of this molten globule is currently unknown and cannot be determined by using NMR spectroscopy, intermediate I_{off} probably contains helices.[13] Elevated protein concentrations[39] and molecular crowding[40] cause severe aggregation of this species.

Anfinsen's thermodynamic hypothesis[41] states that each amino acid sequence codes for a protein that folds to the state that has the lowest free energy. This state is called the native state and is characterized by a unique tertiary fold. In this study it is shown that the equilibrium between native and molten globule apoflavodoxin can be shifted, in the absence of denaturant, from one monomeric species to the other. Full population of the molten globule folding state of apoflavodoxin is possible through covalent introduction of just a single extra oxygen atom in the protein, achieved by substituting F44 by Y44. This substitution leads to significant destabilization of native Y44-apoflavodoxin compared to WT-apoflavodoxin. Upon a mild change in conditions, that is, lowering salt concentration, virtually all protein molecules exist as molten globule, as it has become the lowest energy species. Now characterization of an off-pathway folding intermediate is possible in the absence of denaturant. We show that it has a drastically different topology compared to native protein: it is helical and lacks the parallel β-sheet of native apoflavodoxin. Our observations show that interconversion between two unrelated protein folds can be dictated by a single amino acid sequence.

Experimental Section

Proteins. The single cysteine at position 69 in *A. vinelandii* (strain ATCC 478) flavodoxin II was substituted by an alanine to avoid covalent dimerization of apoflavodoxin. This protein variant is largely similar to wild-type flavodoxin[33,42] and is referred to as WT-flavodoxin. Subsequently, in addition, the phenylalanine at position 44 was substituted by a tyrosine via site-directed mutagenesis. The latter protein variant is referred to as Y44-flavodoxin. Both flavodoxin variants were obtained from transformed *Escherichia coli* cells and purified as described.[33]

To obtain native apoprotein, holoprotein was denatured in 6 M GuHCl. Subsequently, both FMN and denaturant were removed

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via gel filtration on a Superdex 75 column, which is loaded with 100 mM potassium pyrophosphate (KPP_i), pH 6.0. During this removal step, the protein folds to native apoprotein.

KPP_i was the buffer during all experiments, as the use of this buffer gave rise to high-quality NMR spectra of *A. vinelandii* flavodoxin (i.e., sharp NMR resonances were obtained).

Fluorescence. Steady-state fluorescence measurements of denaturant-induced equilibrium unfolding were done at 25 °C on a Cary Eclipse fluorometer (Varian). Excitation was at 280 nm and emission was measured at 340 nm. Protein concentration was 4 μM in 100 mM KPP_i at pH 6.0.

Fluorescence emission spectra were obtained at 25 °C. Excitation was at 280 nm. Protein concentration was 4 μM. The buffer used was 100 mM KPP_i, at pH 6.0, or a 10-fold dilution of this buffer.

Thermal protein unfolding was followed by fluorescence emission. Temperature was increased in a 1.5 mL stirred quartz cuvette (path length 0.4 cm) from 12 to 75 °C at a rate of 1 °C/min. Excitation was at 280 nm and emission was recorded at 340 nm. Protein was in 100 mM KPP_i, pH 6.0, and protein concentration ranged between 2.5 − 3.0 μM.

In all experiments, excitation and emission slits were set to a width of 5 nm.

Far-UV CD. CD measurements were acquired by use of a Jasco J715 spectropolarimeter.

Denaturant-induced equilibrium unfolding was followed at 25 °C in a 2-mm quartz cuvette by measuring ellipticities for a period of 3 min at 222 and 255 nm, and the obtained signals were subsequently averaged. The averaged ellipticity at 255 nm was subtracted from the averaged ellipticity at 222 nm. Protein concentration was 4 μM in 100 mM KPP_i at pH 6.0.

CD spectra of protein in 1-mm quartz cuvettes were obtained by averaging 20 scans and corrected by subtracting spectra of corresponding blank solutions. Protein concentration ranged between 1 and 2 μM. Buffer used was 100 mM KPP_i, at pH 6.0, or a 10-fold dilution of this buffer.

Thermal unfolding of Y44-apoflavodoxin was followed at 210 nm. Temperature was increased from 10 to 75 °C at a rate of 1 °C/min in a 1.5 mL stirred quartz cuvette (path length 0.4 cm). Protein concentration was 3 μM in 100 mM KPP_i at pH 6.0.

Fluorescence Anisotropy. Anisotropy was measured at 25 °C on a Fluorolog 3.2.2 fluorometer (Horiba Jobin Yvon Ltd.). Excitation was at 300 nm with a 1 nm slit, and emission was at 345 nm with a 14 nm slit. For each protein sample, five measurements were done at each setting and the data were subsequently averaged. In the case of denaturant-induced equilibrium unfolding of Y44-apoflavodoxin, protein concentration was 4 μM in 100 mM KPP_i, pH 6.0. In the experiments in which the KPP_i concentration was decreased by diluting the buffer solution, protein concentration ranged between 2.7 and 3.0 μM.

NMR. Gradient-enhanced $^1H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectra were recorded on a Bruker AMX 500 MHz instrument. Sample temperature was 25 °C.

Data Analysis. a. Denaturant-Induced Equilibrium Unfolding. A three-state model for apoflavodoxin equilibrium unfolding was globally fitted to fluorescence emission intensity data at 340 nm, CD data at 222 nm, and fluorescence anisotropy data at 345 nm (see Supporting Information).

b. Thermal-Induced Equilibrium Unfolding. A two-state model of unfolding, in which the change in free energy for thermal-induced protein unfolding is described by the modified Gibbs−Helmholtz equation, was fitted to individual thermal unfolding curves (see Supporting Information).

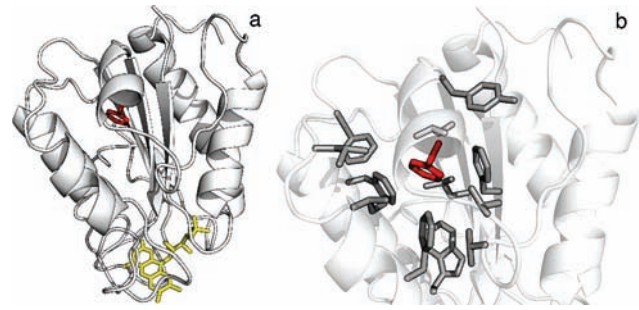

Figure 1. Residue F44 resides in a hydrophobic pocket of WT-flavodoxin. (a) Cartoon representation of WT-flavodoxin in which F44 (red) and FMN (yellow) are shown in stick representation (PDB ID 1YOB). (b) Residues within 5 Å distance of F44 (red) are highlighted.

c. Dissociation Constant of the Y44-Apoflavodoxin−FMN complex. The dissociation constant was determined via quenching of FMN fluorescence upon binding of cofactor to apoprotein[30] (see Supporting Information).

Results and Discussion

Design of Y44-Apoflavodoxin. Residue F44 is a highly conserved amino acid residue in flavodoxins.[43] It is part of the major hydrophobic core of flavodoxin, containing residues that are in a rigid three-dimensional geometry in native protein (Figure 1).[44] Consequently, substitution of F44 by the slightly larger and more hydrophilic residue tyrosine is expected to decrease the stability of the native state of apoflavodoxin against unfolding. It potentially avoids significant population of the native state of Y44-apoflavodoxin under conditions that favor folding. This subtle residue substitution will have much less effect on the stability of the molten globule intermediate of Y44-apoflavodoxin than it has on the stability of native Y44-apoflavodoxin, as this intermediate with exposed hydrophobic groups is highly dynamic.[13,33,39] Unfolded apoflavodoxin is expected to remain largely unaltered upon substituting F44 by Y44, as unfolded molecules are more dynamic than molten globule protein molecules.[37]

FMN Is Tightly Bound to Y44-Flavodoxin. The dissociation constant of the Y44-apoflavodoxin−FMN complex is determined by titrating a solution containing Y44-apoflavodoxin to a solution containing FMN (see Supporting Information). Upon cofactor binding to apoflavodoxin, FMN fluorescence quenches. The corresponding FMN binding curve is shown in Figure S2 in Supporting Information, and the fitted dissociation constant K_D of the Y44-apoflavodoxin−FMN complex is $(4.6 \pm 0.1) \times 10^{-10}$ M. This value differs only slightly from the one that characterizes the WT-apoflavodoxin−FMN complex [i.e., K_D is $(3.4 \pm 0.6) \times 10^{-10}$ M[30]]. Consequently, substitution of F44 by Y44 hardly affects the capacity of apoflavodoxin to bind FMN tightly.

Substitution of F44 by Y44 Does Not Alter the Conformation of (Apo)flavodoxin. Tight FMN binding occurs primarily through a very specific combination and geometry of hydrogen

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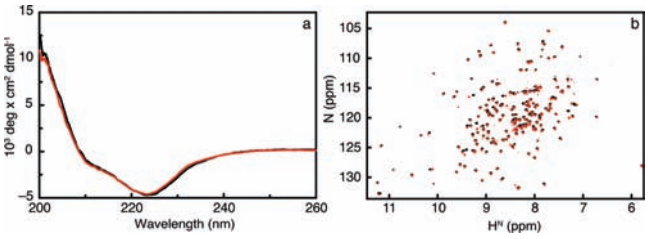

Figure 2. Y44- and WT-flavodoxin have nearly indistinguishable three-dimensional structures. (a) Far-UV CD spectra of WT-flavodoxin (black line) and of Y44-flavodoxin (red line) demonstrate that the secondary structure content of both proteins is identical. (b) $^{1}H-^{15}N$ HSQC spectra of ^{15}N-labeled WT-flavodoxin (black cross peaks) and Y44-flavodoxin (red cross peaks) show that the microenvironments of the backbone amides of sequentially identical residues of both proteins are almost indistinguishable. Sample conditions are 100 mM KPP_i, pH 6.0, at 25 °C.

bonds and aromatic interactions with apoflavodoxin.[45] Consequently, the observation that Y44-apoflavodoxin binds FMN almost as firmly as WT-apoflavodoxin implies that the three-dimensional structures of Y44- and WT-flavodoxin must be similar. Indeed, secondary structure content of both proteins is identical, as revealed by far-UV CD spectroscopy (Figure 2a). In addition, the $^{1}H-^{15}N$ HSQC spectra of both holoproteins are alike (Figure 2b). Only a few, relatively small chemical shift changes due to changing the chemical identity of the side chain of residue 44 are observed. As HSQC spectra are fingerprints of protein conformations, the similarity in cross-peak positions of the backbone amides of Y44- and WT-flavodoxin implies that the corresponding three-dimensional structures are nearly indistinguishable.

Just as observed for flavodoxin, it is expected that introduction of only a single extra oxygen atom into the protein, achieved by substituting F44 by Y44, does not affect the three-dimensional structure of native apoflavodoxin. This structure contains three tryptophans that have specific relative positions and orientations in the hydrophobic core of the molecule.[44] Indeed, in 100 mM KPP_i, pH 6.0, at 25 °C, both protein variants give rise to almost identical fluorescence emission spectra (wavelengths of emission maxima are identical, i.e., 330 nm, and corresponding fluorescence emission intensities differ only slightly). Most importantly, under this experimental circumstance, native WT-apoflavodoxin has a strikingly low fluorescence anisotropy of 0.035 ± 0.003[13] and anisotropy of Y44-apoflavodoxin is comparably low (i.e., anisotropy is 0.038 ± 0.002). The low anisotropy of native WT-apoflavodoxin is due to rapid unidirectional FRET from W167 to W128, as these tryptophans are only 7 Å apart.[34] Consequently, the conformations of native WT- and native Y44-apoflavodoxin must be nearly identical.

Although the substitution of F44 by Y44 does not alter the conformation of native apoflavodoxin, it is expected that this substitution decreases the stability of native protein against unfolding, as is shown below.

Native Y44-Apoflavodoxin Has a Relatively Low Stability against Unfolding and Unfolds According to a Three-State Model. Far-UV CD and fluorescence spectroscopy are used to follow GuHCl-induced equilibrium unfolding of Y44-apoflavodoxin (Figure 3). In contrast to the apparent two-state unfolding behavior of WT-apoflavodoxin detected by fluorescence emission at 340 nm (Figure S1a in Supporting Information),[13] the corresponding unfolding curve of Y44-apoflavodoxin is biphasic (Figure 3a). Both far-UV CD and fluorescence anisotropy unfolding curves of Y44-apoflavodoxin resemble the corresponding unfolding curves of WT-apoflavodoxin. Population of a folding intermediate during Y44-apoflavodoxin folding is further substantiated by the observation that the unfolding curves detected by fluorescence emission and CD do not coincide (Figure 3). In addition, the unfolding curve obtained by fluorescence anisotropy is biphasic.

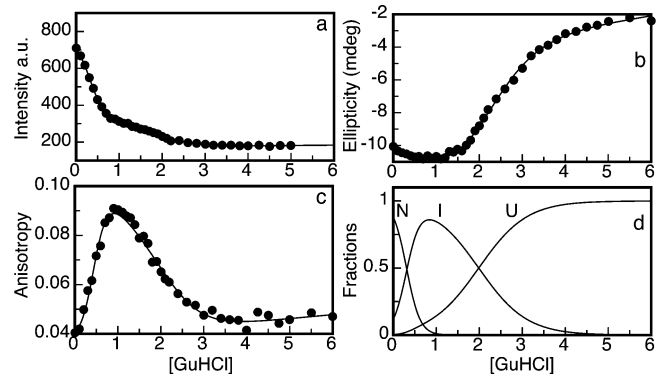

Figure 3. GuHCl-induced equilibrium (un)folding of Y44-apoflavodoxin shows that an intermediate folding state can be almost fully populated. (a) Fluorescence emission at 340 nm with excitation at 280 nm. (b) CD at 222 nm. (c) Fluorescence anisotropy data detected at 345 nm with a slit of 14 nm; excitation is at 300 nm. The solid lines in panels a−c are the result of a global fit of a three-state model for equilibrium (un)folding (see Supporting Information). (d) Normalized populations of native (N), folding intermediate (I), and unfolded (U) apoflavodoxin molecules as a function of denaturant concentration. Sample conditions are 100 mM KPP_i at pH 6.0.

Table 1. Thermodynamic Parameters Obtained from a Three-State Global Fit[a] of Unfolding Data of Y44-Apoflavodoxin[b] and WT-Apoflavodoxin[13] in 100 mM KPP_i

	ΔG (kcal/mol)[c]		m (kcal mol^{-1} M^{-1})[c]	
XY	Y44-apoflavodoxin	WT-apoflavodoxin	Y44-apoflavodoxin	WT-apoflavodoxin
UI	2.11 ± 0.24	3.74 ± 0.49	-1.06 ± 0.07	-1.83 ± 0.19
IN	1.18 ± 0.04	6.70 ± 0.17	-3.73 ± 0.13	-4.40 ± 0.11
UN	3.29 ± 0.24	10.45 ± 0.52		

[a] See Supporting Information. [b] See Figure 3. [c] ΔG_{XY} is the difference in free energy between species X and Y at 0 M denaturant, and m_{XY} is the dependence of ΔG_{XY} on denaturant concentration. Errors shown are standard errors.

The global fit of a three-state folding model (Supporting Information) to the three unfolding data sets presented in Figure 3a−c is good. Whereas the stability of native WT-apoflavodoxin against unfolding is 10.45 kcal/mol, native Y44-apoflavodoxin is severely destabilized compared to WT-apoflavodoxin and has a stability of only 3.29 kcal/mol (Table 1). As predicted, substituting F44 by Y44 affects the stability of the molten globule intermediate only marginally. Unfolding of the Y44-intermediate requires 2.11 kcal/mol, and 3.74 kcal/mol is needed in case of the WT-folding intermediate. Consequently, maximal population of the intermediate is increased from a mole fraction of 0.63 at 1.76 M GuHCl for WT-apoflavodoxin (Figure S1d in Supporting Information) to a mole fraction of 0.86 at 0.84 M denaturant for Y44-apoflavodoxin (Figure 3d). In addition, apoflavodoxin's folding intermediate is now detectable in a wider denaturant range. Actually, the unfolding data of Y44-apoflavodoxin show that at 25 °C, in the absence of GuHCl, already a mole fraction of 0.12 of protein molecules is molten globule.

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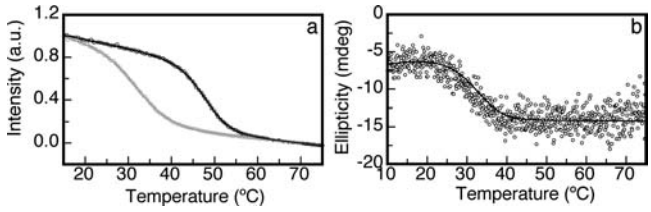

Figure 4. Thermal unfolding of apoflavodoxin shows that substitution of F44 by Y44 destabilizes the protein against thermal unfolding. (a) Thermal unfolding of WT-apoflavodoxin (black) and Y44-apoflavodoxin (gray), as measured by the change in fluorescence emission at 340 nm. (b) Thermal unfolding of Y44-apoflavodoxin as measured by CD at 210 nm. This wavelength is chosen because a much larger change in ellipticity is observed than is seen at 222 nm. Sample conditions are 100 mM KPP_i, pH 6.0, and the heating rate is 1 °C/min.

Thermally Induced Unfolding Experiments Confirm That Substitution of F44 by Y44 Destabilizes Native Apoflavodoxin. Via fluorescence emission (Figure 4a), thermal unfolding of only native protein molecules is monitored, as both folding intermediate and unfolded apoflavodoxin have comparable fluorescence signals. Midpoints of unfolding are 48.2 ± 0.1 °C for WT-apoflavodoxin and 32.9 ± 0.3 °C for Y44-apoflavodoxin. By use of CD data recorded at 210 nm (Figure 4b), it is observed that the thermal midpoint of Y44-apoflavodoxin unfolding is 31.0 ± 1.1 °C, which is close to the one detected by fluorescence emission (Figure 4a). The results confirm that at 25 °C, in the absence of denaturant, a fraction of Y44-apoflavodoxin molecules is present as molten globule.

When KPP_i Concentration Is Decreased from 100 to 10 mM, Y44-Apoflavodoxin Unfolds and Forms the Off-Pathway Molten Globule Intermediate. Although the substitution of F44 by Y44 substantially decreases the stability of native protein against unfolding at 25 °C, GuHCl is still required to obtain a maximal population of the molten globule state in 100 mM KPP_i, pH 6.0 (Figure 3). Denaturant, however, perturbs CD signals at wavelengths below about 210 nm, impeding characterization of the secondary structure content of the folding intermediate of apoflavodoxin.

Decreasing salt concentration is a well-known manner to destabilize folded proteins.[46] Hence, we reduced buffer concentration in the expectation that the equilibrium between native Y44-apoflavodoxin and molten globule would shift toward the latter species. This potential shift in folding equilibrium allows conformational characterization of apoflavodoxin's folding intermediate.

Fluorescence emission of Y44-apoflavodoxin changes drastically when KPP_i concentration is decreased from 100 to 10 mM (Figure 5a). Light scattering experiments show that the decrease in intensity is not caused by protein aggregation (data not shown). The fluorescence emission spectrum of Y44-apoflavodoxin in 10 mM KPP_i strongly resembles the corresponding spectrum of this protein in 100 mM KPP_i containing 0.9 M GuHCl. In the latter condition, Y44-apoflavodoxin is predominantly present as molten globule with a mole fraction of 0.86, whereas native protein is present with a mole fraction of only 0.02 (Figure 3). As fluorescence emission reports about the microenvironments of tryptophan residues, the spectra obtained suggest that in 10 mM KPP_i Y44-apoflavodoxin has similar molten globule-like properties as the off-pathway folding intermediate.

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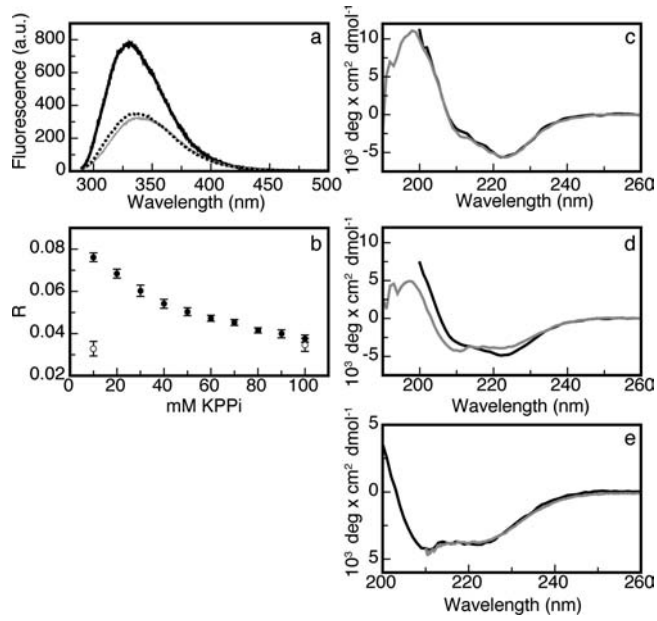

Figure 5. When KPP_i concentration is decreased, Y44-apoflavodoxin forms a molten globule. (a) Tryptophan fluorescence spectra of 4 μM Y44-apoflavodoxin in 100 mM KPP_i (black line), in 10 mM KPP_i (dotted line), or in 100 mM KPP_i with 0.9 M GuHCl (gray line; at 0.9 M GuHCl, mole fractions of native, folding intermediate, and unfolded molecules are 0.02, 0.86, and 0.12, respectively). (b) Fluorescence anisotropy of (●) Y44-apoflavodoxin and (○) WT-apoflavodoxin as a function of KPP_i concentration. (c, d) Far-UV CD spectra of (c) native WT-apoflavodoxin and (d) Y44-apoflavodoxin; protein is in 100 mM KPP_i (black lines) or 10 mM KPP_i (gray lines). (e) Far-UV CD spectra of Y44-apoflavodoxin in 10 mM KPP_i (black line) or in 100 mM KPP_i with 0.9 M GuHCl (gray line). pH of all samples is between 6.0 and 6.5, and temperature is 25 °C.

When KPP_i concentration is decreased from 100 to 10 mM, fluorescence anisotropy of WT-apoflavodoxin does not alter within error and remains at the low value typical for natively folded WT-apoflavodoxin molecules (Figure 5b). Consequently, in 10 mM KPP_i WT-apoflavodoxin is still native. In contrast, in the case of Y44-apoflavodoxin, the same decrease in buffer concentration leads to an increase in fluorescence anisotropy from a value of 0.04 to 0.08. Note that upon equilibrium unfolding of native Y44-apoflavodoxin to folding intermediate at 0.85 M GuHCl, anisotropy increases from 0.04 to 0.09 (Figure 3c). These observations confirm that, in 10 mM KPP_i, Y44-apoflavodoxin is not native but instead is present as molten globule intermediate.

The far-UV CD spectrum of Y44-apoflavodoxin alters considerably when KPP_i concentration is decreased from 100 to 10 mM (Figure 5d). In contrast, in the case of WT-apoflavodoxin, no such change is observed (Figure 5c), as the protein remains in its native state. Consequently, a drastic change in secondary structure content of Y44-apoflavodoxin occurs when buffer concentration is lowered. The far-UV CD spectrum of Y44-apoflavodoxin in 10 mM KPP_i strongly resembles the corresponding spectrum of Y44-apoflavodoxin in 100 mM KPP_i with 0.9 M GuHCl (Figure 5e), the condition at which a mole fraction of 0.86 of Y44-apoflavodoxin molecules is molten globule off-pathway folding intermediate.

Taking all observations together, we conclude that when KPP_i concentration is lowered from 100 to 10 mM, Y44-apoflavodoxin unfolds and forms the off-pathway folding intermediate.

Topology of the Molten Globule Intermediate Differs Drastically from That of Native Apoflavodoxin: It Is Helical. As the molten globule intermediate can be trapped in the absence of

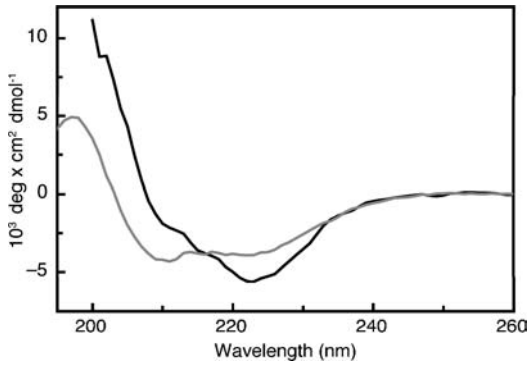

Figure 6. Conformation of Y44-apoflavodoxin in low salt concentration differs drastically from the conformation of WT-apoflavodoxin in its native state. In low salt concentration, native Y44-protein is destabilized and forms a helical molten globule intermediate. Shown are far-UV CD spectra of native WT-apoflavodoxin in 100 mM KPP_i (black line) and of Y44-apoflavodoxin in 10 mM KPP_i (gray line), both at 25 °C. Protein concentration is ~2 μM.

denaturant, its conformational characterization is now possible. Native apoflavodoxin contains a parallel β-sheet that is surrounded by five α-helices. It gives rise to a far-UV CD spectrum typical for flavodoxins; that is, the spectrum has a minimum at 222 nm and a shoulder at 210 nm (Figure 6). In contrast, the far-UV CD spectrum of the Y44-folding intermediate under nativelike conditions is typical for α-helical proteins. The spectrum has minima at 222 and 210 nm and is similar in shape to the far-UV CD spectrum of the apomyoglobin molten globule at pH 4.3, a species that is helical.[47] Consequently, the topology of apoflavodoxin's molten globule drastically differs from the $\alpha-\beta$ parallel topology of native apoflavodoxin.

A Single Polypeptide Sequence Can Code for Monomeric Protein Folds That Are Largely Different. In contrast to most folding intermediates studied to date, the molten globule folding species of apoflavodoxin has a remarkably nonnative character. What causes the off-pathway folding intermediate of apoflavodoxin to be helical?

Upon protein folding, helices are formed much more rapidly than sheets, especially when parallel β-sheets are involved. This rapid helix formation is due to the highly local character of the interactions in helices. In contrast, the residues that form the strands of a parallel β-sheet are separated by many intervening residues,[48,49] as is the case for apoflavodoxin. Unfolded apoflavodoxin contains four regions with reduced flexibility, of which two transiently form native α-helical structures and one forms nonnative α-helical structure.[32] These helices are sufficiently stable to be present about 10% of the time and comprise residues A41−I52, E104−K118, and T160−A169. Rapid formation of α-helices and their subsequent nonnative docking through hydrophobic interactions causes formation of the off-pathway intermediate during apoflavodoxin folding.[32] This docking of helices prevents formation of the parallel β-sheet of apoflavodoxin, and as a result it seems likely that the intermediate is helical. In the study reported here, conclusive data are presented that show that the molten globule intermediate of apoflavodoxin indeed is of helical nature. Most likely, the regions that form helices in unfolded apoflavodoxin are helical in the off-pathway intermediate as well.

Further support for the absence of a β-sheet in the off-pathway folding intermediate of apoflavodoxin is provided by the observed differences in cooperativity of folding of native apoflavodoxin and molten globule. Native apoflavodoxin is formed highly cooperatively,[39] which is inherent to the formation of a parallel β-sheet involving many residues. In contrast, the formation of the off-pathway molten globule of apoflavodoxin is clearly noncooperative.[38] Consequently, this species cannot contain a β-sheet, as is indeed confirmed by the data presented in this study.

Anfinsen's thermodynamic hypothesis[41] states that each amino acid sequence codes for a single tertiary fold with varying amounts of local flexibility. However, monomeric proteins like, for example, prions can assume alternative conformations in a multimeric form.[28,29] The study presented here shows that a single polypeptide sequence can code for monomeric protein folds that are largely different under nativelike conditions. The amino acid sequence of apoflavodoxin codes for the $\alpha-\beta$ parallel topology of the native state as well as for a helical protein species. Upon a mild change of conditions, that is, lowering buffer concentration in the case of Y44-apoflavodoxin, topological switching between both folds occurs and a monomeric protein species with a distinct fold becomes energetically most favorable. Our observations, together with those reported on lymphotactin,[50] show that interconversion between unrelated, monomeric protein structures is likely a common phenomenon in the protein structure universe.

Acknowledgment. The Netherlands Organization for Scientific Research supported this work.

Supporting Information Available: Figures showing GuHCl-induced equilibrium (un)folding data of WT-apoflavodoxin and quenching of FMN fluorescence upon FMN binding to Y44-apoflavodoxin; and description of procedures used to analyze (a) denaturant-induced equilibrium unfolding data, (b) thermal-induced unfolding data, and (c) dissociation constant of the Y44-apoflavodoxin−FMN complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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