Biochemical Characterization of WrbA, Founding Member of a New Family of Multimeric Flavodoxin-like Proteins*

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The protein WrbA had been identified as an *Escherichia coli* stationary-phase protein that copurified and coimmunoprecipitated with the tryptophan repressor. Sequences homologous to WrbA have been reported in several species of yeast and plants. We previously showed that this new family of proteins displays low but structurally significant sequence similarity with flavodoxins and that its members are predicted to share the α/β core of the flavodoxin fold but with a short conserved insertion unique to the new family, which could account for reports that some family members may be dimeric in solution. The general sequence similarity to flavodoxins suggests that the members of the new family might bind FMN, but their wide evolutionary distribution indicates that, unlike the flavodoxins, these proteins may be ubiquitous. In this paper, we report the purification and biochemical characterization of WrbA, demonstrating that the protein binds FMN specifically and is a multimer in solution. The FMN binding constant is weaker than for many flavodoxins, being ~2 μM at 25 °C in 0.1 mM sodium phosphate, pH 7.2. The protein participates in a dimer-tetramer equilibrium over a wide range of solution conditions, with a midpoint at approximately 1.4 μM. One FMN binds per monomer and has no apparent effect on the multimerization equilibrium. WrbA has no effect on the affinity or mode of DNA binding by the tryptophan repressor; thus, its physiological role remains unclear. Although many proteins with flavodoxin-like domains are known to be multimers, WrbA is apparently the first characterized case in which multimerization is associated directly with the flavodoxin-like domain itself.

WrbA was first identified by Somerville and co-workers (1) as a protein that copurified and coimmunoprecipitated with the tryptophan (Trp) repressor, TrpR. By band-shift assay, these workers concluded that WrbA influences the binding of TrpR to its operator sequence and that WrbA alone does not bind to DNA. On this evidence, the protein was named tryptophan (W)-repressor binding protein A. Sequence analysis and homology-based structural modeling (2) suggested that WrbA was very likely to share the α/β twisted open-sheet fold characteristic of flavodoxins. Comparisons with flavodoxins and with the protein sequence data base (3) identified WrbA as the founding member of a new class of flavodoxin-like proteins containing a well conserved insertion of 24 residues following predicted strand β4. Two other *Escherichia coli* proteins, MioC and the hypothetical protein YibB, are members of the new family but are more closely related to flavodoxins than to other family members, and both lack an insertion in this region. This insertion is itself predicted to form an additional α/β segment and is a strong candidate to be the structural element responsible for experimental reports that WrbA (Ref. 1 and this work) and its homolog in yeast, Ycp4 (4), are dimeric in solution. The modeling results also suggested that the protein presents an active site cove that could be suitable for flavin binding but with some notable differences from typical flavodoxins, making FMN binding a weak prediction and implying that experimental characterization of ligand binding by WrbA might reveal interesting features. These intriguing leads, coupled with the possibility that this protein might influence a thoroughly studied metabolic regulon, motivated the present biochemical characterization of WrbA.

MATERIALS AND METHODS

Strains—*E. coli* JM101 (Δlac pro supE thi′ F′traD36 proAB+ lacZΔM15) was obtained from Amersham Pharmacia Biotech. The expression strain *E. coli* CY15071(A DE3) (ΔtrpRthr tnaA2 lacI+ ΔDE3) was constructed (5) by lysogenizing CY15071 (6) with bacteriophage λ (DE3) obtained from Novagen, Inc., according to the manufacturer’s protocol.

Cloning—Genomic DNA was prepared from JM101 with the QiAamp tissue kit (Qiagen), following the manufacturer’s protocol for bacterial growth. 1 μg of DNA was used as template for 25 cycles of polymerase chain reaction amplification. The sequences of the oligonucleotides used as primers were as follows: 5′-CACACATATGGCTAAAGTTCTGGT 3′ (upstream of WrbA gene) and 5′-CACAGATCCTGAACGTATAGGTTGAAC 3′ (downstream of WrbA gene). Start and stop codons are indicated in bold. Added restriction sites for cloning are underlined (NdeI upstream, BamHI downstream). The 596-base pair NdeI-BamHI fragment resulting from polymerase chain reaction amplification was cloned into the corresponding sites of pET3a (New England Biolabs), resulting in plasmid pKGa. The DNA sequence was determined with the chain-terminator method (7). One difference was found from the published sequence (1): bases 711 and 712 of GenEMBL M99166 are given as CG, whereas we read GC. This change also implies a change from Ala to Gly at residue 141 of WrbA protein. Such an alteration is not likely to be the result of polymerase chain reaction amplification, and other members of the family have Gly at that position in the sequence, so it seems likely that the sequence in the data base should reflect this correction.

Protein Analysis—For preparation of total protein extracts, cells were collected, washed with water, lysed by resuspension in the electrophoresis loading buffer (8), boiled for 5 min, and microfuged at 14,000 rpm. The soluble material was used for electrophoresis. Discon-
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Expression and Purification of WrbA—The WrbA gene was amplified from *E. coli* genomic DNA on the basis of the published sequence (1) using the polymerase chain reaction and cloned in plasmid pET3a for expression under the control of *T7* RNA polymerase (16) in host strain CY15071(lDE3) (5), which is deleted in its chromosomal copy of the gene for TrpR (6) to avoid TrpR contamination in protein purification. High level expression of a polypeptide with the expected relative molecular mass of ~21,000 Da was observed after overnight growth in rich medium (Fig. 1A, lane 2). As already reported by Somerville's group (1), we also see that addition of 1 mM isopropyl-thiogalactoside does not increase expression levels, presumably because of the leakiness of the T7 expression system. Identity of the overproduced protein was confirmed by 10 cycles of N-terminal sequencing after blotting of a sample like that in lane 2 of Fig. 1A. As already reported (1), the mature protein starts with Ala following the initial Met.

The purification strategy was based on published methods for flavodoxins (17) in an attempt to retain the predicted flavin cofactor. The dark gray color of the harvested cell paste is typical of cells overexpressing flavoproteins (18). A French press extract of the cell paste was loaded onto a DEAE-cellulose column developed with a linear gradient of NaCl. A deep yellow peak eluted at around 300 mM salt. The pool of yellow fractions had an absorption spectrum typical of flavoproteins (Fig. 1B) and was found to be enriched in WrbA by SDS-polyacrylamide gel electrophoresis (Fig. 1A, lane 3). Trial-scale experiments showed that WrbA bound to Affi-Gel Blue, as well as to Matrix Red A, resins, as do several other nucleotide-binding proteins (19), and could be eluted by salt but not by 10 mM FMN or by 10 mM NAD or NADP, but the flavin cofactor was invariably lost upon elution. A preparative Affi-Gel Blue column developed with a linear NaCl gradient yielded WrbA apoprotein at around 300 mM salt (Fig. 1A, lane 4). Following 50% ammonium sulfate precipitation, the supernatant was found to be essentially pure WrbA apoprotein (Fig. 1A, lane 5). Typical final yields were ~10 mg per liter of cell culture with final purity of at least 96%. The extinction coefficient of the pure apoprotein at 280 nm was determined by the method of Gill and von Hippel (20) to be 22,831 M⁻¹ cm⁻¹.

Identity of Flavin Cofactor—Enriched preparations of WrbA after DEAE-column chromatography were used as the starting material for analysis of flavin content. A sample of the DEAE pool was extracted with 50% methanol and analyzed by reversed-phase column chromatography. The methanol-extract-
able material eluted as a single peak (Fig. 1C, right) with the same retention time as an FMN standard, which was clearly resolved from riboflavin and FAD (Fig. 1C, left). The absorbance spectrum of the methanol extract was indistinguishable from that of the FMN standard (not shown). Estimation of extracted FMN and WrbA protein concentrations in the pool, calculated using their respective extinction coefficients, indicated approximately 1 FMN per 10 monomers.

Reconstitution of WrbA Holoprotein—Pure WrbA apoprotein and an excess of commercial FMN were incubated together and resolved by size-exclusion column chromatography (Fig. 1D). FMN was identified in the elution profile by its absorbance at 450 nm, where WrbA apoprotein has no absorbance, and was found in two peaks, one with the same retention time as WrbA apoprotein and the other with the retention time of free FMN. At similar concentrations, neither FAD nor riboflavin comigrated with WrbA, nor was the retention time of FMN affected by prior incubation with BSA (data not shown). Thus, WrbA binds FMN specifically and reversibly. Equilibration times as short as 5 min yielded equivalent results, indicating a relatively rapid association reaction. Although the facile loss of the cofactor during purification suggests a rapid dissociation rate,

Fig. 1. Purification of WrbA protein and identification of its cofactor. A, purification of WrbA protein, analyzed by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. Lane 1, molecular weight markers; top to bottom: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa. Lane 2, crude extract of E. coli CY15071(DE3) cells overexpressing WrbA from clone pKGWa (sample loaded onto DEAE column). Lane 3, pool of yellow fractions eluting at ~300 mM NaCl from DEAE column. Lane 4, pool of fractions eluting at ~300 mM NaCl from Affi-Gel Blue column. Lane 5, supernatant from 50% ammonium sulfate precipitation of Affi-Gel Blue column pool. Approximately 2.5 μg of total protein was loaded in lane 5, and the detection limit in the gel is ~0.1 μg; thus, the protein is estimated to be at least 96% pure. B, UV-visible absorption spectrum of pooled DEAE-column fractions (equivalent to the sample in lane 3 in panel A). OD, optical density. C, cofactor identity, analyzed by reversed-phase column chromatography. Elution profiles detected by absorbance at 450 nm are shown. Left, commercial standards FAD, FMN, and riboflavin (10 μM each) from left to right, respectively. Right, methanol-extractable material from the DEAE-column pool. The point of injection is marked by the vertical line at the far left of each panel. One horizontal scale mark corresponds to an absorbance of 0.001; one vertical scale mark corresponds to 2.0 μl. The sensitivity settings were dictated by that required for the amount of material in the methanol extract. D, resolution of reconstituted WrbA holoprotein and free FMN by size-exclusion chromatography. The elution profiles detected by absorbance at 280 (upper line) and 450 nm (lower line) are shown for a mixture of 36 μM WrbA apoprotein with 50 μM FMN. The two wavelengths are detected simultaneously but are offset slightly because of the configuration of the recorder pens, as indicated by the point of injection marked by the vertical line at the far left. One horizontal scale mark corresponds to an absorbance of 0.002; one vertical scale mark corresponds to 2.0 μl.
the very minor tailing in the elution profile of the reconstituted holoprotein indicates that the dissociation rate of FMN is slow on the time scale of this column. The amount of bound FMN changed with FMN or protein concentration and reached a plateau at high FMN concentrations, indicating saturation of available binding sites. Quantitation of free and bound species using the peak areas (data not shown) indicated an average stoichiometry of 0.97 FMN per WrbA monomer at saturation (S.D. 0.08; n = 6).

Multimeric State—The elution profiles of both apo- and holoprotein WrbA on the Superdex 75 size-exclusion column used for resolving the reconstituted holoprotein by fast protein liquid chromatography were little changed over a wide range of conditions (flow rate 0.1 or 1.0 ml/min), temperature (−4° or −22 °C), and addition of 1 M NaCl, 0.1% Triton, or various concentrations of FMN), although the elution time decreased slightly as protein concentration increased over the range 0.1 to 10 μM, suggesting a rapid multimeric equilibrium. The protein elutes too close to the molecular weight cutoff of approximately 70 kDa on this column for accurate determination of its apparent molecular weight. Analysis on a TSK-G3000SWxl high pressure liquid chromatography size-exclusion column (molecular mass cutoff ~150,000 Da) gave an apparent molecular mass of ~62,000 Da at an initial WrbA concentration of 8 μM (data not shown), confirming the multimeric state of the protein on a second resin type. The protein-concentration dependence of the gel filtration results is indicative of a system involved in rapid subunit equilibria.

The quaternary structure of WrbA was therefore further examined by analytical ultracentrifugation at several concentrations (Fig. 2A and Table I), which confirmed the presence of a multimeric equilibrium. Simultaneous fitting of the sedimentation equilibrium data collected at different centrifugation speeds was used to derive an average apparent molecular mass (Mav) for each sample, assuming the presence of only a single species. At loading concentrations of 5–10 μM WrbA (monomer), this analysis resulted in an average apparent molecular mass of approximately 74 kDa, implying an association of 3.6 monomers. This result rules out the monomer as the dominant species of WrbA at micromolar protein concentrations and provides support for the existence of a dynamic equilibrium involving at least one higher order species in this range of initial protein concentrations.

To evaluate different possible scenarios for multimerization of WrbA, the square root of variance (Table I) and randomness of residuals, two measures of the goodness of fit, were compared for fitting of the data to various models of subunit equilibrium. Because of literature reports that members of the WrbA family may be dimeric in solution (1, 4), we included both monomer–n-mer and dimer–n-mer models in our analysis. Several models could be excluded because of failure to converge on a solution (monomer–n-mer, where n < 4) or because of poor fit, nonrandomness of residuals, and/or increased square root of variance (all single species models, and all monomer–n-mer or dimer–n-mer models where n > 4). Two models, monomer-tetramer and dimer-tetramer, gave nearly equally good fits as judged by square root of variance and the randomness of residuals. The sedimentation equilibrium data alone cannot distinguish between these two models, as the fits are essentially equivalent. As justified by the experiments reported in the following paragraphs, the fit shown in Fig. 2A is that for the dimer-tetramer model.

The apparent dissociation constants describing these two models are 2.6 × 10−17 M3 for the monomer-tetramer model and 1.4 × 10−6 M for the dimer-tetramer model. To directly compare these two values, we used the relation (Mα−1/2) = 2(1/4 Kd)1/2 introduced by Bujalowski and Lohman (21) to calculate (Mα−1/2), the concentration of total protein (as monomer) at the transition midpoint for the monomer-tetramer model. The value of (Mα−1/2) in this case 3.7 × 10−14 m, can be directly compared with the Kd for the dimer-tetramer model. Thus by either model, the midpoint of the oligomerization transition occurs in the micromolar concentration range.

To determine which of the two models correctly describes the WrbA system, each dissociation constant for tetramer formation was used to calculate the predicted species distributions for monomer, dimer, and tetramer as a function of protein concentration according to each model. The monomer-tetramer model predicts essentially only monomer at protein concentra-

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**Fig. 2. Multimeric state of WrbA apoprotein.** A, equilibrium sedimentation ultracentrifugation. Bottom, radial distribution of WrbA. The solid lines show a global fit of the three data sets to a dimer-tetramer model with Kd = 1.4 μM. Top, residuals for fit of data at each respective speed to dimer-tetramer model. B, Far-UV CD spectrum of WrbA apoprotein in buffer P. Mean residue ellipticity (y axis) is shown for 5.76 μM protein (filled squares) and for 0.36 μM protein (open squares). The spectra were normalized to zero at 250 nm and corrected for the buffer blank. In the 10-cm cell used for the lower concentration sample, absorption interference from the buffer increases the scatter in the data to unacceptable levels at wavelengths lower than about 250 nm.
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**TABLE I**

Analysis of sedimentation equilibrium data

Data were collected at 4 °C with detection at 238 nm for 5 μM, 280 nm for 9 μM, and 285 nm for 10 μM samples and analyzed using the HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut. Analysis was carried out using global fits to data acquired at 6,000, 10,000, and 15,000 rpm for 5 μM protein, at 10,000, 15,000, and 20,000 rpm for 9 μM protein, and at 8,000, 12,000, and 15,000 rpm for 10 μM protein.

| [WrbA] μM | M, m | SS, single species | SRV, square root of variance (×10^4) | lnK_d, natural log of the association constant; average values: 1–4, 38.2; 2–4, 13.5. | 1nK_d, single species |
|----------|-----|------------------|-----------------|--------------------------|-----------------|
| 5.0      | 74,144 ± 3,300 | 4.38 | SS | 3.15 | 4.45 | 4.35 | 4.44 | 39.9 ± 0.8 | 14.0 ± 0.4 |
| 9.0      | 73,288 ± 1,900 | 2.93 | SS | 2.48 | 2.59 | 2.51 | 2.58 |
| 10.0     | 74,360 ± 3,500 | 2.58 | SS | 2.48 | 2.48 | 2.48 | 2.48 |

* SRV, square root of variance (×10^4).
* lnK_d, natural log of the association constant; average values: 1–4, 38.2; 2–4, 13.5.
* Concentration of protein loaded.
* M, the apparent molecular mass in Daltons, determined from single-species analysis.
* SS, single species.
* dimer–n-mer or monomer–n-mer equilibria of increasing order.
* NS, no solution.

The predicted FMN binding ability of WrbA was first verified during purification of the protein from an E. coli strain engineered to overexpress the protein under the control of bacteriophage T7 RNA polymerase. The FMN cofactor was readily lost during purification, suggesting significantly weaker binding than that found for typical flavodoxins (18, 25). Ratios of about 0.1 FMN per WrbA monomer were observed in the first steps of purification, indicating that FMN biosynthesis may be induced
upon overexpression of WrbA and therefore suggesting that FMN is likely to be the physiological cofactor. FMN could be rebound to the apoprotein in 1:1 stoichiometry at high concentrations of protein and FMN. Weak FMN binding with 1:1 stoichiometry was confirmed by fluorescence titration, which yielded a dissociation constant of approximately 2 \( \mu \text{M} \) at room temperature in 10 mM sodium phosphate, pH 7.2. FMN was bound specifically in preference to FAD or riboflavin, strengthening the suggestion that, despite its relatively weak affinity, FMN is the likely physiological cofactor for WrbA. Thus, the prediction of weak but specific FMN binding, based on a structural homology model of WrbA (2), is experimentally confirmed.

The combined results of equilibrium analytical ultracentrifugation and size-exclusion chromatography indicate that the pure apoprotein participates in a rapid dimer-tetramer equilibrium over a wide range of solution conditions, with a mid-point at 1.4 \( \mu \text{M} \) dimer at 4 °C in 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM MgCl\(_2\). Although the apparent dissociation constant for the dimer-tetramer system is similar to the \( K_d \) for FMN binding, the ligand-binding and multimerization equilibria are evidently not strongly coupled, as FMN binding has no effect on the apparent molecular weight of the protein according to the results of size-exclusion chromatography.

Experiments are under way to determine the redox potentials of FMN bound to WrbA. However, the weak binding of the flavin cofactor and the dynamic equilibrium between dimers and tetrarimers have complicated measurements of the redox potential. These unique features, which distinguish WrbA from typical flavodoxins, may confer unusual redox potentials as well. When the values of WrbA redox potentials are established, they may limit the range of potential redox partners of WrbA and thereby help in understanding its physiological role.

Gel mobility shift assays demonstrate that WrbA has no specific effect on the affinity of binding of TrpR to its operator DNA target. Because the operator used for these experiments can bind as many as three TrpR dimers sequentially in single and tandem binding modes (23), we can also conclude from the mobility shift results that the presence of WrbA has no effect on the binding mode. Although many connections are possible between WrbA and tryptophan metabolism, none stands out as obvious, and neither we nor Somerville's group detected any specific effects in growth experiments on various strains of \( E. \) \textit{coli} with or without a functional TrpR protein (Ref. 1 and data not shown). As well, extensive footprinting analysis \textit{in vivo} at various TrpR concentrations using high resolution chemical probes detected no evidence of proteins other than TrpR and RNA polymerase in the regulatory regions of several natural \textit{trp} operators (23). Although the results do not rule out a physiological connection between TrpR and WrbA, it seems possible that the association observed by Somerville and co-workers may have a structural, rather than functional, basis.

The demonstration that WrbA forms multimers has interesting structural and evolutionary implications. Indeed, the structural model (2, 3) suggests strongly that WrbA does not display the typical modular organization of other multimeric flavodoxin-like proteins characterized to date, in which the monomeric flavodoxin-like domain is fused to a multimerization domain (Ref. 26 and references therein). WrbA instead apparently developed its multimerization function by sequence divergence within the fold of the flavodoxin-like domain. According to our alignment (2, 3), two major regions are highly conserved within the WrbA family and are therefore good can-

**FIG. 3.** FMN binding by WrbA, analyzed by fluorescence titration. A constant concentration of FMN was titrated with WrbA and monitored by the quenching of FMN fluorescence. Fluorescence intensity (arbitrary units; y axis) is plotted as a function of WrbA concentration (log units; x axis). Solid curves are calculated for 1:1 binding stoichiometry and dashed curves for stoichiometry of one FMN per WrbA dimer (1:2 stoichiometry), with best-fit apparent binding constant and dashed curves given. Open symbols at left of each panel are experimental values obtained at zero WrbA concentration, for which a log value is undefined, arbitrarily shown at the lowest protein concentration displayed on the log scale. Solid squares indicate data collected using 1 \( \mu \text{M} \) FMN; solid line indicates \( K_d \) 1.98 ± 0.25 \( \mu \text{M} \); dashed line indicates \( K_d \) 0.67 ± 0.12 \( \mu \text{M} \) (dimer). Both fits used a final baseline value of 340 ± 60.

**FIG. 4.** Effect of WrbA on TrpR-DNA binding affinity, analyzed by gel shift assay. The panels show identical titrations with TrpR holoprotein alone (A) or in the presence of additional proteins: WrbA apoprotein at 0.1 mg/ml (5 \( \mu \text{M} \) monomer) in B, WrbA holoprotein (5 \( \mu \text{M} \) each of apoprotein monomer and FMN) in C, and BSA at 0.1 mg/ml in D. In each panel, lane 1 contains no added TrpR, and lanes 2–7 contain TrpR at final concentrations of 0.03, 0.075, 0.15, 0.3, 0.75, and 1.5 \( \mu \text{M} \), respectively, whereas lane 0 (panel C only) contains no added TrpR and no other added protein. Sample and running conditions are described under “Materials and Methods.”
didates to be multimerization elements: the 24-residue insertion and the C-terminal region. Investigation of the WrbA structure might therefore reveal a minimalistic structural unit for the multimerization of this family of proteins. An analogous case might be presented by NADPH-sulfite reductase of *E. coli*. Its isolated flavodoxin-like domain has been reported to form multimers and to bind FMN independently of the larger, FAD binding domain (27). The sequence of the flavodoxin domain aligns without insertions on the sequences of monomeric flavodoxins (26). However, quantitative characterization of the multimerization and FMN binding properties of the isolated domain have not been reported as yet, so its similarity to WrbA cannot be evaluated at present. Both the biochemical properties reported here and the previously reported sequence analysis set WrbA apart from typical flavodoxin-like proteins, and thus imply that further studies on its physiological role may be of significant interest.

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