Redox and Spectroscopic Properties of the *Escherichia coli* Nitric Oxide-detoxifying System Involving Flavorubredoxin and Its NADH-oxidizing Redox Partner*

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Under anaerobic conditions, the flavodiiron NO-reductase from *Escherichia coli* (flavorubredoxin, FlRd) constitutes one of the major protective enzymes against nitric oxide. The redox and spectroscopic properties of the rubredoxin (Rd), non-heme diiron, and FMN sites of flavorubredoxin were determined, which was complemented by the study of truncated versions of FlRd: one consisting only of its rubredoxin module, and another consisting of its flavodiiron structural core (lacking the Rd domain). The studies here reported were performed by a combination of potentiometry with visible and EPR spectroscopies. Moreover, we present the first direct EPR evidence for the presence of the non-heme diiron site in the flavodiiron proteins family. Also, the redox properties of the FlRd physiological partner, the NADH:flavorubredoxin oxidoreductase (FlRd-Red), were determined. It is further shown that the redox properties of this complex electron transfer system are fine-tuned upon interaction of the two enzymes.

The relevance of nitric oxide (NO)3 biology grows alongside its complexity. The physiological aspects of NO lie within a balance, between beneficial and deleterious, both in the eukaryotic and prokaryotic worlds. The production and release of NO by macrophages’ inducible NO synthases constitutes one of the major defense mechanisms from eukaryotic immune systems against pathogen invasion and infection. However, pathogenic organisms have developed subversive mechanisms to counteract the hosts’ immune systems. The discovery of an anaerobic nitric oxide detoxification system in *Escherichia coli* (1, 2) appears to complement the role of flavohemoglobin as an NO scavenger both in aerobic (3, 4) and anaerobic conditions (5). This anaerobic NO detoxification system is constituted by flavorubredoxin (FlRd) (2, 6), a terminal complex modular protein of the flavodiiron protein (FDP) family (7, 8), and its reductase partner (FlRd-Red), an NADH oxidase flavoprotein of the rubredoxin reductase family. The widespread family of flavodiiron proteins shares a common structural core, built by an N-terminal β-lactamase module harboring a non-heme diiron site, fused to a FMN-binding flavodoxin module (9, 10).

The first evidence for the presence of the diiron site arose from the determination of the crystallographic structure of the FDP from *Desulfovibrio gigas*, named rubredoxin:oxygen oxidoreductase (ROO) (9). The center is coordinated by three histidines, two aspartates and one glutamate, in a His79-X-Glu81-X-Asp83-X_gd1-His146-X_gd2-Asp165-X_gd3-His206 motif, which is common to almost all FDPs, with some variability in the spacing between the ligands (8). The irons are bridged by the carboxylate group from Asp165 and by a μ-oxo (or μ-hydroxo) species. The distinctive feature of this novel type of diiron center resides in the structural fold where the ferric ions are embedded. Recently, the crystallographic structure of *Moorella thermoacetica* FDP revealed an almost identical coordination sphere, with the exception that the highly conserved His86 (His84 in ROO) is a ligand to the diiron center, whereas in the structure of ROO a water molecule is a ligand in this position (10). This structural difference cannot be immediately assigned with any mechanistic relevance, because NO binding (revealed in the same work) appears to occur in the *trans* position to these ligands (10). The first coordination sphere of the diiron site in the FDPs family places this metal center in the family of the carboxylate/histidine diiron diiron centers, found in several proteins, like ribonucleotide reductases, methane monoxygenase hydroxylase, hemerythrin, (bacterio)ferritins, and ruberythrin (reviewed in Ref. 11), which have in common the activation of oxygen at the diiron center, coupled to various other reactions (12). Because the two iron atoms are antiferromagnetically coupled, these centers are EPR silent in their fully oxidized FeIII–FeIII state, but have distinctive features at g < 2 in their mixed-valence FeIII–FeII state and a low intensity signal at g ~ 11–15, in parallel mode EPR, in the fully reduced FeII–FeII state (11, 13). EPR is thus a powerful and useful tool to identify and characterize this type of metal centers.

The complexity observed in the modular arrangements of flavodiiron proteins, concerning the presence of extra C-terminal modules, led to their division in sub-classes (8). As all the FDPs found in the genomes of enterobacteria (Class B FDPs), *E. coli* flavoperoxidase bears an extra C-terminal rubredoxin module, which is the entry point to accept electrons from the reduced flavoperoxidase reductase (6), for the subsequent intra-molecular electron transfer steps. This constitutes a specially interesting feature, because in *D. gigas*, rubredoxin is the redox partner for ROO, the FDP of this organism (14). Rubredoxins are small redox proteins generally ranging from 45 to 54 amino acid residues in length, bearing as the sole cofactor one iron atom tetrahedrally coordinated to the sulfur atoms of 4 cysteiny1 residues in two separate Cys-X₁–Cys-Gly segments (15). Rubredoxins are found mostly in archaea and bacteria, mainly anaerobes, although recently eukaryotic rubredoxins have been found in *Guillardia theta* (16) and in the genome of the rodent malaria vector *Plasmodium yoelii* (17). Available biochemical and genetic data suggest the involvement of rubredoxins as reduct component of electron transfer chains in processes such as alkane hydroxylation in *Pseudomonas* (18–20); detoxification of reactive oxygen spe-

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3 The abbreviations used are: NO, nitric oxide; eT, electron transfer; FlRd, flavorubredoxin; Rd, rubredoxin; FDP, flavoperoxidase; FlRd-Red, flavodiiron protein; FlRd-Red, NADH:flavorubredoxin oxidoreductase; Rd-Red, rubredoxin reductase; ROO, rubredoxin:oxygen oxidoreductase; Rd-D, rubredoxin domain; FDR-D, flavodiiron domain.

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Redox Tuning in E. coli Flavorubredoxin-type NO Reductase

cies in Desulfovibrio species (14, 21, 22), Archaeoglobus fulgidus (23), and M. thermoacetica (24); iron metabolism in Desulfovibrio sp. (25); and in photosynthetic related processes (16). The redox properties of rubredoxins from several sources have been the subject of many studies, including site-directed mutagenesis on specific residues thought to be relevant for the modulation of the reduction potential of the [Fe-Cys4] center, namely specific conserved residues close to the ligating cysteine residues (26, 27). As in FlRd, rubredoxin motifs are also found as structural modules in other proteins, as is the case of rubreythrin (28) and M. thermoacetica high molecular weight rubredoxin (29). In most physiological processes where rubredoxins are proposed to act as electron transfer mediators, their reduction is accomplished by NAD(P)H-dependent FAD-binding flavoproteins, therefore named NAD(P):rubredoxin oxidoreductases. These are generally 40- to 45-kDa monomers, with specific sequence fingerprints, both for FAD and NAD(P) binding (30, 31). Studies have been reported on rubredoxin reductases (RdRed) from Pseudomonads (19, 31), Clostridia (32), and Pyrococcus furiosus (33, 34). The Pseudomonas oleovorans electron transfer chain involving an RdRed, an Rd, and a terminal alkane hydroxylase has been thoroughly characterized in terms of the RdRed/Rd redox pair electron transfer kinetics (19), thus providing a comparison model toward the system studied in this work. Here we present the biochemical characterization of the truncated rubredoxin module from flavorubredoxin and a complete redox characterization of the flavorubredoxin-flavorubredoxin reductase system as essential steps for a further kinetic characterization. We also present the first direct EPR evidence for the non-heme diiron center for the flavodiiron protein family.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—E. coli flavorubredoxin (FlRd), flavorubredoxin reductase (FlRd-Red), and the truncated forms of FlRd, consisting of the rubredoxin domain (Rd-D) and of the flavodiiron domain (FDP-D), were overexpressed in E. coli and purified as previously described (2, 6), except for the following modifications: all buffers in the purification of FlRd contained 500 mM phenylmethanesulfonyl fluoride and 18% of glycerol, and the same amount of glycerol was added to all buffers in the FlRd-Red and FDP-D purifications. Glycerol was found to increase significantly the stability of the enzymes, namely by avoiding the loss of the flavin moieties.

Cofactor Analysis—The flavin cofactors from FlRd, FDP-D, and FlRd-Red were analyzed and quantified after acid extraction with tri-chloroacetic acid (35). The iron content was determined by the 2,4,6-tripryidyl-1,3,5-triazine method (36).

Spectroscopic Methods—All UV-visible spectra were recorded in Shimadzu (UV-1603 or Multispec-1501 diode array) spectrophotometers. When required, the spectrophotometers were connected to temperature baths and cell-stirring systems. EPR spectra were collected on a Bruker ESP 380 spectrometer equipped with an ESR 900 continuous-flow helium cryostat from Oxford Instruments.

Redox Titrations—Redox titrations followed by UV-visible spectroscopy were performed as in Ref. 6, using the same set of redox mediators, and at the same concentration. For the redox titrations of FlRd and FDP-D followed by EPR spectroscopy, the same mediators were used, although at a concentration of ∼80 μM. A combined platinum Ag/AgCl electrode was used, calibrated at 25 °C with a saturated quinhydrone solution at pH 7.0. All potentials are quoted against the standard hydrogen electrode. The titrations were carried out at 25 °C in 50 mM Tris-HCl, 18% glycerol, pH 7.5. Experimental data analysis was performed using MATLAB (Mathworks, South Natick, MA) for Windows. For the Rd-domain titration analysis a single one-electron Nernst curve was used to fit the experimental data. Two consecutive one-electron steps were considered for the FAD_red ← FAD_sq ← FAD_ox transitions in the FlRd-Red titration analysis, corresponding to the three possible redox states of the flavin cofactor. For each of the three redox states the molar extinction coefficients used at 456 nm were: ε_red = 14,500 M⁻¹·cm⁻¹, ε_red = 4,500 M⁻¹·cm⁻¹, and ε_red = 3,400 M⁻¹·cm⁻¹, and ε_red = 2,700 M⁻¹·cm⁻¹ (37). The same extinction coefficients and analysis strategy were used for the spectral changes of the FMN cofactor in the FlRd-Red titrations. The data obtained by monitoring the non-heme diiron center EPR-active species (the mixed-valence FeIII–FeII) was fitted as the intermediate species of two consecutive one-electron step processes.

RESULTS AND DISCUSSION

The several recombinant intact and truncated proteins were successfully purified. The addition of glycerol enhanced the stability of the flavin moieties, leading to higher FMN and FAD contents of the respective enzymes, namely ∼0.7 mol of FMN/mol of FlRd and ∼1 mol of FAD/mol FlRd-Red. The iron content of the proteins was ∼3 mol of Fe/mol of FlRd, ∼2 mol of Fe/mol of FDP-D, and ∼1 mol of Fe/mol of Rd-D, indicating a complete iron load in each protein.

EPR Spectroscopy on the Iron Centers of Flavorubredoxin—To characterize the rubredoxin module (Rd-D) of flavorubredoxin isolated from the other cofactors (the FMN and the non-heme diiron center), a truncated version of FlRd was obtained, consisting of its C-terminal 79 residues. The EPR spectrum of Rd-D (not shown) is identical to the one obtained for the whole protein in its fully oxidized state, with resonances at g = 9.4, g = 4.8, and g = 4.3 (see Fig. 1A, line b), which were previously assigned to two slightly different conformations of the high-spin (S = 5/2) ferric Rd center (6). Although the shape and properties of the FlRd EPR spectrum have already been discussed, the fact that it is identical to the Rd-D one suggests that no significant change in the [Fe-Cys4] center structure is produced upon truncating FlRd.

Direct EPR evidence for the diiron center in flavodiiron proteins was never before obtained, due to the anti-ferromagnetic coupling of the iron nuclei, confirmed by Mössbauer spectroscopy (29). In their fully oxidized and fully reduced states, these centers have integer spins, and are thus EPR silent in a perpendicular field. However, the fully reduced species can be identified by a low intensity signal at g ~ 11–15 in parallel field EPR, characteristic of an S = 4 spin ground state (11, 13). It was thus expected that the detection of a mixed-valence FeIII–FeII species could provide direct EPR evidence for the presence of the diiron center in FlRd. This was investigated both in the intact FlRd and in a truncated form of FlRd, consisting of the flavodiiron structural core (FDP-D), i.e. structurally similar to ROO and all Class A FDPs. The first EPR evidence was obtained through mild chemical reduction, by anaerobically incubating FDP-D with sub-stoichiometric amounts of sodium dithionite, in the absence of redox mediators. Anaerobic conditions were maintained by continuous argon flushing on the surface and by the oxygen-scavenging glucose oxidase/catalase system. A rhombic signal was observed with g values at 1.93, 1.88, and 1.82, very similar in shape and g values (TABLE ONE) to those observed for S = 1/2 mixed-valence non-heme diiron centers from the hydroxylase component of methane monooxygenase. By varying the temperature (not shown), it was observed that the signal displays its maximal intensity at ~7 K, and that it virtually disappears above 25 K, due to the increasing population of the higher energy spin states and line broadening. Anaerobic redox titrations followed by EPR were carried out, both with intact FlRd and the FDP-D, to study the diiron center reduction potentials and their modulation by the other cofactors and/or the presence of the redox partner (FlRd-Red).
Redox Tuning in E. coli Flavorubredoxin-type NO Reductase

Comparison of the spectroscopic (EPR) and redox properties of non-heme diiron sites from different proteins

| Protein  | Organism       | EPR spectra* | Redox potentials | Observations                                                                 | References |
|----------|----------------|--------------|-----------------|------------------------------------------------------------------------------|------------|
| FlRd     | E. coli        | m.v./g: 1.95, 1.80 and 1.74 red.: g = 11.3 | −20 mV; −90 mV +20 mV; −50 mV (w/ FlRd–Red) | Observed only in the course of redox titration with sodium dithionite, in the presence of redox mediators | This work  |
| FDP-D    | E. coli        | m.v.: g = 1.92, 1.88 and 1.82 red.: g = 11.3 | 0 mV; −50 mV | Obtained by mild chemical reduction with sodium dithionite | This work  |
| AOX      | A. thaliana    | m.v.: g = 1.86, 1.67 and 1.53 red.: g = 15 | | Obtained with 2 mmo dithionite, 1 mmo PMS, and reoxidation by O2 | (41)       |
| MMOH (Bath) | Methylococcus capsulatus | m.v.: g = 1.95, 1.88 and 1.78 red.: g = 15 | +48 mV; −135 mV | Obtained with dithionite at room temperature, signal appears after 2 min. Components B and C decrease the potential of +48 mV to −200 mV; with propylene, the potentials shift to over +150 mV | (42, 43) |
| MMOH (OB3b) | Methylosinus trichosporium | m.v.: g = 1.94, 1.86 and 1.75 red.: g = 15 | +76 mV; +21 mV −52 mV; −115 mV (w/MMOB) | Partially reduced by anaerobic addition of dithionate | (44, 45) |

* m.v., mixed-valence state ([Fe(II)/Fe(III)].

Regarding the FDP-D titration, an immediate observation was the different shape and g values of the diiron center spectrum (Fig. 1B), with respect to the one obtained by mild chemical reduction. In this condition, the g values are 1.95, 1.80, and 1.74. Heterogeneity in the shape of diiron centers’ spectra has often been observed (e.g. Ref. 38), either for a single protein or among orthologues, arising essentially from differences in the way the mixed-valence species was obtained. Nevertheless, the temperature dependence of the signal from the samples of the FDP-D redox titration displayed identical features as the one described above (not shown), which indicates that the intrinsic relaxation properties of the diiron center remain unaltered in the presence of redox mediators, thus suggesting that only a minor perturbation was induced at the diiron center.

The mixed-valence diiron center from intact flavorubredoxin was detected only by performing the above mentioned redox titration but not by mild chemical reduction. In Fig. 1A (line a) are presented the EPR signals for the metal centers of partially reduced FlRd. Whereas the signal from the rubredoxin module still corresponds to its fully oxidized state, an extra signal is displayed with g values < 2 corresponding to the mixed-valence diiron center spectrum. The g values and shape for the latter signal are identical to the signal of the truncated flavodiiron domain, FDP-D.

Further EPR evidence for the non-heme diiron center arises from the low intensity signal observed by parallel mode EPR in the fully reduced differential (Fe(II)–Fe(II)) state, at g ~ 11.3. (Fig. 1A, inset). Under the same conditions, resonances for the reduced (S = 2) rubredoxin site were not observed.

Redox Properties of FlRd-Red—The characterization of the redox parameters of the system here described was performed both with the isolated components and with stoichiometric mixtures of the latter. Anaerobic redox titrations of FlRd-Red were performed using a chemical reductant, sodium dithionite, or the physiological electron donor for this enzyme, NADH. Reduction of the protein resulted in a bleaching of its spectroscopic features, namely the two flavin bands with maxima at 380 and 454 nm (Fig. 2). Both with dithionite and NADH as reductants, the experimental data, representing absorbance at 454 nm versus reduction potential, were fitted with a Nernst curve for two consecutive one-electron steps (Fig. 2, inset). With dithionite as reductant, the fitted potentials were −255 ± 15 mV for FADred/FADred and −285 ± 15 mV for FADred/FADred (Em ~ 270 ± 15 mV), whereas for the NADH titration, the reduction potentials were −220 ± 15 mV for FADred/
Redox Tuning in E. coli Flavorubredoxin-type NO Reductase

FAD$_{sq}$ and $-260 \pm 15$ mV for FAD$_{sq}$/FAD$_{red}$ ($E_m = -240 \pm 15$ mV). In both cases, the potentials for the two consecutive one-electron steps are so close, that no intermediary semiquinone species was significantly stabilized and detected. The determined $E_m$ values are close to the one observed for P. oleovorans NADH:rubredoxin oxidoreductase (31). The fact that the $E_m$ value with NADH is $-30$ mV upshifted with respect to the potential measured with dithionite suggests that NAD binding may slightly modulate the reduction potential of the FAD cofactor. As above described, FlRd-Red appears to have specific sequence motifs for NAD binding, and this specificity may contribute to an increase of the driving force for electron transfer from the physiological reductant. The values here reported are quite different from the ones previously obtained for FlRd-Red (6), purified in the absence of glycerol. As stated above, the addition of glycerol has contributed to the obtaintion of FlRd-Red batches fully loaded with FAD and stabilized the flavin moiety throughout the purification procedures. Therefore, we assign this difference of the redox potentials to the higher homogeneity of the protein batches here studied in comparison to the other previously reported.

Redox Properties of the Rubredoxin Domain of Flavorubredoxin—As previously discussed, the overlapping spectral features of the flavin and rubredoxin cofactors of flavorubredoxin hamper the deconvolution of their isolated redox properties. The redox behavior of the Fe-Cys$_4$ center from the rubredoxin domain of flavorubredoxin was initially studied in the intact protein, by performing redox titrations, followed both by visible and EPR spectroscopies (6). The unusually low reduction potential of $-140$ mV was assigned to a series of amino acid substitutions in key residues that appear to modulate the reduction potentials of rubredoxins. To analyze the extent of these substitutions on the reduction potential of the center, i.e. apart from the influence of the other redox centers and the whole protein environment, we have constructed a truncated form of FlRd consisting of its C-terminal rubredoxin module (Rd-D) 79 amino acids long. The spectral features of Rd-D (Fig. 3A) are almost identical to those observed for rubredoxins, with maxima at 380 nm and 484 nm, and a broad shoulder $-570$ nm. Data from an anaerobic redox titration of the Rd module were best fitted with a Nernst curve for a one-electron process with a reduction potential of $-123 \pm 15$ mV (Fig. 3A, inset). This value confirms the exceptionally low reduction potential for this rubredoxin module, and a minor influence of the FlRd modules on this potential. This observation enforces the proposed effect of point natural substitutions in redox relevant residues, in lowering the potential of the Rd center from FlRd, with respect to canonical rubredoxins. Taking into account that the global charge of the Rd center changes from $-1$ to $-2$ upon reduction, a decrease in the potential of the truncated domain would be expected if the center was buried and hidden from the solvent in the intact FlRd. The fact that this reduction potential is almost identical both in the truncated and native proteins suggests that the level of solvent exposure must be similar in the intact and in the truncated Rd modules.

Because we demonstrated previously that the electron entry point of FlRd is its Rd module (6), we went on to check whether the presence of the redox partner FlRd-Red changed the redox properties of the Rd domain, suggesting the formation of an electron transfer complex. For that purpose we titrated anaerobically a 1:1 mixture of FlRd-Red and Rd-D (Fig. 3B), using NADH as reductant and, because both FlRd and Rd-D do not accept electrons directly from NADH, their reduction is exclusively accomplished by reduced FlRd-Red. Although the spectral features of FlRd-Red (containing FAD) and Rd-D are again overlapping, the large separation of their reduction potentials allowed the deconvolution of each redox curve, as observed in the inset in Fig. 3B. To assess the formation of the protein complex, we ran the two proteins (separately and in a 1:1 mixture) through an analytical gel filtration column (not shown). Despite the poor resolution inherent to this method, we were able to observe an increase in the area of the higher molecular mass peak, concomitant with a decrease in area of the peaks correspondent to the isolated proteins. Although this does not account for 100% complex formation, these results are biased by the fact that the proteins were run through the column in their fully oxidized states, whereas in the redox titrations NADH is present (the complex is expected to occur between NADH-reduced FlRd-Red and oxidized FlRd). The data for FlRd-Red were fitted with a Nernst plot for two consecutive one-electron processes, as described for the isolated FlRd-Red titrations. However, although the fitted mean potential is almost identical to the isolated protein ($-238 \pm 15$ mV against $-240 \pm 15$ mV), the potentials appear to be inverted for each transition, i.e. the best fit is obtained with a potential for the first transition lower (FAD$_{ox}$/FAD$_{sq}$, $-250 \pm 15$ mV) than that of the second (FAD$_{sq}$/FAD$_{red}$, $-220 \pm 15$ mV). This means in
fact that the electron transfer process from NADH to FlRd-Red occurs most probably as a two electron process, and that this effect is enhanced by the presence of the redox partner, Rd-D in this case. We thus checked for the best fit with two identical reduction potentials ($E^0 = 123$ mV), although the fitted curve was not as adequate as the one described above (data not shown). More striking was the redox upshift from $-123 \pm 15$ mV to $-65 \pm 15$ mV observed for the Rd-D, when titrated in the 1:1 mixture with FlRd-Red. Many redox partners form more or less stable complexes, in which the reduction potentials of the cofactors are changed solely by the presence of one another (e.g. Refs. 39 and 40).

**Effect of the NADH:Flavorubredoxin Oxidoreductase on the Redox Properties of Its Redox Partner Flavorubredoxin**—With the previous observations in mind, we pursued to complete the redox characterization with the intact flavorubredoxin. FlRd was titrated anaerobically (Fig. 4A) as previously (6) with the exception that 18% of glycerol were present in the work here described. With the complete knowledge of the visible features of

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**FIGURE 3.** Redox properties of the rubredoxin domain (Rd-D) of *E. coli* flavorubredoxin. A, optical transitions measured upon reduction of 20 $\mu$m Rd-D with sodium dithionite (in 50 mM Tris-HCl, 18% glycerol, pH 7.5). Arrow points to the absorbance bleach at 484 nm used for the analysis of the redox process. Inset, Absorbance at 484 nm fitted with a reduction potential of $-123$ mV. B, changes in the visible spectra of a stoichiometric mixture of Rd-D and FlRd-Red (in 50 mM Tris-HCl, 18% glycerol, pH 7.5, each protein at 20 $\mu$m) upon reduction with NADH, at 25 °C. Inset, normalized absorbance versus measured reduction potential for each redox active species: hollow circles (•) for FlRd-Red and hollow diamonds for Rd-D (224). Lines a and b were calculated with reduction potentials of $-250$ mV and $-220$ mV (a) and $-65$ mV (b).
the Rd center, we were able to deconvolute the data for the Fe-Cys₄ center in the Rd domain, which were fitted with a Nernst plot for a one-electron transition (Fig. 4A, inset), with a reduction potential of −123 ± 15 mV. Because this value is identical to the one measured for the truncated Rd-domain, it was possible to subtract the spectra of the Rd-D titration to the ones of the intact FlRd titration, where the corresponding experimental redox values matched. In this manner, it became possible to obtain a matrix comprising solely the spectral features of the flavodiiron core obtained by subtracting the rubredoxin contribution from the whole flavobacterioflavin titration. Solid line, oxidized FMN moiety (+45 mV); dashed line, partially reduced FMN moiety (−77 mV); dotted line, reduced FMN moiety (−179 mV). Arrows depict the absorbance changes used to monitor the redox changes. Inset, normalized absorbance at 390 nm minus 458 nm as a function of the reduction potential, fitted with reduction potentials of −40 mV and −130 mV.

To complete the characterization of the redox properties of FlRd, we titrated the non-heme diiron center, following by EPR spectroscopy the absorbance at 390 nm as a function of the redox potential. Whereas the semiquinone radical had already been observed in the previous report (6), the fitted potentials (Fig. 4B, inset), are markedly different from the ones then observed (−40 ± 15 mV against −140 mV for the FMNₜₐₜ/FMNₐₜ step and −130 ± 15 mV against 180 mV for the FMNₜₐₜ/FMNₐₜ step, in this work and the previous (6), respectively). This may result from several factors, such as the inclusion of 18% glycerol in the buffers (which may contribute to the stabilization of the semiquinone radical), or a higher iron incorporation in the present work with respect to previous protein batches. These values are closer to the reduction potentials measured for D. gigas ROO, and altogether −80 mV higher than those for M. thermoacetica FDP (29).
Redox Tuning in E. coli Flavorubredoxin-type NO Reductase

FIGURE 5. Modulation of the redox properties of E. coli flavorubredoxin by its partner NADH:flavorubredoxin oxidoreductase. A, changes in the visible spectra of a stoichiometric mixture of FlRd and FlRd-Red (in 50 mM Tris-HCl, 18% glycerol, pH 7.5, each protein at 20 μM) upon titration with NADH, at 25 °C. Inset, spectrum of the stoichiometric mixture of FlRd and FlRd-Red minus the sum of their isolated spectra. B, normalized absorbance versus reduction potential data for the FAD moiety from FlRd-Red (hollow squares □) and the Rd (hollow circles ○) and FMN (solid circles ●) centers of FlRd. Fitted curves corresponding to Nernst plots with −250 and −220 mV (fit for hollow squares □), −40 mV and −130 mV (fit for solid circles ●) and −65 mV (fit for hollow circles ○). C, normalized EPR signal intensities (averaged from $g_{max}$ and $g_{med}$) corresponding to the appearance of the mixed-valence non-heme diiron center in FlRd upon one-electron reduction and its disappearance upon full reduction to the di-ferrous state. Solid circles (●), isolated FlRd; hollow circles (○), FlRd stoichiometrically mixed with FlRd-Red. Data fitted with −20 mV and −90 mV (solid circles ●) and +20 mV and −50 mV (hollow circles ○).
rhombic signal at $g < 2$ characteristic of the mixed-valence $\text{Fe}^{\text{III}}–\text{Fe}^{\text{II}}$ centers (Fig. 1B). The measured data yield bell-shaped curves, corresponding to the appearance (one-electron reduction of the fully oxidized $\text{Fe}^{\text{III}}–\text{Fe}^{\text{III}}$ to $\text{Fe}^{\text{III}}–\text{Fe}^{\text{II}}$) of the $g < 2$ EPR signal and its subsequent disappearance (one-electron reduction of $\text{Fe}^{\text{III}}–\text{Fe}^{\text{II}}$ to the fully reduced $\text{Fe}^{\text{II}}–\text{Fe}^{\text{II}}$). The fitted potentials for FlRd ($−20 \pm 20$ mV for the $\text{Fe}^{\text{III}}–\text{Fe}^{\text{III}}$/Fe$^{\text{III}}–\text{Fe}^{\text{II}}$/Fe$^{\text{II}}–\text{Fe}^{\text{II}}$ step and $−90 \pm 20$ mV for the $\text{Fe}^{\text{III}}–\text{Fe}^{\text{II}}$/Fe$^{\text{II}}–\text{Fe}^{\text{II}}$ step, Fig. 5C, solid circles) are slightly lower than the ones observed for FDP-D ($0 \pm 20$ mV and $−50 \pm 20$ mV, not shown), the truncated form of FlRd consisting of its flavodiiron core (i.e. lacking the rubredoxin domain). This observation reflects the fact that the rubredoxin module influences the redox properties of the diiron center, possibly due to several factors, such as blockage of solvent accessibility, electrostatic interactions, conformational changes, or a combination of these factors.

The redox titrations of FlRd and FlRd-Red were performed also at different pH values, from pH 6.5 to 8.5, following their optical properties. The data revealed that the potentials of FAD from FlRd-Red and of FMN and Rd from FlRd are clearly pH-dependent in this pH range, all of them decreasing by more than 60 mV per pH unit (data not shown). A more detailed study was hampered by the instability of the proteins below pH $\sim 6$.

Having established the redox properties of all cofactors in FlRd, we tested the effect of the FlRd-Red on the latter, by titrating both proteins combined in stoichiometric amounts, and following the redox active species both by visible and EPR spectroscopy, at pH 7.5, close to the expected value of the E. coli cytoplasm. In the titration followed by visible spectroscopy, the data comprise absorption features from three almost overlapping redox centers: the FAD from FlRd-Red, the FMN from the flavodoxin module and the $[\text{Fe–Cys}_4]$ center from the Rd module (Fig. 5A). In this titration the spectrum of the oxidized mixture of the two components is slightly different from the sum of their isolated spectra (Fig. 5A, inset). Interestingly, this slight spectral change has been observed for the mixture of rubredoxin reductase and rubredoxin from Pseudomonas oleovorans, and Lee et al. (19) propose that this could result from minor structural perturbations of the combined partner proteins. As in the titration of FlRd-Red with the Rd domain, deconvolution of the FlRd-Red data were immediate (Fig. 5B, hollow squares), because its reduction potential is significantly lower than those of the FlRd centers. In fact, the reduction potentials used to fit the data with two consecutive one-electron Nernst curves were identical to the ones for the titration of FlRd-Red with Rd-D ($\text{FAD}_{\text{ox}}/\text{FAD}_{\text{red}}$: $−250 \pm 15$ mV and $\text{FAD}_{\text{ox}}^\prime/\text{FAD}_{\text{red}}^\prime$: $−220 \pm 15$ mV). Using the reduction potential for FlRd-Red and the spectra of its oxidized and reduced forms (Fig. 2), it was possible to create a matrix of the FlRd-Red spectra as part of the titration of the mixture. For this purpose, a fraction of oxidized and reduced FlRd-Red was assigned to each experimental potential of the mixture titration, and concomitantly a spectrum with the contribution of FlRd-Red to the overall spectrum. After subtraction of the FlRd-Red matrix to the one corresponding to the mixture titration, we obtained a matrix consisting solely of the FlRd spectra in the course of the titration. In the same manner as in the FlRd titration, we were able to isolate the data for the Rd domain and to fit it with a one-electron Nernst curve with a reduction potential of $−65 \pm 15$ mV (Fig. 5B, hollow circles), identical to the reduction potential of the Rd-D measured in the presence of FlRd-Red. With identical reduction potentials for the Rd-D, both in the intact and truncated FlRd (in the presence of the partner FlRd-Red) we proceeded to isolate the reduction potentials of the FMN in the FlRd-Red/FlRd titration by subtracting a matrix with the exclusive contribution of the Rd-D to the one containing the FlRd data (the latter already obtained after subtraction of the FlRd-Red contribution, as described above). To obtain the isolated Rd-D matrix, we took the data from Fig. 3B and subtracted the FlRd-Red contribution, as described above for the FlRd-Red/FlRd titration. By subtracting the Rd-D matrix to the FlRd matrix (both obtained after subtracting the
**CONCLUSION**

The nitric oxide detoxification system from *E. coli*, consisting of flavorubredoxin (FlRd) and its NADH oxidizing partner, was studied in terms of its redox properties, contributing to the proposal of inter- and intramolecular electron transfer (eT) mechanisms, coupling NADH oxidation to NO reduction. Besides studying the intact proteins, we used truncated versions of FlRd, namely one form lacking the C-terminal rubredoxin (Rd) module, and another consisting solely of the Rd domain. Moreover, we used EPR spectroscopy to monitor the redox properties of the non-heme diiron center from FlRd, a novelty for the flavodioxygen proteins family.

The NADH:flavorubredoxin oxidoreductase (FlRd-Red) is here established as a member of the rubredoxin reductases (RdReds) family. The determined redox properties are consistent with this protein to accept consecutively two electrons from NADH, without the formation of a partially reduced semiquinone species. In the presence of its partner (FlRd), this two-electron eT character is enforced by a possible inversion of its reduction potentials.

More striking are the changes in reduction potentials of the iron centers (but not of the flavin moieties) in flavorubredoxin when in the presence of the partner FlRd-Red, which strongly suggests the formation of a protein complex. As depicted in Fig. 6, the reduction potential of the rubredoxin site is upshifted due to the presence of FlRd-Red, still allowing eT to the oxidized FMN but hampering eT to the one-electron reduced flavin semiquinone, as $\Delta \psi$ then becomes much larger (approximately $-60$ mV). With the parallel upshift observed in the reduction potentials for the two consecutive transitions of the non-heme diiron center, the flavin semiquinone then has an effective reduction potential that allows eT to the diiron center to occur without the need of having the fully reduced flavin hydroquinone to transfer electrons to the mixed-valence diiron site. It is commonly observed that interaction between redox partners often results in the formation of more or less stable electrostatic complexes. Upon formation of these complexes, efficient eT will benefit from a favorable thermodynamic drive provided by an upward directionality of the reduction potentials of each eT species, from the donor to the acceptor side.

The observations reported in this work indicate that fast and effective electron transfer appears to be ensured through a bypass of the second transition of the flavin (from the semiquinone to the hydroquinone forms), because the semiquinone species appears to be sufficiently functional to reduce both iron's, as judged by the thermodynamic data. This effect seems to be enforced by the upshift observed in the reduction potential of the electron entry point, the rubredoxin module, which creates a barrier for eT from Rd to the flavin semiquinone. The fine tuning, which appears to impose electrons to be delivered (via the flavin semiquinone) one at a time onto the non-heme diiron center, the site of nitric oxide reduction, may be related with the complex chemistry of NO or simply to avoid the plausible formation of even more reactive species. NO is not expected to cause physiologically relevant changes in the reduction potential of the FlRd-Red/FlRd system, because it was previously demonstrated that it should only bind to the fully reduced diiron center of FlRd (6, 10). Further studies will aim to understand the nature of the redox modifications imposed on the redox cofactors of FlRd by the presence of FlRd-Red and how this fine-tuning of the thermodynamic parameters may affect the NO reduction mechanism.

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