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Flavodoxin cofactor binding induces structural changes that are required for protein–protein interactions with $NADP^+$ oxidoreductase and pyruvate formate-lyase activating enzyme ☆

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ABSTRACT

Flavodoxin (Fld) conformational changes, thermal stability, and cofactor binding were studied using circular dichroism (CD), isothermal titration calorimetry (ITC), and limited proteolysis. Thermodynamics of apo and holo-Fld folding were examined to discern the features of this important electron transfer protein and to provide data on apo-Fld. With the exception of fluorescence and UV–vis binding experiments with its cofactor flavin mononucleotide (FMN), apo-Fld is almost completely uncharacterized in *Escherichia coli*. Fld is more structured when the FMN cofactor is bound; the association is tight and driven by enthalpy of binding. Surface plasmon resonance binding experiments were carried out under anaerobic conditions for both apo- and holo-Fld and demonstrate the importance of structure and conformation for the interaction with binding partners. Holo-Fld is capable of associating with $NADP^+$-dependent flavodoxin oxidoreductase (FNR) and pyruvate formate-lyase activating enzyme (PFL-AE) whereas there is no detectable interaction between apo-Fld and either protein. Limited proteolysis experiments were analyzed by LC-MS to identify the regions in Fld that are involved in conformation changes upon cofactor binding. Docking software was used to model the Fld/PFL-AE complex to understand the interactions between these two proteins and gain insight into electron transfer reactions from Fld to PFL-AE.

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1. Introduction

Flavodoxins are small acidic proteins that utilize a flavin mononucleotide cofactor (FMN) for electron transfer reactions. Flavodoxins are widespread in bacteria and are present in some red and green algae [1–3]. In higher eukaryotes, proteins with sequence homology to flavodoxin and its reductase are fused to multidomain proteins as in methionine synthase reductase, which reductively activates methionine synthase [4–6]. Crystal structures are available for holo-Fld from *Escherichia coli*, however there is no structure for apo-Fld [7]. Fld binds the cofactor FMN with an equilibrium constant of 1 nM and a 1:1 stoichiometry, using multiple hydrogen bonds, salt bridges, and stacking interactions between the isoalloxazine ring and aromatic residues [7]. These interactions are extremely important for determining the reduction potentials of the FMN cofactor once bound to the protein [8,9]. Flavodoxins are ideal electron donors for a number of biological redox reactions, including those catalyzed by the radical *S*-adenosylmethionine (SAM) superfamily enzymes due to their low reduction potentials. Several studies have shown that flavodoxin (Fld) is capable of activating pyruvate formate-lyase activating enzyme and anaerobic ribonucleotide reductase activating enzyme (PFL-AE and RNR-AE) as well as biotin synthase (BioB), presumably by reducing the [4Fe–4S] cluster that binds and reductively cleaves SAM [10–12]. The details of these interactions, however, are not well understood.

In addition to interacting with PFL-AE, RNR-AE, and BioB, *E. coli* Fld serves as an electron transfer partner with $NADP^+$-dependent flavodoxin reductase (FNR), methionine synthase, ferredoxin, pyruvate dependent flavodoxin oxidoreductase, and probably other unknown proteins, and thus plays integral roles in metabolism [10]. Residues involved in the binding site interface of Fld with SAM binding domain of methionine synthase and FNR have been mapped onto the crystal structure of *E. coli* Fld, showing several residues in common, all located near the FMN binding site [13]. These results suggest that both methionine synthase and FNR bind the same face of Fld and thus presumably bind sequentially [13]. Further support for sequential binding to a single face of Fld comes from the observation of competitive binding between FNR and methionine synthase in spectrophotometric assays [14].

Pyruvate formate-lyase activating enzyme (PFL-AE) is a 28 kDa monomer that contains a [4Fe–4S] cluster and utilizes *S*-adenosylmethionine (SAM) to activate pyruvate formate-lyase (PFL), a central enzyme in anaerobic glycolysis [15]. The SAM cofactor coordinates the unique iron of the [4Fe–4S] cluster via amino and carboxylate moieties [16,17]. In the reduced $[4Fe–4S]^+$ state, the cluster donates an electron to SAM, thus promoting homolytic cleavage of the S – C(5′)

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bond to generate methionine and a 5′-deoxyadenosyl radical; this adenosyl radical abstracts a hydrogen atom from G734 of PFL to generate the active enzyme [15,18,19]. Knappe and coworkers have shown that Fld can be used in the activation of PFL, presumably as an electron donor for PFL-AE [20]. When the crystal structure of PFL-AE was solved, a conserved region opposite to the PFL binding site was proposed to act as the Fld binding site on PFL-AE [21].

In this work we provide insights into the apo- and holo-forms of *E. coli* Fld, and their interactions with protein partners, PFL-AE and FNR. Fld unfolding studies were performed with apo- and holo-Fld to probe differences in protein stability and folding thermodynamics as a result of cofactor binding. Limited proteolysis experiments analyzed using LC-MS were used to determine the regions of Fld that were involved in conformational changes. We utilize surface plasmon resonance under anaerobic conditions to examine the interactions between the air-sensitive PFL-AE and Fld and FNR. Findings in the present study indicate that Fld cofactor binding increases protein stability and results in conformational changes that are required for protein–protein interactions.

2. Materials and methods

2.1. Cloning, expression, and purification of Fld and FNR

PFU-Turbo DNA polymerase and restriction enzymes NcoI and BamHI were purchased from New England Biolabs. FMN was purchased from MP Biomedicals, LLC, and was used without further purification. Fld (*fldA*) and FNR (*fpr*) were separately cloned using PCR to amplify the *fldA* and *fpr* genes from *E. coli* strain B genomic DNA. The PCR products were separately inserted into pET-14b using BamHI and NcoI restriction sites that were incorporated into the ends of the PCR products. The *fldA* and *fpr* genes were completely sequenced at Nevada Genomics Center. The purified DNA was used to transform the BL21(DE3)pLysS expression cell line. For protein expression, cells were grown in LB media at 37 °C in Fernbach flasks with agitation at 250 rpm. When cultures reached an O.D. of 0.8, protein production was induced by addition of IPTG to 100 μM final concentration. Cells were harvested 3 h later by centrifugation at 6000 rpm for 10 min.

Cells were lysed by chemical lysis (~23 g of cells in 50 mL of lysis buffer: 20 mM HEPES, 1% w/v Triton X-100, 5% w/v glycerol, 10 mM $MgCl_2$ pH 7.2 with 9 mg PMSF dissolved in 100 μL methanol, 8 mg lysozyme, 1 mg of DNAse and RNAse; cells were lysed for 1 h followed by centrifugation at 18,000 rpm for 30 min) and Fld and FNR were purified using ammonium sulfate precipitation. Fld and FNR precipitated out in the 60–100% fraction and were subsequently purified using a Superdex-75 size exclusion column (Fig. S1). Fractions of the highest purity with the most intense color were pooled and concentrated and then flash frozen and stored at −80 °C (Fig. S2). Holo-Fld was quantified using a previously determined extinction coefficient of $\varepsilon_{467\ nm} = 8250\ M^{-1}\ cm^{-1}$ [22].

Apo-Fld was prepared using trichloroacetic acid precipitation as described previously [23]. This technique has been used in several previous studies to prepare apo-Fld [7,23–26]. Apo-Fld was quantified using $\varepsilon_{280nm} = 30{,}900\ M^{-1}\ cm^{-1}$, which was calculated using the protparam tool in ExPASy (http://www.expasy.org). The concentration of the FMN cofactor was determined using $\varepsilon_{445nm} = 12{,}500\ M^{-1}\ cm^{-1}$ [27]. To ensure that the apo-Fld was capable of cofactor binding, a single titration of concentrated apo-Fld was added to a UV–vis cuvette containing FMN to show the 20 nm shift in λ_{max} from 445 nm to 465 nm, characteristic of holo-Fld formation (Fig. S3) [22]. PFL-AE was grown and purified as described previously [28]. An extinction coefficient of $\varepsilon_{280nm} = 39{,}400\ M^{-1}\ cm^{-1}$ was calculated using ExPASy and correlated well with results from Bradford protein assays [29] after applying a previously-determined correction factor of 0.65 [30].

2.2. CD spectroscopy

Secondary structure and thermal unfolding experiments were carried out using circular dichroism spectroscopy (CD). Spectra were measured with a Jasco-710 spectropolarimeter using either 0.1 mm or 1 cm pathlength cuvettes. Far-UV measurements were in the range of 195–260 nm and near-UV measurements were in the range of 240–800 nm. Sensitivity was 100 millidegrees, data pitch was 0.1 nm, using a continuous scan mode with a speed of 100 nm/min, response was 4 s, bandwidth was 1.0 nm, with an accumulation of 3 scans. The buffer used for secondary structure and thermal unfolding experiments was 20 mM HEPES, 50 mM NaCl, pH 8.0. Protein concentrations of 30 μM were used for far-UV measurements and 30 μM or 75 μM for near-UV and visible region measurements. The Fld unfolding curves were independent of protein concentration under the conditions studied. Temperature was maintained for thermal unfolding experiments using an endocal refrigerated circulating water bath from NESLAB model RTE-5. Temperature was increased or decreased at a rate of 0.5 K/min for unfolding or folding, respectively.

Circular dichroism binding studies were performed on a Jasco-810 spectropolarimeter using a 1 cm cuvette. Scans were taken from 240 to 800 nm using the same parameters as above. The buffer used for CD binding experiments was 20 mM HEPES, 10 mM NaCl, pH 7.4. In these experiments FNR was held constant at 30 μM while Fld was titrated into the FNR. Spectra were also measured for the Fld titration into buffer alone and the contribution of both proteins was subtracted out of the mixture to create difference spectra to visualize changes in visible and near-UV regions as a result of binding. All CD experiments were run in triplicate.

2.3. Analysis of thermal unfolding data

Thermal denaturation of Fld was assessed at pH 8.0 using far-UV (222 nm), near-UV (296 nm), and visible regions (496 nm) of circular dichroism. Thermal unfolding curves were first fit to a two state unfolding model to obtain initial thermodynamic parameters of unfolding (Eqs. (1) and (2)). The spectroscopic signals of the native (S_N) and unfolded (S_U) states with respective slopes m_N and m_U, are presumed to vary linearly with temperature. The change in enthalpy, heat capacity, and thermal melting point of the transition corresponds to ΔH_m, ΔC_p, and T_m, respectively. The ΔC_p for Fld unfolding was not reported since it has been determined to be unreliable [31,32]. Apo-Fld and holo-Fld unfolding curves were subsequently fit to a three state unfolding model using a global fit (Eq. (3)). ΔG for the three state unfolding model follows the expressions analogous to Eq. (2). The signal of the intermediate (S_I) and its associated slope (m_I) at T = 0 K are also presumed to vary linearly in relation to temperature. ΔG_1 and ΔG_2 correspond to the differences in free energy between the native/intermediate and intermediate/unfolded equilibria, respectively. Thermodynamic parameters of unfolding are reported with subscripts NI, IU, and NU corresponding to the transition from native to intermediate, intermediate to unfolded, and native to unfolded Fld; NU is reported as the sum of NI and IU.

2.4. Isothermal titration calorimetry

Experiments were performed on a VP-ITC (Microcal LLC, Northampton, MA) using 25 μM apo-Fld in the cell with 600 μM FMN cofactor in the syringe. Experimental parameters were as follows: 26 injections, reference power of 10 μcal/s, initial delay of 300 s, stir speed of 510 rpm, feedback mode/gain was high and automatic with fast equilibration. Injection duration was 24 s, spacing was 300 s, and filter period was 2 s. All experiments were performed in triplicate at either 25 °C or 37 °C. All binding isotherm data were analyzed using a best fit to a single-site binding model by Marquardt nonlinear least-squares analysis (Origin 5.0) with VP-ITC software.

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