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The Long and Short Flavodoxins

I. THE ROLE OF THE DIFFERENTIATING LOOP IN APOFLAVODOXIN STRUCTURE AND FMN BINDING*

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Flavodoxins are well known one-domain α/β electron-transfer proteins that, according to the presence or absence of a ~20-residue loop splitting the fifth β-strand of the central β-sheet, have been classified in two groups: long and short-chain flavodoxins, respectively. Although the flavodoxins have been extensively used as models to study electron transfer, ligand binding, protein stability and folding issues, the role of the loop has not been investigated. We have constructed two shortened versions of the long-chain *Anabaena* flavodoxin in which the split β-strand has been spliced to remove the original loop. The two variants have been carefully analyzed using various spectroscopic and hydrodynamic criteria, and one of them is clearly well folded, indicating that the long loop is a peripheral element of the structure of long flavodoxins. However, the removal of the loop (which is not in contact with the cofactor in the native structure) markedly decreases the affinity of the apoflavodoxin-FMN complex. This seems related to the fact that, in long flavodoxins, the adjacent tyrosine-bearing FMN binding loop (which is longer and thus more flexible than in short flavodoxins) is stabilized in its competent conformation by interactions with the excised loop. The modest role played by the long loop of long flavodoxins in the structure of these proteins (and in its conformational stability, see López-Llano, J., Maldonado, S., Jain, S., Lostao, A., Godoy-Ruiz, R., Sanchez-Ruiz, Cortijo, M., Fernández-Recio, J., and Sancho, J. (2004) *J. Biol. Chem.* 279, 47184–47191) opens the possibility that its conservation in so many species is related to a functional role yet to be discovered. In this respect, we discuss the possibility that the long loop is involved in the recognition of some flavodoxin partners. In addition, we report on a structural feature of flavodoxins that could indicate that the short flavodoxins derive from the long ones.

The flavodoxins are electron transfer proteins involved in both photosynthetic and non-photosynthetic reactions, which carry a molecule of non-covalently bound FMN as their only redox center (1, 2). Soon after their discovery, it was realized that they could be isolated in two sizes and were accordingly divided in two classes: 1) the short-chain flavodoxins (*i.e. Clostridium beijerincki* and *Desulfovibrio vulgaris* flavodoxins) and 2) the long-chain ones (*i.e. Synechococcus* sp. (strain PCC 7942) and *Anabaena* sp. (strain PCC 7119) flavodoxins). Once the x-ray structures of representatives of the two groups became available (3–6), the structural difference was seen to be due to the presence in long flavodoxins of an extra loop that splits the fifth strand of the central β-sheet (Fig. 1). Because many of the functional and thermodynamic properties of short and long flavodoxins are similar (redox potentials, affinity for the FMN redox cofactor, and so forth), it is not clear yet what role the extra loop of the long flavodoxins may play. In our laboratory, we have used the holoform (6) and apoform (7) of the flavodoxin from *Anabaena* as models to investigate protein folding (8), protein stability (9–16), and protein/ligand interaction (17–20) and a wealth of thermodynamic and kinetic information is now available on this protein. In this work, we investigate the influence of the long loop of *Anabaena* flavodoxin on the structure and cofactor binding of the apoprotein by deriving and studying two shortened variants where the long loop has been removed by site-directed mutagenesis. Although one of the shortened variants displays a somewhat altered structure compatible with some local unfolding, the second one is clearly well folded, indicating that the loop is not required for the correct folding of the long flavodoxins. Surprisingly, the affinity of the shortened protein for its cofactor is severely reduced despite the fact that the loop makes no contacts with the FMN in the structure of the holoflavodoxin. From a comparison of the structures of short and long flavodoxins, we propose that this could be related to a role played by the long loop in stabilizing the native conformation of the adjacent tyrosine-bearing loop involved in FMN binding. Additionally, a structural analysis of the conformation of the fifth β-strand in short and long flavodoxins points to the possibility that short flavodoxins derive from the long ones.

MATERIALS AND METHODS

Site-directed Mutagenesis, Protein Expression, Purification, and Quantitation—Oligonucleotide-directed mutagenesis of the flavodoxin gene cloned in the plasmid pTrc99a (21) was performed by a modification of the method of Deng and Nickoloff (22). The mutagenic oligonucleotide used to delete residues 119–139 to produce the shorter mutant flavodoxin, Δ(119–139), with Gly^{118} adjacent to Gly^{140} was 5′-GATTATCTTCATCAAGAGCTAGTCCGCCGACAGTTTTACCACCAC-3′. To delete residues 120–139 and produce Δ(120–139) with Tyr^{119} adjacent to Gly^{140}, the oligonucleotide used was 5′-GATTATCTTCATCAAGAGCTAGTCCATAGCCGACAGTTTTACCAC-3′.

Mutant plasmids were identified by direct sequencing. The expression and purification of flavodoxin mutants were done essentially as described for the wild type protein (23). The purity of each flavodoxin

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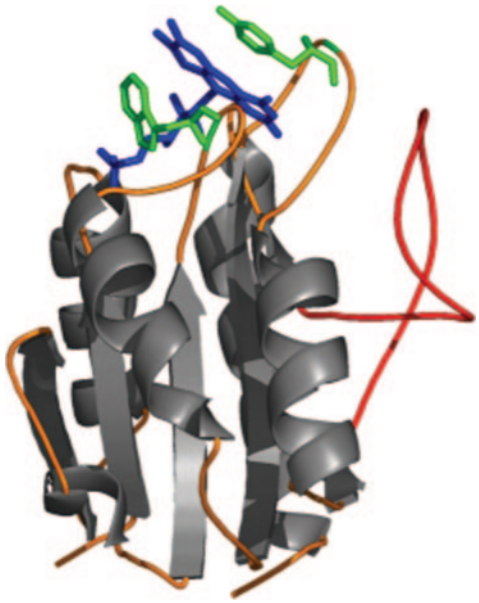

FIG. 1. ***Anabaena* holoflavodoxin ribbon diagram showing the bound FMN cofactor (*blue*), the loops bearing the Trp^{57} and Tyr^{94} binding residues (loops in *orange*, residues in *green*) and the long loop characteristic of long flavodoxins (in *red*).**

preparation was confirmed by SDS-polyacrylamide gel electrophoresis. The mutants were purified as apoproteins, as they lost the FMN prosthetic group along the purification. The concentration of the mutants was determined from the absorbance at 280 nm (24) using an extinction coefficient of 25,590 M^{-1} cm^{-1} for Δ(119–139) and of 27,000 M^{-1} cm^{-1} for Δ(120–139).

Absorbance, Fluorescence, Circular Dichroism, and ^1H NMR Spectra—Absorbance spectra were recorded at 298.2 ± 0.1 K in a Kontron Uvikon 860 spectrophotometer. Corrected steady-state fluorescence emission at 298.2 ± 0.1 K in the 300–450-nm range was obtained on a SLM 8000D spectrofluorometer with excitation at 295 nm. Far-UV and near-UV circular dichroism spectra at 298.2 ± 0.1 K were recorded in a Jasco 710 spectropolarimeter using a 0.1- and 1-cm cuvette, respectively. ^1H NMR spectra were acquired on a Bruker AMX-600 pulse spectrometer operating at a proton frequency of 600.13 MHz. One-dimensional spectra were recorded at 298.2 ± 0.1 K using 32-K data points zero-filled to 64 K before performing the Fourier transformation. Water suppression was achieved by selective presaturation. The temperature of the NMR probe was calibrated with methanol. Samples for NMR experiments were prepared at 1–2 mM protein concentration in 5 mM phosphate buffer, pH 7.0, containing 10% D_2O. Sodium [3-trimethylsilyl 2,2,3,3-$^2H_4$] propionate (TSP) was the internal reference.

Determination of the Apparent Molecular Weight of Δ(119–139) and Δ(120–139)—The apparent molecular weights of Δ(119–139) and Δ(120–139) apoflavodoxins were determined by automated molecular exclusion chromatography in fast protein liquid chromatography (Amersham Biosciences) with a Superose 12HR 10/30 column equilibrated in 50 mM sodium phosphate, pH 7, containing 100 mM NaCl. The flow rate was 0.5 ml/min.

FMN Binding to Δ(120–139) Apoflavodoxin—The interaction of the FMN cofactor with Δ(120–139) apoflavodoxin was studied fluorimetrically (the binding of FMN to apoflavodoxin strongly quenches its fluorescence emission) and by molecular exclusion chromatography. The FMN used was 98% pure according to reverse-phase high pressure liquid chromatography. The fluorescence experiments were carried out in a Kontron SMF 25 fluorimeter (excitation at 445 nm, emission from 500 to 600 nm) at 298.2 ± 0.1 K in the darkness. The cofactor (0.9 ml of 0.2 μM FMN in 5 mM sodium phosphate, pH 7) was mixed with 0.1 ml of 530 μM Fld Δ(120–139) in the same buffer, and the mixture was allowed to reach equilibrium for 5 min before an emission spectrum was recorded. In the molecular exclusion experiments, an fast protein liquid chromatography apparatus (Amersham Biosciences) was used, equipped with a Superose 12HR 10/30 column equilibrated in 5 mM sodium phosphate, pH 7, containing 150 mM NaCl. The flow rate was 0.5 ml/min, and the absorbance at 464 nm was followed. A mixture of 344 μM Δ(120–139) and 820 μM FMN preincubated in the same buffer for 90 min was loaded in the column.

Time-resolved Fluorescence and Fluorescence Anisotropy Measurements—Time-resolved fluorescence intensity and depolarization measurements at 298.2 ± 0.1 K were performed in 50 mM sodium phosphate buffer, pH 7.0, using the time-correlated single photon-counting technique. The experimental setup and analysis was similar to the one described previously (25). Total fluorescence decays at different emission wavelengths were globally analyzed, assuming a four-exponential model. Anisotropy decays were described by a biexponential function. Details of the analysis will be described elsewhere. Time-resolved fluorescence anisotropy provides a direct measurement of the hydrodynamic volume of proteins in solution, because the global rotational correlation times determined, ϕ_G, are related to hydrated molecular volumes, V_H, and solution viscosities, η, by the Stokes-Einstein relationship: $\phi_G = \eta_H^V/kT$.

RESULTS

Spectroscopically Based Structural Characterization of Δ(119–139) and Δ(120–139) Apoflavodoxins—Several spectroscopic and hydrodynamic properties of Δ(119–139) and Δ(120–139) apoflavodoxins have been compared with those of the entire protein. Because both the fluorescence and near-UV CD spectra of wild type apoflavodoxin seem dominated by contributions from Trp^{120} (13) and this residue is no longer present in the shortened flavodoxins, we will compare the spectroscopic properties of the shortened apoflavodoxins with those of the W120F apoflavodoxin mutant (termed pseudo wild type, pWT),[1] which is a more appropriate reference than wild type.

We have first compared the fluorescence emission spectra of the shortened variants with that of pWT apoflavodoxin (Fig. 2*A*). The deletion mutants display a more quenched tryptophan emission, especially the Δ(120–139) variant, and slight differences in the emission spectra at $\lambda < 340$ nm relative to pWT. The relative amplitudes, determined at different emission wavelengths, and the fluorescence lifetimes were used to resolve the emission spectra associated with individual decay constants. The decay-associated spectra (26) displayed in all of the cases the same pattern (data not shown). The maximum of the emission spectra associated with the longest lifetime (5.1 ns for pWT and 4.7 ns for both deletion mutants) was ~345 nm. This lifetime (that has been associated to Trp^{57})[2] is the one that contributes most to the slight differences observed for the three protein variants.

The exposure of aromatic residues to solvent in the shortened and in the full-length pWT apoflavodoxin has been further investigated by recording absorbance difference spectra (native minus denatured) in the near-UV. The shape of the difference spectra (Fig. 2*B*) is the same in the three proteins, indicating a substantial aromatic residue burial in all of them. The two truncated apoflavodoxins show quite similar difference spectra, although the intensity of the shorter Δ(119–139) is a bit lower, which is suggestive of a greater exposure of its aromatic residues in the folded conformation.

The far- and near-UV CD spectra of the short flavodoxins are compared with those of pWT in Figs. 2, *C* and *D*, respectively. The far-UV spectra indicate that the two shortened apoflavodoxins contain substantial helical structure with helical contents not very different from that of pWT. The greater (in absolute value) mean residue ellipticity of Δ(120–139), relative to pWT, is simply due to its reduced number of loop residues. In fact, its molecular ellipticity is almost the same (98% pWT). As for Δ(119–139), its helical content is 77% that of pWT and its deeper and blue-shifted minimum of around 207 nm confirms that it is somewhat less structured. This is also suggested by the near-UV CD data (Fig. 2*D*), whereas both pWT and Δ(120–139) display distinct spectra in this region, indicating that they

[1] The abbreviations used is: pWT, pseudo wild type; FNR, ferredoxin oxidoreductase.

[2] M. P. Lillo and J. Sancho, manuscript in preparation.

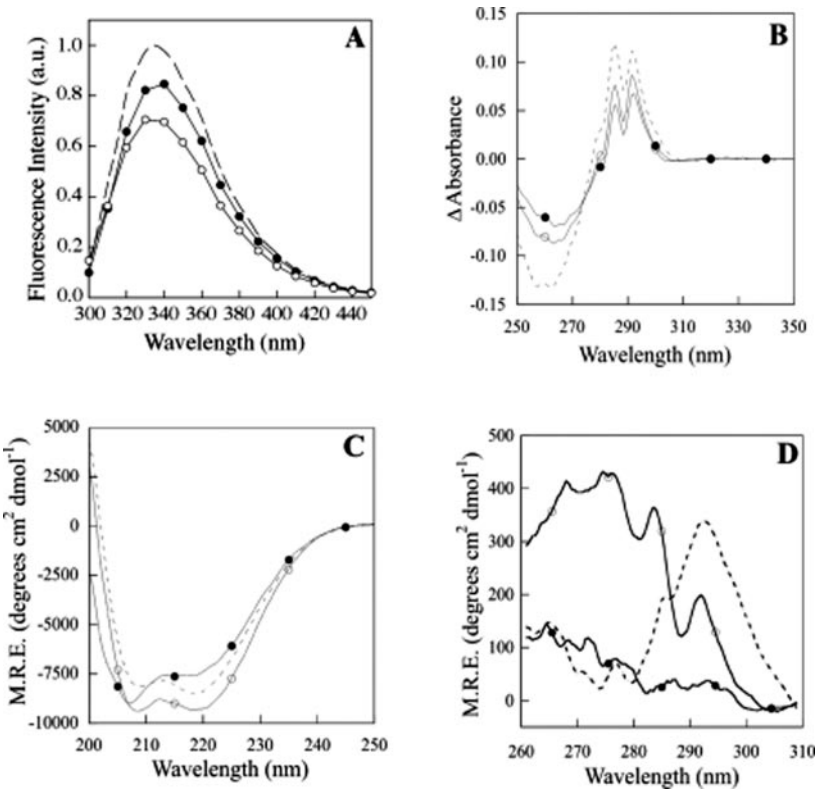

FIG. 2. **Spectroscopic characterization of shortened and full-length apoflavodoxins in 5 mM sodium phosphate, pH 7.** *A*, corrected emission spectra. *Solid circles*, Δ(119–139); *open circles*, Δ(120–139); *dashed line*, pWT. Protein concentration: 5 μM. *B*, near-UV absorbance difference spectra (native minus denatured in 4 M urea). *Solid circles*, Δ(119–139); *open circles*, Δ(120–139); *dashed line*: pWT. Protein concentration: 20 μM. *C*, far-UV spectra. *Solid circles*: Δ(119–139); *open circles*: Δ(120–139); *dashed line*: pWT. Protein concentration: 20 μM. *D*, near-UV spectra. *Solid circles*, Δ(119–139); *open circles*, Δ(120–139); *dashed line*, pWT. Protein concentration: 40 μM.

present some well defined tertiary interactions involving aromatic residues, Δ(119–139) shows a flat spectrum deprived from any peak. However, Because, in a protein with few tryptophan residues, this is not conclusive evidence of a lack of well folded tertiary structure, we have acquired ^{1}H NMR spectra of the two shortened apoflavodoxins (Fig. 3). The large dispersion of the NH amide protons (10.5–6.0 ppm) and the presence of signals at $\delta < 0.5$ ppm and in the 5.0–6.0-ppm spectral region clearly indicate that the two shortened variants are substantially well folded and point to a loss of local interactions in Δ(119–139) rather than to a more global denaturation.

Hydrodynamically Based Structural Characterization of Δ(119–139) and Δ(120–139) Apoflavodoxins—In addition to the spectroscopic characterization, molecular exclusion chromatography experiments have been performed. Both mutant flavodoxins are eluted from the gel filtration column as single peaks (data not shown) whose elution volumes are concentration-independent from 5 to 200 μM. This finding indicates that the truncated flavodoxins are monomeric. The elution volumes, slightly higher than that of pWT, indicate that the shortened proteins are similarly compact as the entire protein as much as gel filtration experiments can estimate. From a calibration of the column, we estimate the difference in molecular mass between the shortened apoflavodoxins and the pWT at 1.5 kDa, the theoretical difference being 2.5 kDa.

To obtain a finer picture of the apparent size of the variants in solution, we have determined their rotational correlation times, which are linearly dependent on their hydrodynamic volume. Because the apoflavodoxin variants contain three tryptophan residues, their fluorescence emission will be depolarized by internal local motions, characteristic of each tryptophan microenvironment, and by the overall global protein rotation. To understand the contribution of individual tryptophans to the anisotropy decay, we have performed a complete fluorescence intensity and anisotropy characterization of the wild type protein and of a set of different tryptophan apoflavodoxin mutants.[2] The size and shape of the apoflavodoxin variants then have been estimated from their global rotational correlation times. The ϕ_G measured for pWT (6.9 ns, data not shown) agrees well with that determined for wild type apoflavodoxin (7.1 ns) and with the theoretical predictions from the Stokes-Einstein relationship (assuming a hydrated volume of 3.2 nm^3 estimated from the x-ray structure of the wild type protein). The global rotational correlation time for Δ(120–139) is also the same within the error (6.7 ns). In contrast, Δ(119–139) displays a correlation time of 8.1 ns, ~20% higher than that of pWT, which confirms that the global structure of Δ(119–139) is less compact than that of Δ(120–139).

FMN Binding to Fld Δ(120–139) Flavodoxin—Both Δ(119–139) and Δ(120–139) are purified as apoproteins, the FMN prosthetic group being lost in the purification. We have tried to reconstitute the holoform of the natively folded Δ(120–139) apoflavodoxin by adding FMN to it. When the binding of the cofactor was followed fluorimetrically at a 300 Δ(120–139)/FMN molar ratio, a small change in the emission maximum of FMN that shifted from 525 to 523 was observed (data not shown). This indicates that FMN can still bind to Δ(120–139) apoflavodoxin but that the affinity is much lower than in the wild type full-length protein. To estimate a value for the affinity of the complex, we performed size-exclusion experiments where a mixture of FMN and Δ(120–139) incubated for 90 min was filtered through a Superose 12HR 10/30 column. Two absorbance peaks at 464 nm were detected (Fig. 4), one corresponding to the elution of free FMN and a second minor one corresponding to the elution volume of Δ(120–139). Precise calculation of equilibrium binding constants from size-exclusion chromatography experiments is admittedly difficult, because the complex that is formed during sample incubation may dissociate during the chromatography unless the k_{off} is very low. A lower limit for the K_b can nevertheless be estimated assuming that no significant dissociation has taken place in the column and that the concentrations of the different species calculated from the absorbance of their corresponding peaks represent those in the loaded sample. The fact that the elution

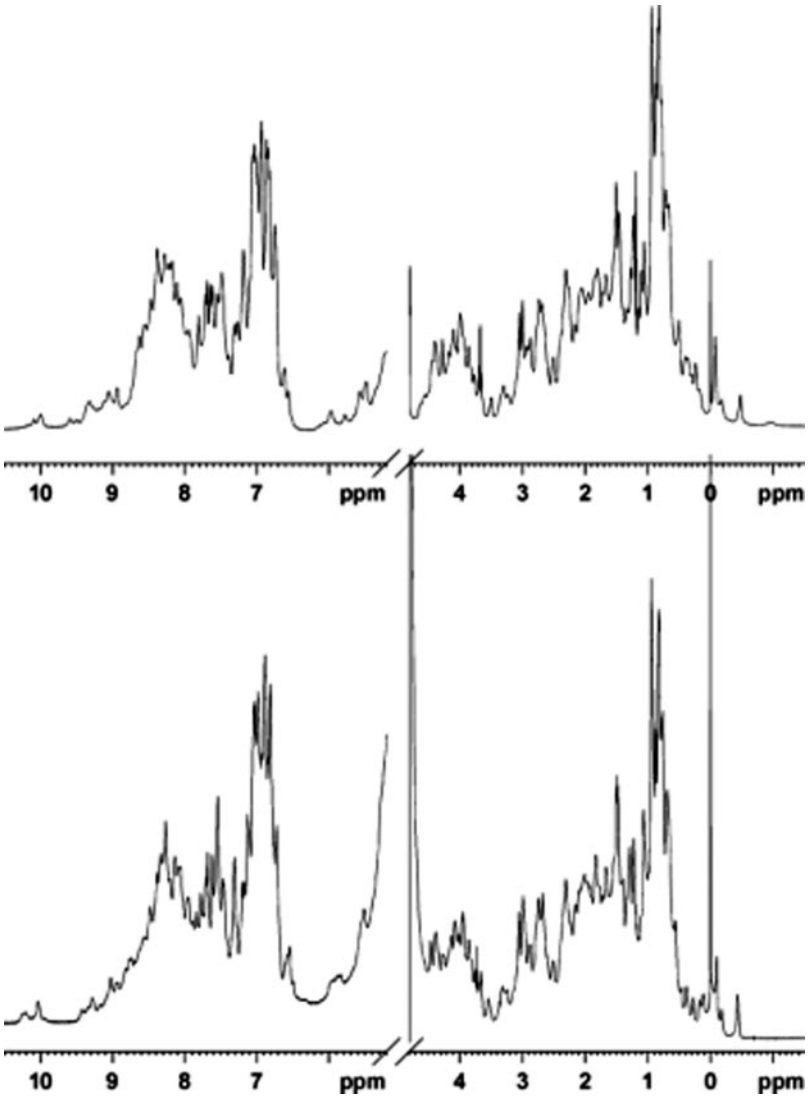

FIG. 3. **^{1}H NMR spectra of shortened apoflavodoxins.** The spectra were recorded in H_2O/D_2O (9:1), 5 mM sodium phosphate, pH 7.0, and 25 ± 0.1 °C. *Upper spectrum*: Δ119–139 (2.7 mM). *Lower spectrum*: Δ120–139 (1.3 mM).

volumes of the peaks observed in the chromatogram of the Δ(120–139)/FMN mixture are very similar to those of the separately loaded molecules suggests that the approximation is not too gross in this case. From the absorbance of the different peaks, we estimate that K_b is larger than 2849 M^{-1} ($K_d < 350$ μM), corresponding to an affinity of the complex greater than −4.7 kcal mol^{-1}. Similar numbers are obtained if the areas of the peaks rather than their actual maximal absorbances are used in the calculation (data not shown). The affinity of the wild type apoflavodoxin/FMN complex is certainly greater (18) (approximately −12.5 kcal mol^{-1} at the same ionic strength).

DISCUSSION

Insights into the Structure of Apoflavodoxin Lacking the Long Loop Characteristic of Long Flavodoxins—Among the long flavodoxins, the sequence of the long loop splitting the fifth β_5 strand is similarly conserved as it is the sequence of the rest of the protein (data not shown). This finding suggests that the loop may play a role from either the structural or the functional point of view. To assess the structural relevance of the loop, we have constructed two shortened apoflavodoxins where either the Gly^{118} or the Tyr^{119} wild type residue has been connected to the wild type Gly^{140} residue, thus removing the long loop from the *Anabaena* apoflavodoxin. The expression yields of the two variants were high, and unlike other shortened apoflavodoxin fragments previously produced in the laboratory (15), they can be purified in the conventional way used to purify wild type flavodoxin; although they are obtained in the apoform.

The low resolution hydrodynamic characterization performed by size-exclusion chromatography indicates that both shortened forms are monomeric and that their apparent molecular weights are consistent with those of compact conformations rather than markedly unfolded ones. Their compactness is also evidenced by the tryptophan fluorescence emission spectra and the near-UV absorbance difference spectra (Fig. 2, *A* and *B*) that show that the aromatic residues, specifically the tryptophans, are not fully exposed to solvent. In addition, both shortened apoflavodoxins contain a substantial secondary structure (Fig. 2*C*). However, small differences are observed that indicate that Δ(119–139) is somewhat less compact, its absorbance difference spectrum is less intense, its helical content is reduced, and its near-UV CD spectra are flat. Consistent with this spectroscopic evidence, our analysis of the global rotational correlation times[2] indicates that, although Δ(120–139) and pWT are similarly compact, Δ(119–139) is somewhat expanded. Nevertheless, we notice that, in the NMR spectra (Fig. 3), both shortened apoflavodoxins display low field resonances, peak dispersion, and line-widths similar to those of well folded proteins. This means that Δ(119–139) is mostly in a native-like conformation, probably not far from the one described for the wild type protein (7, 9), and therefore, the

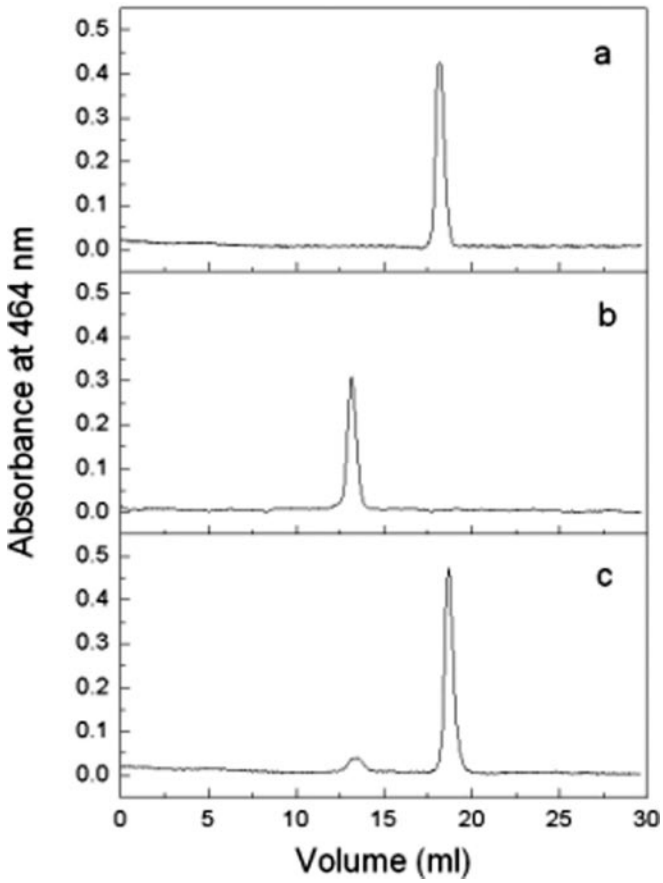

FIG. 4. **Estimation of the binding constant between Δ(120–139) and FMN by size-exclusion chromatography using a Superose 12HR 10/30 column equilibrated in 5 mM sodium phosphate, pH 7.** *a*, FMN elution. *b*, Δ(120–139) elution. *c*, elution of a mixture of 344 μM Δ(120–139) and 820 μM FMN preincubated for 90 min. The absorbance of the FMN at 464 nm (chromatograms *a* and *c*) or that of the apoprotein at 280 nm (chromatogram *b*) was recorded.

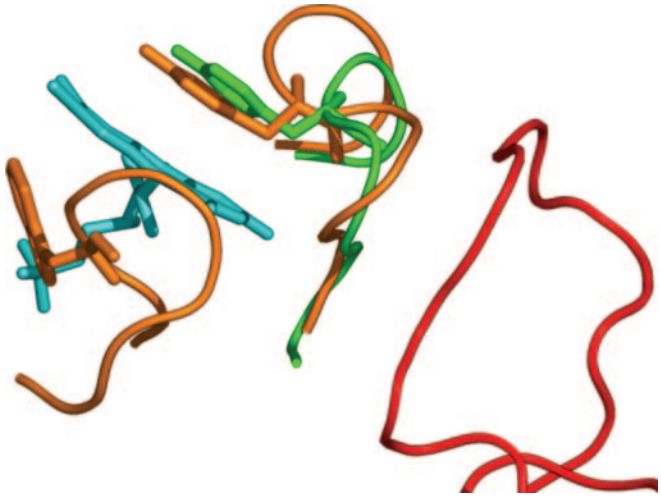

FIG. 5. **A close view to the FMN binding loops (in *orange*) in the long *Anabaena* flavodoxin (Protein Data Bank code 1FLV) showing the Trp^{57} and Tyr^{94} binding residues.** The loop splitting the fifth β-strand in long flavodoxins is shown in *red*. Superimposed is also shown in *green* the loop bearing the equivalent tyrosine residue of the short *D. vulgaris* flavodoxin (Protein Data Bank code 1J8Q). The tyrosine loop of the *Desulfovibrio* flavodoxin is shorter than the equivalent one in the *Anabaena* flavodoxin.

greater size and lack of near-UV CD signal observed in Fld Δ(119–139) may be related to the local unfolding of a small region of the protein, possibly involving an α-helix.

The way the two variants were designed may help to explain their structural differences. They were simply designed to provide alternative ways for the protein to accommodate the removal of the loop. In principle, the nitrogen of Gly^{140} is at very similar distances from the carbonyl carbons of Gly^{118} (5.16 Å) and of Tyr^{119} (5.00 Å). To make a canonical β-strand out of the two bits connected (strands β_{5a}, 115–117, and β_{5b}, 140–143) and make it pair with strand β_4 (residues 81–89), only one spacing residue (Gly^{118}) is required. In this respect, the shorter mutant Δ(119–139) contains all of the residues necessary to form a fifth strand of the appropriate length. The fact that it folds to a close to native but locally expanded conformation is the likely consequence of an insufficient stabilization of the hypothetical canonical fifth β-strand formed by splicing residues 118 and 140. This makes the shortened protein relax into a less compact conformation where, as judged from the reduced helical content, one α-helix is probably unwound. Helices 4 and 5 flanking the long loop are the likely candidates. In contrast, the native-like spectroscopic properties of the longer variant Δ(120–139) illustrate that even the connection is "too long" to form a canonical strand. The greater flexibility afforded by the extra residue allows the mutant protein to fold into a native conformation. In fact, the inspection of the available x-ray structures of short flavodoxins (from *Desulfovibrio vulgaris*, Protein Data Bank code 1J8Q, and from *Clostridium beijerinckii*, Protein Data Bank code 2FDX) shows that their fifth β-strand contains a bulge at precisely the same point where it appears split by the long loop in the long flavodoxins.

Importantly, the correct folding of the shortened Δ(120–139) apoflavodoxin clearly illustrates the non-relevance of the extra loop of long flavodoxins for adopting a well folded conformation. Thus, this long loop appears to play a peripheral role from the structural point of view.

The Long Loop, the Tyrosine-bearing Loop, and FMN Binding—We have investigated the interaction of FMN with shortened flavodoxin using the Δ(120–139) variant, which is natively folded. Somewhat surprisingly, the affinity of Δ(120–139) apoflavodoxin for the prosthetic group is drastically reduced, despite the fact that none of the residues of the loop make direct contact with FMN in holoflavodoxin. It should be noticed, however, that several long loop residues are in contact (Fig. 5) with the short loop that bears the highly conserved and functionally important tyrosine residue (Tyr^{94} in *Anabaena* flavodoxin) that lays on one face of the FMN in the vast majority of flavodoxins of the known structure (17) and stabilizes the functional complex (18). It is clear that even subtle changes around the tyrosine loop could produce the observed decrease in the affinity for FMN. The fact that the fluorescence of Trp^{57} located at the other loop involved in FMN binding (Figs. 1 and 5) is altered in the shortened apoflavodoxins may reveal a further propagation of conformational changes to this more distant FMN binding loop. Importantly, we notice that, in short flavodoxins, the tyrosine loop is shorter than in long flavodoxins (by one or two residues; see Fig. 5). This should reduce in short flavodoxins the conformational flexibility of the tyrosine loop to a point that no additional interactions with the missing long loop would be needed to fix the side chain of the redox active and FMN binding important tyrosine residue in its functional conformation. Thus, the long loop itself should not be regarded as an essential feature for the binding of the cofactor. Short flavodoxins that lack it manage to fold the tyrosine loop similarly to long flavodoxins (Fig. 5) and indeed bind FMN tightly. Nevertheless, the long loop seems to be used by long flavodoxins as a conformational helper to stabilize the FMN binding competent conformation of their longer tyrosine bearing loop, thus increasing the affinity of the functional complex.

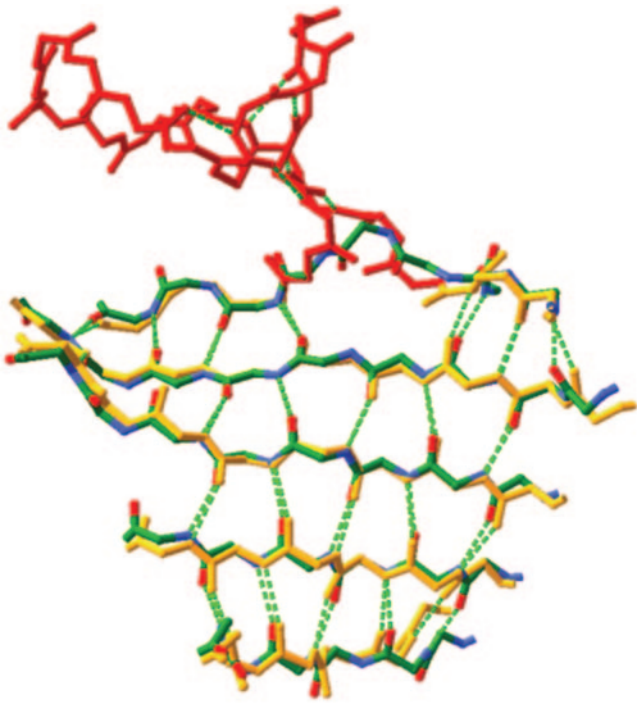

FIG. 6. **Superposition of the central β-sheets of the long flavodoxin from *Anabaena* PCC 7119 (Protein Data Bank code 1FTG) and of the short-chain flavodoxin from *D. vulgaris* (Protein Data Bank code 1J8Q) performed with DeepView.** The short flavodoxin is depicted in CPK colors, whereas the long flavodoxin is depicted in *orange* with the exception of the long loop depicted in *red*. A clear bulge is observed in the fifth β-strand of the short flavodoxin, precisely where it appears split by the long loop in the long flavodoxins. We propose that this is vestigial evidence of a loop removal event. The short flavodoxin from *C. beijerinckii* (Protein Data Bank code 2FDX) displays the same pattern as that from *Desulfovibrio* (data not shown).

Work is in progress in our laboratory to try to increase the FMN affinity of Δ(120–139) by replacing its tyrosine loop by equivalent loops of short flavodoxins.

A Structural Hint on a Possible Derivation of Short Flavodoxins from the Long Ones—So far, it is not clear which version of the flavodoxin gene (the long or the short one) is older. To investigate this issue, we have performed a phylogenetic analysis of the available short and long flavodoxin sequences (data not shown). The analysis indicates that horizontal transfer of the flavodoxin gene has been frequent, which complicates the interpretation. However, from a completely different perspective, we have noticed a structural feature of the short-chain flavodoxins that might be consistent with their derivation from the long ones. As has been mentioned, the fifth β-strand of short flavodoxins contains a bulge at precisely the point where the loop of the long flavodoxins departs from the β-sheet. We show in Fig. 6 a superimposition of the β-sheets of the long *Anabaena* flavodoxin (Protein Data Bank code 1FTG) and of the short flavodoxin from *Desulfovibrio* (Protein Data Bank code 1J8Q). The β-sheets are very similar and can be superimposed very well. Significantly, in the short flavodoxin, the hydrogen-bonding pattern between strands 4 and 5 is clearly distorted because the fifth strand contains a bulge. The short flavodoxin from *Clostridium* contains the same bulge at the same position (data not shown). We notice that the bulge is located exactly where the long flavodoxins display the long loop that split their fifth strand. In principle, it is possible that an ancient short flavodoxins would have contained a central β-sheet that was regular in all of them but in the presence of a bulge and that the long loop was later inserted precisely at the bulge. Nevertheless, we think more probably that the bulge simply represents a vestigial remain of the lost loop of the more ancient long flavodoxins.

Is There Room for a Specific Role of the Flavodoxin Long Loop?—The presence of this fairly long loop in so many flavodoxins and the fact that its sequence is similarly conserved as that of the rest of the protein (data not shown) together with the small influence of the loop in the overall structure and stability of the protein (see also Ref. 37 for details) suggests that the loop could play an unknown functional role rather than a structural one. Looking for hints, we have performed a BLAST search for short and nearly exact matches of the loop sequence in other proteins, but no close homologues of the loop are evident (data not shown). On the other hand, given the location of the loop in the structure, not very far from the redox active site of the protein, where the FMN group is tightly bound (Fig. 1), we speculate that one possible role of the loop could be that of providing binding residues for flavodoxin partner proteins. Several of these have been identified in the different metabolic pathways where the flavodoxins are involved. In *Escherichia coli*, flavodoxin is required for the reductive activation of cobalamin-dependent methionine synthase (27), for biotin synthesis (28), and for the anaerobic activation of both ribonucleotide reductase (29) and pyruvate-formate lyase (30). In nitrogen-fixing bacteria, such as *Anabaena*, flavodoxin is the electron donor for the nitrogenase iron-containing protein (31). In addition, flavodoxins can replace ferredoxin under certain conditions and have been shown to be substrates of the NADPH:ferredoxin oxidoreductase (FNR) of cyanobacteria (32) and of *E. coli* (33), of the pyruvate-ferredoxin oxidoreductase of *Clostridium pasteurianum* (34), and of the enzymes of dissimilatory sulfate reduction (35). Experimental evidence that the flavodoxin long loop could be involved in the interaction of *E. coli* flavodoxin with methionine synthase has already been provided by NMR mapping (36). According to the chemical shift perturbations observed upon flavodoxin binding to methionine synthase, three residues of the flavodoxin long loop could be in contact with the enzyme in the complex. In the same study, the flavodoxin/FNR complex was also investigated. Significantly, a larger number of loop residues (six or more) were found to change their chemical shift upon binding to FNR. In fact, in the flavodoxin/FNR complex, the long loop is the flavodoxin region where the largest chemical shift changes take place upon complex formation (36). Thus, it seems that the flavodoxin long loop could play an important role in the recognition of non-photosynthetic FNRs. In addition, our preliminary phylogenetic analysis (data not shown) indicates that photosynthetic FNR is consistently associated to long flavodoxin organisms. Thus, a possibility exists that the flavodoxin long loop is similarly involved in the recognition of photosynthetic FNRs.

CONCLUSIONS

Although the loop splitting the fifth β-strand in long-chain flavodoxins does not contain FMN binding residues and it is not essential for the proper folding of the apoprotein, it seems very important to stabilize the adjacent Tyr^{94}-bearing FMN binding loop to the extent that *Anabaena* apoflavodoxin no longer binds FMN with high affinity when the long loop is removed. This should not be taken to imply that the long loop chief role is to assist in FMN binding, because the short flavodoxins can also bind FMN very efficiently. Given the conservation of the long loop among flavodoxins and its small influence on the structure and stability of the protein (see also Ref. 37), we suggest that it might serve to interact with flavodoxin partners.

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REFERENCES

1. Mayhew, S. G., and Tollin, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed) Vol III, pp. 389–426, CRC Press, Inc., Boca Raton, FL
2. Ludwig, M. L., and Luschinsky, C. L. (1992) in *Chemistry and Biochemistry of flavoenzymes* (Müller, F., ed) Vol III, pp. 427–466, CRC Press, Inc., Boca Raton, FL
3. Ludwig, M. L., Andersen, R. D., Mayhew, S. G., and Massey, V. (1969) *J. Biol. Chem.* **244,** 6047–6048
4. Watenpaugh, K. D., Sieker, L. C., Jensen, L. H., Legall, J., and Dubourdieu, M. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 3185–3188
5. Smith, W. W., Pattridge, K. A., Ludwig, M. L., Petsko, G. A., Tsernoglou, D., Tanaka, M., and Yasunobu, K. T. (1983) *J. Mol. Biol.* **165,** 737–753
6. Rao, S. T., Shaffie, F., Yu, C., Satyshur, K. A., Stockman, B. J., Marley, J. L., and Sundaralingam, M. (1992) *Protein Sci.* **1,** 1413–1427
7. Genzor, C. G., Perales-Alcon, A., Sancho, J., and Romero, A. (1996) *Nat. Struct. Biol.* **3,** 329–332
8. Fernández-Recio, J., Genzor, C. G., and Sancho, J. (2001) *Biochemistry* **40,** 15234–15245
9. Genzor, C. G, Beldarrain, A., Gomez-Moreno, C., Lopez-Lacomba, J. L., Cortijo, M., and Sancho, J. (1996) *Protein Sci.* **5,** 1376–1388
10. Fernández-Recio, J., Romero, A., and Sancho, J. (1999) *J. Mol. Biol.* **290,** 319–330
11. Langdon, G. M., Jimenez, M. A., Genzor, C. G., Maldonado, S., Sancho, J., and Rico, M. (2001) *Proteins* **43,** 476–488
12. Irun, M. P., Garcia-Mira, M. M., Sánchez-Ruiz, J. M., and Sancho, J. (2001) *J. Mol. Biol.* **306,** 877–888
13. Irun, M. P., Maldonado, S., and Sancho, J. (2001) *Protein Eng.* **14,** 173–181
14. Maldonado, S., Lostao, A., Irún, M. P., Fernández-Recio, J., Genzor, C. G., González, E. B., Rubio, J. A., Luquita, A., Daoudi, F., and Sancho, J. (1998) *Biochimie* (*Paris*) **80,** 813–820
15. Maldonado, S., Jimenez, M. A., Langdon, G. M., and Sancho, J. (1998) *Biochemistry* **37,** 10589–10596
16. Maldonado, S., Irun, M. P., Campos, L. A., Rubio, J. A., Luquita, A., Lostao, A., Wang, R., Garcia-Moreno, E. B., and Sancho, J. (2002) *Protein Sci.* **11,** 1260–1273
17. Lostao, A., Gómez-Moreno, C., Mayhew, S. G., and Sancho, J. (1997) *Biochemistry* **36,** 14334–14344
18. Lostao, A., El Harrous, M., Daoudi, F., Romero, A., Parody-Morreale, A., and Sancho, J. (2000) *J. Biol. Chem.* **275,** 9518–9526
19. Lostao, A., Daoudi, F., Irún, M. P., Ramón, A., Fernández-Cabrera, C., Romero, A., and Sancho, J. (2003) *J. Biol. Chem.* **278,** 24053–24061
20. Casaus, J. L., Navarro, J. A., Hervas, M., Lostao, A., De La Rosa, M. A., Gomez-Moreno, C., Sancho, J., and Medina, M. (2002) *J. Biol. Chem.* **277,** 22338–22344
21. Amann, E., Ochs, B., and Abel, K. J. (1998) *Gene* (*Amst.*) **69,** 310–315
22. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem.* **200,** 81–88
23. Fillat, M. F., Borrias, W. E., and Weisbeek, P. (1991) *Biochem. J.* **280,** 187–191
24. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326
25. Lillo, M. P., Cañadas, O., Dale, R. E., and Acuña, A. U. (2002) *Biochemistry* **41,** 12436–12449
26. Donzel, B., Gauduchon, P., and Wahl, P. (1974) *J. Am. Chem. Soc.* **96,** 801–808
27. Osborne, C., Chen, L. M., and Matthews R. G. (1991) *J. Bacteriol.* **173,** 1729–1737
28. Sanyal, I., Gibson, K. J., and Flint D. H. (1996) *Arch. Biochem. Biophys.* **326,** 48–56
29. Bianchi, V., Eliasson, V. R., Fontecave, M., Mulliez, E., Hoover, D. M., Matthews, R. G., and Reichard, P. (1993) *Biochem. Biophys. Res. Commun.* **197,** 792–797
30. Sawers, G., and Watson, G. (1998) *Mol. Microbiol.* **29,** 945–954
31. Fillat, M. F., Sandmann, G., and Gomez-Moreno, C. (1988) *Arch. Microbiol.* **150,** 160–164
32. Smillie, R. M. (1965) *Biochem. Biophys. Res. Commun.* **20,** 621–629
33. Blaschkowski, H. P., Never, G., Ludwig-Festl, M., and Knappe, J. (1982) *Eur. J. Biochem.* **123,** 563–569
34. Fitzgerald, M. P., Rogers, L. J., Rao, K. K., and Hall, D. O. (1980) *Biochem. J.* **192,** 665–672
35. Peck, H. D., Jr., and LeGall, J. (1982) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **298,** 443–466
36. Hall, D. A., Vander Kooi, C. W., Stasik, C. N, Stevens, S. Y., Zuiderweg, E. R. P, and Matthews, R. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9521–9526
37. López-Llano, J., Maldonado, S., Jain, S., Lostao, A., Godoy-Ruiz, R., Sanchez-Ruiz, Cortijo, M., Fernández-Recio, J., and Sancho, J. (2004) *J. Biol. Chem.* **279,** 47184–47191