Flavodoxins play central roles in the electron transfer involving various biological processes in microorganisms. The mioC gene of *Escherichia coli* encodes a 16-kDa flavodoxin and locates next to the chromosomal replication initiation origin (oriC). Extensive researches have been carried out to investigate the relationship between *mioC* transcription and replication initiation. Recently, the MioC protein was proposed to be essential for the biotin synthase activity *in vitro*. Nevertheless, the exact role of MioC in biotin synthesis and its physiological function *in vivo* remain elusive. In order to understand the molecular basis of the biological functions of MioC and the cofactor-binding mechanisms of flavodoxins, we have determined the solution structures of both the apo- and holo-forms of *E. coli* MioC protein at high resolution by nuclear magnetic resonance spectroscopy. The overall structures of both forms consist of an α/β sandwich, which highly resembles the classical flavodoxin fold. However, significant differences are observed between the two forms, especially the stabilization of the FMN-binding loops and the notable extension of secondary structures upon FMN binding. Structural comparison reveals fewer negative charged and aromatic residues near the FMN-binding site of MioC, as compared with that of flavodoxin 1 from *E. coli*, which may affect both the redox potentials and the redox partner interactions. Furthermore, the backbone dynamics studies reveal the conformational flexibility at different time scales for both apo- and holo-forms of MioC, which may play important roles for cofactor binding and electron transfer.

Flavodoxins are small FMN-binding proteins found mainly in prokaryotes that are involved in the electron transfer chain reactions of various biological processes, including photosynthesis (1, 2), nitrate reduction (3), methionine synthesis (4–7), biotin synthesis (8–11), and activation of certain enzymes, such as pyruvate-formate lyase (13) and (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate synthase (14). It has been established by potentiometry that electrons flow from NADPH to flavodoxin reductase and then to flavodoxin, which subsequently transfers the electron to the downstream targets (15). Eukaryotes and most prokaryotes also contain proteins carrying domains homologous to flavodoxins and flavodoxin reductase in a single polypeptide chain, such as the NADPH-cytochrome P450 oxidoreductase (CPR) (2) (16, 17), nitric-oxide synthase (NOS) (18), methionine synthase reductase (19), and sulfite reductase (20, 21). These domains function in a similar fashion as the flavodoxins and flavodoxin reductase proteins in prokaryotic cells. Extensive efforts have been devoted to understanding the cofactor binding, redox partner interaction, and electron transfer mechanisms of flavodoxins and flavodoxin-like domains as well as the folding and stability of these proteins (22–26). However, the detailed mechanisms are not clear yet and remain to be further elucidated.

Flavodoxins are classified into the long-chain and short-chain groups according to the presence or absence of a 20-amino acid loop that splits the fifth β-strand of the central β-sheet (25, 26). The *Escherichia coli* protein MioC is identified as a flavodoxin with 147 amino acid residues and belongs to the short-chain group. The mioC gene is located next to the chromosomal replication initiation origin (oriC) of the *E. coli* genome, and the transcription of *mioC* runs through the oriC site (27). Based on its peculiar chromosomal location, extensive efforts were dedicated to establishing the relationship between *mioC* transcription and DNA replication initiation (28–37). However, these experimental results are often contradictory, and no ultimate conclusions have been reached thus far. Recently, it was suggested that MioC protein plays an essential role in promoting the biotin synthase (BioB) activity *in vitro* (11). Two other essential protein components for keeping BioB activity were previously identified to be *E. coli* flavodoxin 1 (FldA) and flavodoxin reductase (10). It was also shown that both MioC and FldA are needed in the *in vitro* activity assays, and neither of them can substitute the function of the other (11). Intriguing questions are thus raised. What exact role does MioC play in biotin synthesis? Does it act up- or downstream of FldA in the electron transfer chain, or does it act parallel? Why

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*This research was supported by National Natural Science Foundation of China Grant 30125009 (to B. X.) and Grant 30325010 (to C. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

*The atomic coordinates and structure factors (code 2HNA and 2HNH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).* [The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2.]

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*2 The abbreviations used are: CPR, NADPH-cytochrome P450 oxidoreductase; FldA, flavodoxin 1; DTT, dithiothreitol; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; NOS, nitric-oxide synthase; r.m.s., root mean square.*

**Solution Structures and Backbone Dynamics of a Flavodoxin MioC from *Escherichia coli* in both Apo- and Holo-forms**

**IMPLICATIONS FOR COFACTOR BINDING AND ELECTRON TRANSFER**

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are two flavodoxins needed in this reaction system, and how do they distinguish their redox partners and their biological roles? Is MioC essential for biotin synthesis in vivo, and what other potential physiological roles may MioC play? In order to elucidate these issues, one needs to answer the following two fundamental questions. Does E. coli MioC exhibit similar or different molecular properties asFldA? What are the structural and dynamic features that might determine the roles of MioC in the biotin synthesis and other redox activities?

Since MioC and FldA share low sequence identity and belong to the short-chain and long-chain flavodoxin groups, respectively, the structural and dynamic diversities may give rise to the different redox activities and functions of the two proteins. In an effort to obtain insights into the molecular mechanism of the biological functions, we have determined the solution structures of both the apo- and holo-forms of MioC by high resolution NMR spectroscopy. Both structures show the typical flavodoxin fold consisting of an α/β sandwich. However, the protein structure is considerably stabilized, and the secondary structures are extended upon FMN binding. In addition, backbone dynamics studies of the apo- and holo-forms in conjunction with structural analysis and titration experiments provide further insights into the molecular mechanisms of cofactor binding and electron transfer of flavodoxins.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of the Recombinant MioC**—The mioC gene was cloned into pET21a expression vector and expressed in E. coli BL21 (DE3) strain (Novagen). The culture was allowed to grow in LB medium, centrifuged, and resuspended in M9 minimal medium with antibiotics (ampicillin) and 15NH₄Cl in the presence or absence of 13C₆-glucose for preparations of [13C]15N-labeled or [15N]-labeled samples, respectively (38). The MioC protein was purified by ion exchange chromatography (DEAE) and subsequently gel filtration (Superdex-75) using an ÄKTA FPLC system (Amersham Biosciences). The purity was determined to be greater than 95% as judged by SDS-PAGE. The apo-form of MioC was prepared by denaturing the purified protein using 5% trichloroacetic acid (39), followed by refolding the protein in a buffer containing 30 mM sodium phosphate (pH 7.0), 30 mM NaCl, and 20 mM dithiothreitol (DTT). The holo-form of MioC was prepared by refolding the protein in a buffer containing 30 mM sodium phosphate (pH 7.0), 30 mM NaCl, and excess fully oxidized FMN (50 mM). 5% D₂O was added for preparations of the NMR samples, and 2,2-dimethyl-2-silapentanesulfonic acid was added as the internal chemical shift reference.

**NMR Spectroscopy**—The NMR experiments were carried out at 25°C on Bruker Avance 500- and 800-MHz spectrometers equipped with four RF channels and triple resonance probes with pulsed field gradients. The chemical shifts were referenced to internal 2,2-dimethyl-2-silapentanesulfonic acid. All NMR spectra were processed using NMRPipe (40) and analyzed using NMRView (41). Two-dimensional [13C]-15N and [15N]-edited HSQC spectra were collected with 24 transients per increment and a recycle delay of 3000 ms. Complete spectral assignments were obtained with 2,2-dimethyl-2-silapentanesulfonic acid as the internal chemical shift reference.

**Backbone 15N Relaxation Measurements**—The backbone 15N relaxation parameters of the longitudinal relaxation rates (R₁), transverse relaxation rates (R₂), and steady-state heteronuclear (1H)-15N NOE values of the apo- and holo-forms of MioC were measured, respectively (57). The experiments were performed on a Bruker Avance 800-MHz NMR spectrometer at 25°C. A spectral width of 1160.7 Hz for the 1H dimension was used for both the apo- and holo-MioC, whereas for 15N dimension, spectral widths of 2595.0 and 2676.1 Hz were used for the apo- and holo-MioC, respectively. For the R₁ and R₂ measurements, 1024 (1H) and 100 (15N) complex data points were collected with 24 transients per increment and a recycle delay of 2.7 s. The delays used for the R₁ experiments were 10 (×2), 100, 200, 500, 800, 1100, 1500, 2000, 2500, and 3190 ms, and those used for the R₂ experiments were 8 (×2), 16, 32, 48, 64, 88, 112, 136, and 176 ms. The relaxation rate constants were obtained by fitting the peak intensities to a single exponential function.
using the nonlinear least squares method as described (58).

The \(^1\text{H}-\text{\textsuperscript{15}N}\) NOE experiments were performed in the presence and absence of a 3-s proton presaturation period prior to the \textsuperscript{15}N excitation pulse and using recycle delays of 2 and 5 s, respectively (59). 64 transients were used for each experiment.

RESULTS

NMR Characterizations of the Apo- and Holo-forms of E. coli MioC—The overexpression and purification procedures of the recombinant MioC were similar to that previously described (11). The elution with bright yellow color from the ion exchange column was further purified by gel filtration chromatography. However, the two-dimensional \textsuperscript{15}N-edited HSQC spectrum of the directly purified sample showed an extra set of peaks, indicating a mixture of inhomogeneous compositions.

The trichloroacetic acid precipitation and subsequent refolding procedure were used to prepare the apo-form of MioC (39). The corresponding two-dimensional \textsuperscript{15}N-edited HSQC spectrum showed a unique set of cross-peaks with chemical shift distribution corresponding to a reasonably well folded protein (Fig. 1A). However, the backbone amide signals of 21 nonproline residues were missing, including Ala\textsubscript{2}, Gly\textsubscript{9}, Thr\textsubscript{11}, Leu\textsubscript{12}, Gly\textsubscript{14}, Ala\textsubscript{15}, Glu\textsubscript{55}, Ser\textsubscript{125}, Asp\textsubscript{127}, His\textsubscript{128}, Asp\textsubscript{133}, Ala\textsubscript{135}, and Glu\textsubscript{136}. Half of the missing residues are located in regions connecting the secondary structures of the central core and the FMN-binding loops, suggesting the intermediate con-
Structures and Dynamics of E. coli MioC

After the holo-form of MioC was exchanged using the NMR buffer without free FMN molecules, the bound FMN molecules gradually left off from the protein. The HSQC spectrum of the sample prepared in this way also showed two sets of peaks, indicating that FMN is noncovalently bound to MioC and the apo- and holo-forms of MioC are in an exchanging process. Taken together, the existence of two sets of peaks instead of one in the HSQC spectra indicates that the exchange of apo- and holo-forms of MioC is in the slow regime. When the molar ratio of FMN/protein reached and went beyond 1, the set of peaks corresponding to the apo-form was completely unobservable, and the HSQC spectra were identical to that of the holo-form. These results suggest a 1:1 binding between MioC and FMN with high affinity, which is in agreement with the previously characterized nanomolar range of $K_d$ values commonly observed for flavodoxins (60).

The composite chemical shift changes of individual residues in the two forms are plotted in Fig. 1D. Residues exhibiting the largest chemical shift changes are clustered around the three FMN-binding loops: the P-loop (residues Gly9–Gly14), the 50s loop (residues Ser67–Gln76), and the 90s loop (residues Ser47–Asp64), and the 90s loop (residues Ser55–Asp64). In addition, five backbone NOE contacts between residues Ile88 and Gly89 and Leu121, Lys122, and Ile123 were assigned to establish the short strand β5′ for residues Leu121–Ile123. However, this
Structures and Dynamics of E. coli MioC

TABLE 1
Structural statistics of the apo- and holo-forms of MioC

|                          | Apo-form | Holo-form |
|--------------------------|----------|-----------|
| Distance restraints      |          |           |
| Intraresidue unambiguous NOEs | 1526    | 1821      |
| Sequential unambiguous NOEs | 746     | 804       |
| Medium range unambiguous NOEs | 540     | 500       |
| Long range unambiguous NOEs | 706     | 882       |
| Total unambiguous NOEs    | 3518    | 4007      |
| Total ambiguous NOEs      | 1283    | 1674      |
| Dihedral angles (φ and ψ) | 128     | 158       |
| Intermolecular NOEs       | 0       | 7         |
| r.m.s. deviation from mean structure (Å) |          |           |
| Secondary structure backbone atoms | 0.57 ± 0.10 | 0.48 ± 0.11 |
| Secondary structure heavy atoms | 0.98 ± 0.12 | 1.01 ± 0.13 |
| All backbone atoms        | 1.43 ± 0.26 | 0.97 ± 0.18 |
| All heavy atoms           | 1.92 ± 0.28 | 1.46 ± 0.21 |
| Energy (kcal/mol)         |          |           |
| Mean AMBER energy         | -7065.22 ± 15.91 | -7548.59 ± 11.19 |
| NOE distance restraints violation energy | 15.61 ± 1.69 | 13.85 ± 1.71 |
| Torsion angle restraints violation energy | 0.64 ± 0.12 | 0.85 ± 0.14 |
| Restraints violations     |          |           |
| Distance (>0.3 Å)         | 0       | 0         |
| Dihedral angle (>5°)      | 0       | 1         |
| Ramachandran statistics (%)|        |           |
| Residues in most favored regions | 73.0     | 79.3     |
| Residues in additional allowed regions | 22.6 | 19.1 |
| Residues in generously allowed regions | 3.4 | 1.3 |
| Residues in disallowed regions | 1.0 | 0.2 |

short strand is highly flexible in the apo-form (see below). Furthermore, the three FMN-binding loops and the 120s loop are highly mobile and adopt a flexible conformation, in accordance with the lack of NOE contacts and high internal flexibility (see below).

The holo-form of MioC also contains four α-helices (residues Gly14–Ala29 (α1), Ser67–Gln76 (α2), Ile103–Asn111 (α3), and Ala135–Leu146 (α4)) and five β-strands (residues Ile4–Ser8 (β1), Thr33–His37 (β2), Ser97–Ser95 (β3), Ala82–Gly91 (β4), Lys115–Gln116 (β5), and Leu121–Asn124 (β5’)). Residues from Leu41 to Asp43 form a short 310 helix (H1). The core structure is similar to that of the apo-form, but the secondary structures are significantly extended and stabilized. In particular, the FMN-loops are straightened up and fixed in a relatively rigid conformation for the accommodation of FMN.

Binding-induced Secondary Structure Formation—Although the core structures of the apo- and holo-forms of MioC are similar, significant differences are observed. An overlay of the ribbon diagrams of the mean structures (energy-minimized using AMBER) of apo- and holo-forms of MioC is shown in Fig. 3A. The backbone r.m.s. deviation between the two forms is 4.63 Å for all residues, and it is 2.30 Å after excluding the four loops near the cofactor-binding sites, indicating the FMN-binding loops are involved in the largest conformational changes. The secondary structures in the holo-form are generally longer than that in the apo-form. The most significant secondary structure extensions are observed for strands β3 and β4, extending two and three residues further toward the FMN-binding pocket. These two strands are directly connected to the 50s and the 90s loops, which are responsible for coordination of the isoalloxazine ring of the FMN cofactor. The short strand β5’ is flexible and not well defined in the apo-form of MioC, whereas it is fully established and stabilized in the holo-form. The α-helices are also extended. Both helices α1 and α4 extend two residues toward the FMN-binding site. In addition to the binding-induced secondary structure extension and stabilization observed at local structures of the FMN-binding site, a small helix H’ from residue Leu40 to Leu44 is also induced after binding of the FMN molecule. In contrast, this region adopts a looplike structure in the apo-form, and there were not sufficient NOE contacts obtained to establish a helical structure.

The induced formation of secondary structures and the significant stabilization of the overall structure of MioC after FMN binding is mostly responsible for the high affinity toward the FMN molecule and may also be important for maintaining the stable MioC-FMN complex and its biological activity in vivo.

Structural Comparisons of the Apo-form of E. coli MioC with the Crystal Structures of Apo-flavodoxins—For a full understanding of the molecular mechanism of cofactor binding, structures of both the holo-form and apo-form are required. Previous investigations on flavodoxin structures generally employed the crystallographic approach. Crystal structures of flavodoxins in the FMN-bound state from various organisms have been reported (60–65), whereas much less information is available for apoflavodoxins. Two crystal structures of the long-chain apoflavodoxins from Anabaena and Helicobacter pylori were reported (22, 66). In both structures, the P-loop was stabilized by the recruitment of an anion (a sulfate ion for Anabaena apoflavodoxin and a chloride ion for H. pylori apoflavodoxin). All of the three FMN-binding loops showed similar rigid conformations as observed in the holoflavodoxins, whereas only the 50s loop appeared to move closer to the gap left by the cofactor (22, 66). Two other crystal structures of apo-WrbA proteins, which also belong to the long-chain flavodoxin group, have been determined recently (67). The FMN binding loops in these structures also showed rigid conformations, and no significant conformational changes were observed upon FMN binding.
Our structure of the apo-form of MioC is the first solution structure reported for apoflavodoxins and, moreover, the first structure of the apo-form of short-chain flavodoxins thus far. The backbone cross-peaks were missing in the two-dimensional HSQC spectrum and could not be assigned for 10 residues (Gly9, Gly14, Ala15, Glu16, Ser55, Asp64, Arg93, Ala101, Ile102, and Asp103) connecting the FMN-binding loops and the central β-sheet, which suggests possible hinge motions. In contrast, most of the residues located in the loops could be observed and assigned (except for residues Thr11 and Leu12 in the P-loop and Cys99 in the 90s loop), although very limited NOE contacts were observed due to the flexibility in these regions. The backbone r.m.s. deviation is 3.7 ± 0.9 Å for the FMN-binding loops, whereas it is only 0.8 ± 0.2 Å for the secondary structures. These results indicate that the FMN-binding loops of the apo-form of MioC are flexible in solution, which are in good agreement with the previous solution NMR characterizations of the structural properties of apo-forms of long-chain flavodoxins (68, 69).

Fig. 3, B and C, show the structural comparisons of the apo-form of MioC (energy-minimized mean structure) with the crystal structures of apoflavodoxin from Anabaena (Protein Data Bank entry 1FTG; green) and H. pylori (Protein Data Bank entry 2BMV; yellow) in ribbon diagrams, respectively. D–F, structural overlays of the holo-form of MioC (red) with the crystal structures of the flavodoxin from Anabaena (Protein Data Bank entry 1RCF; cyan), the FMN-binding domain of the cytochrome P450BM-3 from B. megaterium (Protein Data Bank entry 1BVY, F chain; light gray), and the FMN-binding domain of the NADPH-cytochrome P450 oxidoreductase from Rat (Protein Data Bank entry 1AMO; sky blue). The images were generated using MOLMOL (54).

FIGURE 3. Structural comparisons. A, an overlay of the ribbon diagrams of the apo-form (blue) and the holo-form (red) of E. coli MioC. B and C, structural overlay of the apo-form of MioC (blue) with the crystal structures of apoflavodoxin from Anabaena (Protein Data Bank entry 1FTG; green) and H. pylori (Protein Data Bank entry 2BMV; yellow) in ribbon diagrams, respectively. D–F, structural overlays of the holo-form of MioC (red) with the crystal structures of the flavodoxin from Anabaena (Protein Data Bank entry 1RCF; cyan), the FMN-binding domain of the cytochrome P450BM-3 from B. megaterium (Protein Data Bank entry 1BVY, F chain; light gray), and the FMN-binding domain of the NADPH-cytochrome P450 oxidoreductase from Rat (Protein Data Bank entry 1AMO; sky blue). The r.m.s. deviation values for the 126 and 124 aligned Cα residues between the apo-form of MioC and the two crystal structures are 3.1 and 3.2 Å, respectively, as determined by DaliLite (70). The secondary structures in the apo-form of MioC are less well defined than that in both crystal structures of the apoflavodoxins. In addition, the FMN-binding loops in the apo-form of MioC do not show a straightened up conformation as observed in the crystal structures of apoflavodoxins and holoflavodoxins. The solution structures of the apo-form of MioC are relatively loose, especially at the FMN-binding site (see Fig. 2A). Since it was also observed that the FMN binding loops in the apo-form of long-chain flavodoxins were flexible in solution (68, 69), this may be a common feature for all flavodoxins. It is likely that the structural flexibility is required for the cofactor-binding loops to sample different conformations and thus facilitate the recognition and binding with the FMN molecule.

Structural Comparisons of the Holo-form of E. coli MioC with Other Flavodoxins and Flavodoxin-like Domains—The solution structure of the holo-form of MioC shows a compact flavodoxin-like fold similar to other flavodoxins and flavodoxin-like domains in multidomain proteins. A search against the Protein Data Bank by DALI (71) found structures showing high
The electrostatic potential at the FMN-binding site of MioC is shown in Fig. 4A. For comparison, the cofactor binding site of *E. coli* flavodoxin FldA and the flavodoxin-like domain of rat CPR are shown in Fig. 4, B and C, respectively. The FMN binding pocket is generally surrounded by many negatively charged residues, which are thought to be important factors for modulating the redox potentials of FMN (72, 73). *E. coli* FldA has a total of five negatively charged residues located in the 50s and 90s loops that are in proximity with the isoalloxazine ring of FMN, whereas *E. coli* MioC has only three negatively charged residues in these loops. In addition, a positively charged residue Arg\(^{93}\) locates in the 90s loop next to the negatively charged Glu\(^{94}\) in MioC, which may neutralize the negative charge to a certain degree. Therefore, the electrostatic charge of MioC is much less negative compared with FldA around the flavin ring.

Similar to other flavodoxins, MioC contains the conserved aromatic residues Tyr\(^{95}\) and Phe\(^{98}\) in the 90s loop. In the 50s loop, the aromatic residue Trp\(^{57}\) in *E. coli* FldA is substituted by a histidine residue in MioC. This position is also occupied by an aromatic residue tyrosine in both cytochrome P450BM-3 from *Bacillus megaterium* (Tyr\(^{536}\)) and rat CPR (Tyr\(^{140}\)). Although not enough NOE contacts between FMN and MioC were obtained and the orientation of the molecule FMN was ill defined, the local backbone conformation of MioC is quite similar to other well characterized flavodoxins, suggesting that MioC may bind FMN in a similar manner as other flavodoxins. Notably, the *E. coli* FldA has a total of six aromatic residues surrounding the FMN molecule (residues Trp\(^{57}\), Tyr\(^{58}\), Tyr\(^{59}\), Tyr\(^{94}\), Tyr\(^{97}\), and Phe\(^{98}\)). These aromatic residues form a hydrophobic wall that separates the flavin ring of FMN from the solvent (60). In contrast, the *E. coli* MioC protein does not contain any other aromatic residues except His\(^{97}\), Tyr\(^{95}\), and Phe\(^{98}\), as mentioned above. The positions equivalent to residues Tyr\(^{58}\), Tyr\(^{59}\), and Tyr\(^{97}\) in FldA are occupied by the short side-chain residues Gly\(^{58}\), Ala\(^{59}\), and Thr\(^{97}\) in the MioC structure. These differences may play a role in determining the different redox partners that MioC and FldA interact with, and thus the biological processes they are involved in. These structural properties may also affect the redox potentials.

Interestingly, the aforementioned structural features at the FMN-binding site of MioC are also shared by the flavodoxin-like domains in cytochrome P450BM-3 from *B. megaterium* and the flavodoxin-like domains of mammalian CPR. The FMN-binding domains of these multidomain proteins also contain fewer aromatic residues and are less negatively charged. Furthermore, both of these flavodoxin-like domains contain a positively charged lysine residue at the position equivalent to Arg\(^{93}\) in MioC. The electrostatic potential at the protein surface may act in orienting the protein in protein-protein interactions (74). Therefore, a similar pattern of charge distribution between MioC and these flavodoxin-like domains may
suggest similar redox partner-interacting surfaces and thus similar biological functions.

**Backbone Relaxation Parameters and Model-free Analysis**

It is well understood that the biological function of a protein is strongly dependent on its structure and dynamics, and the changes of both structure and dynamics play critical roles in many biological processes, such as ligand binding, protein-protein interactions, and enzyme catalysis (75–77). Proteins mostly undergo submillisecond time scale conformational changes upon ligand binding, and it is of importance to recognize the change of dynamic properties associated with this process. NMR is a well established technique to provide the site-specific motional information with exquisite time resolution (78). Therefore, insights obtained from dynamics may have potential implications in understanding of the biological functions (76).

In order to characterize the dynamic properties of the apo- and holo-form of MioC and obtain further insights into the molecular mechanisms of cofactor binding and electron transfer, we have determined the $^{15}$N backbone relaxation parameters, including the longitudinal relaxation rates $R_1$, transverse relaxation rates $R_2$, and heteronuclear Overhauser effect $^{1H}$-$^{15}$N NOE values for both forms of MioC using freshly prepared samples. During the analysis of the relaxation data, 86 of 147 residues were used for the apo-form, whereas 115 were used for the holo-form of MioC. The unanalyzed residues include the proline residues, the ones that were not assigned and those that overlapped or were too weak to be accurately analyzed. The experimental data of the apo- and holo-form for MioC are shown in Fig. 5A.

Overall, the protein adopts a rigid structure in both forms as reflected by the high $^{1H}$-$^{15}$N NOE values (>0.75) for most of the residues in the core secondary structures. The 50s loop and the 120s loop are relatively flexible in the apo-form of MioC, as reflected by the relatively low $^{1H}$-$^{15}$N NOE values (≤0.7). Many residues in the 90s loop were unanalyzed due to weak signals and thus poor data quality for the determination of relaxation rates ($R_1$ and $R_2$). In addition, many backbone cross-
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peaks were missing in these regions, especially for residues in the P-loop. This suggests that the intermediate conformational exchanges on submillisecond time scales, which result in the line broadening and the disappearing of the cross-peaks. Upon binding to FMN, the P-loop, the 90s loop, and part of the 50s loop became more rigid, since many new cross-peaks appeared for residues in these regions, which showed close to average $^{1}H$,$^{15}N$ NOE values and relaxation rates. However, motional flexibilities still exist for some parts of the loops, especially the 50s loop and the 120s loop, and will be discussed below.

The precise determination of motional anisotropy is essential for analyzing the relaxation data, particularly for the characterization of chemical or conformational exchanges (79). The motional anisotropy can be described by the rotational diffusion tensor. The ratios of the principle components of the inertia tensors of the apo- and holo-forms of MioC calculated from the solution structures are (1.0:80.0:75) and (1.0:77.0:66), respectively, suggesting motional anisotropy for both forms. The rotational diffusion tensors of apo- and holo-forms of MioC were determined following the general procedures (80). A total of 63 and 95 residues were used for the apo- and holo-forms to determine the rotational diffusion tensors, respectively. The results indicated that the diffusion tensors for both forms were best represented the axially symmetric model, demonstrating the motional anisotropy. For the apo-form of MioC, the rotational correlation time is $\tau_m = 7.57 \pm 0.04$ ns, and the diffusion anisotropy is $D_1/D_2 = 1.09 \pm 0.03$. For the holo-form of MioC, the rotational correlation time is $\tau_m = 7.27 \pm 0.03$ ns, and the diffusion anisotropy is $D_1/D_2 = 1.14 \pm 0.03$. The results suggest that both forms of the MioC protein exist as monomers in solution under our present experimental conditions, which was also observed during the MioC purification procedure using gel filtration (data not shown).

Model-free analysis was performed to extract the dynamic parameters from the experimentally determined relaxation data (81, 82), and the axially symmetric diffusion model was used during the calculations. The calculations were carried out by using the experimental data, the uncertainties, and the energy-minimized mean structures for both forms of MioC as input. Five models with increasing complexity (M1, $S^2; M2, S^2, \tau_J; M3, S^2, R_{ex}; M4, S^2, \tau_J, R_{ex}; M5, S^2, S^2, \tau_J$) were used iteratively to reproduce the experimental data until confidence reached within 95% (83). The confidence level was estimated using 300 Monte Carlo simulations per run in combination with $\chi^2$ and F-statistic analysis. The amide bond length was fixed at 1.02 Å, and the $^{15}N$ chemical shift anisotropy value of ~175 ppm was used during the calculations. The optimized internal mobility parameters (the generalized order parameter $S^2$, the fast internal motions on the picosecond to nanosecond time scales $\tau_J$, and the conformational exchanges $R_{ex}$ on the micro- to millisecond time scales) for both apo- and holo-forms of MioC are shown in Fig. 5B.

For the apo-form of MioC, a total of 63 residues were assigned to model M1, with an average $S^2 = 0.86 \pm 0.03$. Ten residues (Ala$^{23}$, Glu$^{27}$, Ala$^{46}$–Ile$^{49}$, Leu$^{51}$, Lys$^{115}$, Glu$^{119}$, and Leu$^{121}$) were assigned to model M2, with an average $S^2 = 0.76 \pm 0.03$ and internal motions on the picosecond to nanosecond time scales. Nine residues (Ser$^8$, Val$^{118}$, Ala$^{19}$, Gly$^{38}$, Trp$^{50}$, Ile$^{53}$, Gly$^{91}$, Leu$^{139}$, and Val$^{143}$) were assigned to model M3, with an average $S^2 = 0.80 \pm 0.04$ and conformational exchanges ($R_{ex}$) on the microsecond to millisecond time scales. Three residues (Asp$^{129}$, Ile$^{130}$, and Gly$^{140}$) were assigned to model M4 with an average $S^2 = 0.81 \pm 0.05$, whereas one residue (Lys$^{147}$) was assigned to model M5.

For the holo-form of MioC, a total of 95 residues were assigned to model M1, with an average $S^2 = 0.88 \pm 0.03$. Twelve residues (Asp$^3$, Thr$^{13}$, Ala$^{46}$–Ile$^{49}$, Thr$^{97}$, Gly$^{113}$, Glu$^{119}$, Asp$^{129}$, Trp$^{142}$, and Leu$^{145}$) were assigned to model M2, with an average $S^2 = 0.76 \pm 0.03$ and internal motions on the picosecond to nanosecond time scales. Seven residues (Tyr$^{17}$, Ala$^{59}$–Ile$^{62}$, Ile$^{90}$, and Ile$^{125}$) were assigned to model M3, with an average $S^2 = 0.84 \pm 0.04$ and conformational exchanges on the microsecond to millisecond time scales. No residues were assigned to model M4, and one residue (Lys$^{147}$) was assigned to model M5.

DISCUSSION

**Internal Dynamics**—Although much information is available on the structures of flavodoxins, there are no systematic dynamic characterizations for either the apo-form or the holo-form thus far. For a clear overview, the extracted dynamic parameters are mapped onto the MioC structures of the apo- and holo-forms and are shown in Fig. 6. Overall, the core structures of apo- and holo-forms of MioC show a similar rigidity, indicated by the similar average $S^2$ values for residues assigned to M1. However, as shown in Fig. 7, a closer examination of the differences of the $S^2$ values between the two forms of MioC demonstrates that the holo-form is less mobile than the apo-form, which is also indicated by the fact that many more residues in the holo-form of MioC were assigned to M1 by the model-free analysis. For the apo-form of MioC, significant conformational exchanges on the microsecond to millisecond time scales as well as the internal motions on the picosecond to nanosecond time scales were observed around the FMN binding loops, indicating that these loops may sample multiple conformations in solution in the absence of FMN. In addition, the line broadening for the signals of residues connecting the loops to the central structural core also suggests conformational exchanges on the submillisecond time scales, which may enable the loops to adopt the open and closed conformations, offering the entrance of the FMN molecule into the binding pocket. Upon binding to FMN, the conformational exchanges of the MioC protein are largely diminished, especially those residues in the loops around the binding site. The residues connecting the loops to the structural core are stabilized and mostly are not involved in conformational changes, and residues located in the P-loop and 90s loop regions are greatly stabilized as well. On the other hand, the holo-form of MioC is not completely rigid, and the motional flexibilities at submillisecond time scales still exist for some residues in the loops. Residues Ala$^{39}$–Ile$^{62}$ in the 50s loop were assigned but could not be analyzed in the apo-form due to the poor data quality, an indication of conformational exchanges. These residues could be analyzed in the holo-form and were shown also involved in conformational exchanges on the microsecond to millisecond time scales. This fact suggests that the conformational exchanges still exist in this region even
after the FMN binding. Interestingly, residues Ala$^{46}$–Ile$^{49}$ located in the central β-strand show internal motions on the picosecond to nanosecond time scales in both the apo- and holo-forms. Since this strand directly connects to the 50s loop, the internal motions may be coupled to the conformational exchanges occurring in the 50s loop. In addition, residues in the

FIGURE 6. Ribbon diagrams representing the dynamic properties of E. coli MioC. A and B, ribbon diagrams of the apo- (A) and holo- (B) forms of E. coli MioC representing the generalized order parameter $S^2$ values with colors ranging from yellow to red and blue corresponding to $S^2$ values from 0.4 to 0.85, and $>0.85$. C and D, ribbon diagrams of the apo- (C) and holo- (D) forms of E. coli MioC representing the internal motions on the picosecond to nanosecond time scales with colors ranging from yellow to red and magenta corresponding to $\tau_e$ values from 10 to 100 ps and $>100$ ps. E and F, ribbon diagrams of the apo-form (E) and holo-form (F) of E. coli MioC representing the residues with conformational changes ($R_{ex} > 1$ s$^{-1}$) on the microsecond to millisecond time scales colored in blue. The missing residues caused by the conformational exchanges are colored in black. The figures were generated using MOLMOL (54).
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Figure 7. Differences of the generalized order parameter $S^2$ between the apo- and holo-forms of E. coli MioC. The difference was determined using $\Delta S^2 = S^2_{\text{holo}} - S^2_{\text{apo}}$ for the same residue in both the apo- and holo-forms of MioC and is shown by the filled bar. The open bars show the residues that were missing or unanalyzed for the apo-forms but were analyzed for the holo-form of MioC.

120s loop, which is near the binding site but are not bound with FMN, also show conformational flexibilities. Residue Asp$^{129}$ shows fast internal motion on subnanosecond time scales, whereas residue Ile$^{125}$ shows conformational exchanges on the microsecond to millisecond time scales. Notably, the backbone signals of many nonproline residues in this loop (Leu$^{126}$, His$^{127}$, Glu$^{132}$, Asp$^{133}$, and Ala$^{135}$) were still missing in the holo-form, strongly suggesting intermediate conformational exchanges. Since the FMN-binding site is also involved in the redox partner interaction and subsequently the electron transfer (74, 84), the motional flexibility observed in this region of the holo-form of MioC may be required for redox partner recognition.

**Functional Implications**—Previous studies have established certain structural determinants for the modulation of the FMN redox potentials (72, 73). It was argued that the negative charges near the flavin ring destabilize the anionic hydroquinone and thus result in a lower E1 (semiquinone/hydroquinone) potential (60, 72, 73). In addition, it was proposed for the E. coli FldA that the hydrophobic walls formed by the aromatic rings may also lower the E1 value (60). Since MioC has much less negative charge and fewer aromatic residues around the cofactor-binding site, it may have a higher E1 potential than FldA. However, further investigations are required to clarify this issue.

Our dynamics results showed that the conformational exchanges on the microsecond to millisecond time scales are maintained for some residues in the 50s loop of the holo-form. Since flavodoxins act as electron donors in many biological processes, the protein conformation is expected to change during the oxidation and reduction of the FMN molecule. It was demonstrated by earlier studies that residues 58–60 have to experience a backbone flip upon the reduction of FMN from the fully oxidized state to the semiquinone state (85, 86), and the corresponding redox potential (E2) was shown coupled to local conformational flexibility (87, 88). The conformational exchanges on microsecond to millisecond time scales observed for the holo-form of MioC might be important for the protein to sufficiently fine tune to different redox states of the bound molecule. Moreover, it is highly possible that different dynamic properties of the FMN binding loops may be another fundamental factor affecting the redox potentials besides the structural and electrostatic properties.

In the FMN-bound form, the flavodoxins are able to shuttle electrons from the FAD-containing flavodoxin reductase to diverse downstream targets (15). Several studies were also carried out to investigate the interaction surface of flavodoxins with their redox partners (74, 84). It was shown that flavodoxins interact with both the upstream and downstream redox partners using a similar interface, which primarily consists of the FMN-binding loops, and ternary complex formation is excluded (74). Large conformational changes are expected as the flavodoxin dissociates from its upstream redox partner and subsequently interacts with the downstream partner (74). The conformational flexibility on the slow time scales observed for the 50s loop may also be required for the redox partner switching process.

In addition, it has been suggested that residues in the additional loop in the long-chain flavodoxins (25, 26) and the acidic residues located in the loop connecting the fifth β-strand and the following helix of rat CPR (17, 89, 90) are not directly involved in FMN binding but may also play a role in redox partner interactions. It is interesting to notice that the backbone signals of residues in the 120s loop are still largely missing in the two-dimensional HSQC spectrum of the holo-form, indicative of the intermediate conformational exchanges. Furthermore, the PEDPAE motif of this loop is present only in the MioC protein subfamily, and the proline and negatively charged residues are highly conserved among different bacterial species. The presence of two proline residues shortens the last helix and makes the 120s loop of MioC significantly longer and more mobile than other flavodoxins previously reported (60–65). Although it does not bind to FMN directly, we propose that this loop may represent a unique feature of the MioC protein subfamily and thus may play a specific role in redox partner recognition.

**Evolutionary Implications**—Our current results show that the three-dimensional structure of MioC is similar to that of the FMN-binding domains of multidomain protein CPR and shares similar characteristics around the FMN-binding site. The E. coli genome contains at least four genes predicted to encode flavodoxins: fldA, fldB, mioC, and yqcA. The first two genes, fldA and fldB, encode proteins belonging to the long-chain flavodoxin group, whereas MioC and YqcA proteins belong to the short-chain group. Interestingly, flavodoxin-like domains found in multidomain proteins from mammals are more homologous to the short-chain group in amino acid sequences. When searching for protein sequences from human genome by BLAST (91), the MioC protein sequence shows the highest similarity with the FMN-binding domain from CPR (sequence identity of 31% and similarity of 46% with 5% gaps), whereas YqcA shows the highest similarity with the FMN-binding domain of methionine synthase reductase (sequence identity of 34% and similarity of 48% with 3% gaps). In contrast, FldA shows 27% sequence identity (42% sequence similarity with 25% gaps) with CPR and 25% sequence identity (39% sequence similarity with 19% gaps) with methionine synthase reductase, whereas FldB shows no sequence identity with protein domains from human or other eukaryotic organisms. Although FldA is an essential protein for E. coli growth and it has long been accepted that FldA acts as the electron donor to various
enzymes like methionine synthase in *E. coli*, few investigations have been performed, and there is a lack of insight into the roles of other flavodoxins, especially the short chain flavodoxins MioC and YqCA.

It has been proposed that the long-chain flavodoxins may precede short-chain flavodoxins during evolution (25, 26). These short chain flavodoxins may take the place of FldA in specific biological pathways or under special conditions. Future detailed investigations on the short-chain flavodoxins will shed light on the evolution and functions of the flavodoxin family.

**Conclusion**—We have determined the solution structures of an *E. coli* short-chain flavodoxin MioC in both the apo- and holo-forms at high resolutions by NMR spectroscopy. Our results show the extensive secondary structure formation and overall stabilization upon FMN binding. The backbone dynamics of apo- and holo-forms of MioC provide detailed descriptions of the backbone conformational mobility of flavodoxins for the first time. Moreover, our results suggest that conformational flexibility is important for both cofactor binding and redox partner interactions. Our current studies provide further insights into the molecular mechanisms of the FMN binding and electron transfer of flavodoxins.

**Acknowledgments**—All NMR experiments were carried out at the Beijing Nuclear Magnetic Resonance Center, Peking University. We thank Weibin Gong for kind help in this project.

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