Molecular dissection of a putative iron reductase from *Desulfotomaculum reducens* MI-1

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Abstract

Desulfotomaculum reducens MI-1 is a Firmicute strain capable of reducing a variety of heavy metal ions and has a great potential in heavy metal bioremediation. We recently identified Dred_2421 as a potential iron reductase through proteomic study of *D. reducens*. The current study examines its iron-reduction mechanism. Dred_2421, like its close homolog from *Escherichia coli* (2, 4-dienoyl-CoA reductase), has an FMN-binding N-terminal domain (NTD), an FAD-binding C-terminal domain (CTD), and a 4Fe-4S cluster between the two domains. To understand the mechanism of the iron-reduction activity and the role of each domain, we generated a series of variants for each domain and investigated their iron-reduction activity. Our results suggest that CTD is the main contributor of the iron-reduction activity, and that NTD and the 4Fe-4S cluster are not directly involved in such activity. This study provides a mechanistic understanding of the iron-reductase activity of Dred_2421 and may also help to elucidate other physiological activities this enzyme may have.

Keywords

Iron reduction, NADH, *Desulfotomaculum*, pfam00724, old yellow enzymes, NADH:flavin oxidoreductase

Introduction

Heavy metal and radionuclide contamination in subsurface sediments is a threat to the environment and human health [1]. Bioremediation utilizing indigenous bacterial strains capable of reducing metal ions has attracted intensive interest as a way to remove the contamination [1, 2]. The reduction of certain soluble metal and radionuclide ions leads to the production of their insoluble form, and thus facilitates their immobilization and subsequent removal from the contaminated site. Such reduction can result directly from biological catalysis by microbial organisms [3]. Alternatively, it may involve both biological and abiotic reactions [4, 5].

The best-studied bacteria strains that have radionuclide bioremediation potential are dissimilatory iron (III)-reducing bacteria (DIRB) [6] and sulfate-reducing bacteria (SRB) [7]. The former group includes species of the *Geobacteraceae* and *Shewanellaceae* families, for example, *Geobacter sulfurreducens*, *G. metallireducens*, *Shewanella putrefaciens*, and *S. oneidensis*, all of which are Gram-negative [8, 9]. For the latter, there have been 60 genera comprised of more than 220 species of SRB reported, including both Gram-positive and Gram-negative species [7]. Gram-negative bacteria have been the main focus in the studies of microbial metal reduction, and many enzymes and electron-transfer pathways have been identified from these studies [9-11]. In contrast, the Gram-positive strains has not been well explored. As more and more environmentally relevant Gram-positive bacteria are being recognized as potential metal reducers [12, 13], their potential application to bioremediation becomes increasingly attractive and requires a better understanding of their mechanisms of metal reduction.

Recently, we reported the proteomic and biochemical studies of potential metal reductases from an environmentally relevant Gram-positive SRB strain, *Desulfotomaculum reducens* MI-1 [14]. Isolated from a heavy metal-contaminated site, *D. reducens* can reduce a variety of heavy metal ions, including uranium (VI), chromium (VI), manganese (IV), and iron (III) [15]. Therefore, *D. reducens* provides a valuable model and tool for heavy-metal bioremediation. To understand its mechanisms of metal

reduction, we identified a few putative metal reductases, including a soluble protein, Dred_2421, and a potentially membrane-associated protein complex, Dred_1685 and Dred_1686 [14].

Dred_2421 is annotated as a member of the NADH:flavin oxidoreductase (NFO) family (pfam00724) [16]. However, this annotation is not based on the full length protein sequence, but rather based on the fact that it contains NADH- and flavin-binding motifs [17]. Proteins in this family have been shown to be strikingly diverse, with many protein subfamilies catalyzing completely unrelated reactions [18-23]. Moreover, many biochemically characterized NFO proteins are relatively small, ranging from 10 to 30 kilo-Dalton (kDa) in molecular weight [21-23], while Dred_2421 is a relatively large protein (668 amino acids, ~72 kDa). Homologs of small NFOs have also been annotated in a DIRB strain *G. sulfurreducens*, including GSU1371 and GSU3330 [24].

We reported that Dred_2421 can reduce a series of metal and radionuclide ions, including iron (III), chromium (VI), and uranium (VI), in an NADH-dependent manner [14]. Its large size compared to other NFO proteins, raises a question of how electrons are transferred from NADH to iron within this large entity. Dred_2421 is highly homologous to a specific group of well-known prokaryotic proteins, namely 2, 4-dienoyl-CoA reductases (DCR) [17]. Most of the homologs of Dred_2421 have not been well studied. The best-studied homolog is the DCR from *E. coli* (EcDCR), which has been biochemically and structurally characterized [25-27]. EcDCR (672 amino acids, ~73 kDa) has a similar size to Dred_2421, and the two proteins share a 36% identity and 54% similarity [28]. Therefore, the crystal structure of EcDCR provides a reasonable structure model to study Dred_2421. Using this model as a guideline, we investigated the contribution of different structural domains to the iron-reduction activity of Dred_2421. The results provide valuable information for the mechanism of iron-reduction by Dred_2421.

Materials and Methods

Cloning and construct designing

Genes encoding for the domain variants of Dred_2421 were amplified by polymerase chain reaction (PCR) using the expression construct of Dred_2421 [14] as the template. GSU1371 and GSU3330 were amplified using the genomic DNA of *G. sulfurreducens* PCA as the template. EcDCR was amplified using *E. coli* genomic DNA as the template. The genes were cloned into the expression vector pET28b (Invitrogen). To generate the construct for Compact-CTD, overlapping PCR was used. Nucleotide sequences encoding for residues 375-523 and 553-668 were each amplified using the Dred_2421 construct as the template. The primers used for 375-523 were: 375-523-fwd: atat cat atg cctggtaaaattctagtattggggccggtg; 375-523-rev: tcc TCCTCCTCCTCC ttcagccagtaataagactgtttctacccc aacg. The primers for 553-668 were: 553-668-fwd: gaa GGAGGAGGAGGA ggaactaaaagattacagttctgga gatggccaacg; 553-668-rev: atat ctcgag ttaatatattaatcgctcattataagcctgggtgattgcatcc. The restriction enzyme recognition sequences are indicated by underlines. The capitalized letters are part of the overlapping sequence and represent the four-glycine loop. A second round of PCR was performed using these two PCR products as the templates and the primers 375-523-fwd and 553-668-rev. The resultant PCR product was then cloned into pET28b.

Protein expression, purification, and analysis

Protein expression and purification were performed in *E. coli* Rosetta2 cells as described [14] with a few exceptions. For NTD variants, 0.13 mM of riboflavin was added to the media at the time of protein expression induction. Proteins were typically stored in 20 mM Tris pH 8, 500 mM NaCl, 10% (v/v) glycerol, but for all domain variants, 20% glycerol was included in the purification and storage buffers.

To check the flavin molecules bound to the proteins, proteins (typically 100 μ M) were boiled at 95 °C for 5 minutes. Denatured proteins were removed by centrifugation. The supernatant was checked by reversed-phase HPLC at 260 nm (Kinetex™ 5 μ m C18 100 Å, 150 x 4.6 mm, 10% (v/v) to 30% acetonitrile in 15 minutes). 100 μ M of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Sigma) were each used as the flavin standards.

106

107 To check for the presence of the 4Fe-4S cluster, 100 μ M of Full length Dred_2421 was scanned from 200
108 nm to 800 nm on a Cary 300 UV-Vis spectrophotometer to check for absorption at around 420 nm that is
109 characteristic of 4Fe-4S clusters.

110

111 **Flavin reconstitution of NTD and CTD variants**

112 To reconstitute the flavin-bound domain variants, the proteins were incubated with 1 mM of
113 corresponding flavin molecules (FMN for NTD and FAD for CTD) overnight at 4 °C. Then excess flavin
114 molecules were removed by passing the mixture through a gel filtration column (Superdex 75 16/60, GE
115 Healthcare) pre-equilibrated in 20 mM Tris pH 8, 500 mM NaCl, 20% glycerol.

116

117 **Iron reduction assays**

118 Iron-reduction activity of heterologously expressed proteins was confirmed using a colorimetric
119 ferrozine-based assay [29]. A typical reaction setup contains 2 mM NAD(P)H, 0.5 mM ferrozine, and 5
120 μ M enzyme in 20 mM Tris pH 8, 100 mM NaCl, 10% glycerol. Fe (III)-nitrilotriacetic acid (NTA) (0.5
121 mM) was added to start the reaction. Reaction was monitored by either visually observing the purple
122 color formation or recording the absorbance at 562 nm on a spectrophotometer.

123

Results

Domain analysis of Dred_2421 based on sequence homology

Given the identity (36%) and similarity (54%) between Dred_2421 and EcDCR (Figure S1), we used the crystal structure of EcDCR as a model to analyze Dred_2421. The structure of EcDCR consists of an N-terminal domain (NTD) and a C-terminal domain (CTD), with the former bound to flavin mononucleotide (FMN) and the substrate 2, 4-dienoyl-CoA, and the latter bound to flavin adenine dinucleotide (FAD) and NADPH [26]. A 4Fe-4S cluster is bound between the two domains (Figures 1A, 1B, and S1). HPLC traces showed that full length Dred_2421 also contains both FAD and FMN molecules (Figure 1C). It also showed an absorbance peak at 420 nm (Figure 1D), which is characteristic of a 4Fe-4S cluster. These results confirmed that Dred_2421 is highly similar to EcDCR. Based on the sequence homology of these two proteins, we divided Dred_2421 into two domains, NTD (residues 1-325, Figures 1E and S1) and CTD (residues 326-668, Figures 1G and S1, Table S1). A BLAST search showed that the NTD is highly homologous to a large family of FMN-containing proteins called Old Yellow Enzymes (OYEs), while the CTD contains characteristic NAD(P)H- and FAD-binding motifs [17]. To understand the role of each domain in iron reduction, we purified and characterized the two domains separately.

Purification and analysis of Dred_2421 NTD

Although Dred_2421 was expressed and purified in a soluble form [14], the NTD of Dred_2421 under the same conditions was insoluble. We reasoned that the interactions between the NTD and CTD help stabilize the NTD (Figure 1A). Without such interactions, the individual domains would be unstable. To prevent precipitation of NTD, we included 20% (v/v) glycerol in the cell lysis buffer and all other buffers in the purification procedure, which led to successful purification of soluble NTD. However, to our surprise, the purified NTD was colorless, indicating the absence of FMN, which is bound to the NTD in the full length protein (Figure 1A). The absence of FMN was confirmed by HPLC results (Figure 2A).

We reasoned that the NTD of Dred_2421 might lack some important interactions from downstream residues that support the binding of FMN. Using EcDCR structure as a model, we hypothesized that residues 341-351 of Dred_2421 are involved in FMN binding (Figure S1). Therefore, we generated a longer truncate to include these residues. This longer truncate, which consists of residues 1-367, was named long-NTD (Figures 1F and S1). Long-NTD was similarly purified as a soluble protein in the presence of 20% glycerol. However, the new truncate was still colorless and lacked FMN (Figure 2A).

We also tried to supplement the *E. coli* growth medium with riboflavin to facilitate the synthesis and loading of the flavin molecules [27], but still did not get FMN loaded onto either NTD or long-NTD. Incubating either NTD or Long-NTD with FMN and then removing excess FMN by desalting column did not lead to a protein with FMN bound either.

Characterization of two homologs of NTD

Since both NTD variants did not have FMN bound, we could not test its iron reduction ability directly. The NTD of Dred_2421 is highly homologous to OYEs. OYEs have been shown to catalyze diverse reactions [18-20], and many OYEs can reduce non-physiologically molecules (“opportunistic electron acceptors”) [18], such as dioxygen. We therefore asked whether all OYEs can reduce iron. If this was the case, we might expect the NTD of Dred_2421 to have similar activity. Two OYEs, GSU1371 and GSU3330, were identified in *Geobacter sulfurreducens* PCA [24], which is a Gram-negative bacterium and one of the predominant metal-reducing bacteria studied. Both are highly homologous to the NTD of Dred_2421 (32% identity for GSU1371, and 36% identity for GSU3330, Figure S1). Therefore, we tested their iron-reduction activities in order to provide some indirect insights into the activity of the NTD of Dred_2421.

Both purified GSU1371 and GSU3330 had FMN molecule bound (Figure 2A). Ferrozine-based colorimetric assay showed that while GSU1371 showed a strong iron-reducing activity (as indicated by

the purple color) in the presence of NADH but not NADPH, GSU3330 did not show significant activity with either electron donor (Figure 2B). Thus, iron-reducing activity is not a universal feature of OYEs.

CTD of Dred_2421 shows a similar iron-reducing activity to the full length enzyme

Similar to the NTD, the CTD (326-668) (Figure 1G) of Dred_2421 was purified in a soluble and stable form in the presence of 20% glycerol. It showed a bright yellow color after metal-affinity purification, indicating a tightly bound flavin molecule. The flavin molecule was confirmed to be FAD by HPLC (Figure 3A), consistent with the prediction based on the EcDCR model. Interestingly, CTD showed a strong iron-reduction activity (Figure 3B) in an NADH-dependent manner. As a more quantitative method, a real-time monitoring of the reactions at 562 nm showed little difference between the activities of CTD and full length Dred_2421 (Figure 3C). This result suggested that CTD is the main contributor to the iron-reduction activity of Dred_2421.

The CTD truncate contains the binding motifs (residues 326-367) for the 4Fe-4S cluster (Figure 1G). To check whether the iron-sulfur cluster is involved in iron-reduction, we generated a shorter CTD variant (residues 375-688) without the iron-sulfur cluster-binding motif. However, this variant was unstable and precipitated out even in the presence of 20% glycerol. In the structure of EcDCR, we observed that a “bump”, composed of a short α -helix and a long loop (Figure 1G), is protruding out from the core structure of CTD. It corresponds to residues 524-552 in Dred_2421. In the full length protein, this bump interacts with the NTD. Without the NTD, this bump has no interaction partners, which may contribute to the instability of the short CTD (375-688). Therefore we designed another CTD variant by replacing the bump structure with a short linker composed of four glycine residues, which leads to a compact core structure of only 269 amino acids, containing residues 375-523 and 553-668, linked by the four-glycine loop. We named the new variant Compact-CTD (Figure 1H).

200 In contrast to the longer CTD variant, purified compact-CTD was stable in the presence of 20% glycerol.
201 However, it only showed a weak yellow color, indicating a relatively low occupancy of FAD. Fortunately,
202 by incubating Compact-CTD with FAD and then removing excess FAD by a desalting column, we
203 obtained a Compact-CTD protein whose FAD level is comparable to that of the longer CTD (Figure 3A).
204 This reconstituted Compact-CTD showed a strong iron-reduction activity, similar to both CTD and full
205 length Dred_2421 (Figure 3B and 3C). These results further confirm that the CTD reductase activity
206 alone is comparable to the full length enzyme and suggested that the iron-sulfur cluster is not required for
207 transferring the electron to Fe (III).

208
209 In an attempt to compare Dred_2421 and EcDCR on their biochemical and enzymatic profiles, we
210 expressed and purified EcDCR under the same conditions as those for Dred_2421. To our surprise, the
211 purified EcDCR only had FAD, but not FMN (Figure 4A). The reason why this occurred was not clear,
212 although it might suggest an intrinsic low affinity to FMN by the N-terminal domain of this family of
213 protein. Interestingly, with FAD bound to the CTD but no FMN bound to the NTD, this DCR serves as a
214 serendipitous control for the CTD of Dred_2421. Our results showed that this “incomplete EcDCR” did
215 not reduce iron (Figure 4B), suggesting that iron reduction is not a universal activity for the FAD-binding
216 CTD in this family of proteins, and highlighting the importance of this activity of the CTD of Dred_2421.

Discussion

Desulfotomaculum reducens MI-1 is an excellent example of a Firmicute that can act as a metal reducer and have a great potential in reducing heavy-metal contaminants in the subsurface sediments. Identifying and characterizing the responsible metal reductases in this organism would enable a better understanding of its metal-reduction mechanism and facilitate the identification of relevant biomarkers. Dred_2421 was recently identified as a potential iron reductase from *D. reducens* MI-1. Based on the crystal structure of its homolog in *E. coli*, we predicted that Dred_2421 consists of an FMN-binding N-terminal domain, an FAD-binding C-terminal domain, and a 4Fe-4S cluster-binding motif between the two domains. In the current study, we investigated the contribution from each of these components to the iron-reduction activity to understand the mechanism of iron reduction by Dred_2421.

We created two NTD variants, residues 1-325 and 1-367, respectively. But neither variant we purified had FMN bound and thus we could not test its iron reduction activity. However, it is highly conceivable that the NTD does not contribute directly to the iron-reduction activity of the full length Dred_2421. This conclusion is based on three pieces of evidence. First, our studies on two OYEs (GSU1371 and GSU3330), which are close homologs to the NTD, suggested that iron-reduction is not a universal activity of this FMN-binding domain; Second, the two CTD variants have similar activity to the full length Dred_2421, suggesting that CTD is the main contributor of the iron-reduction activity. Third, in EcDCR, the 4Fe-4S cluster bridges the N- and C-terminal domains and likely transfers the electron from CTD to NTD. Our results with the Compact-CTD showed that, the iron-sulfur cluster is not required for the iron-reduction activity. This implies that electrons are not transferred beyond the CTD in the reduction of iron, and thus the NTD should not be involved.

The reason why these NTD variants did not bind FMN when produced heterologously is unknown. However, this might be a common property among Dred_2421 and its homologs, for example, EcDCR. Our results showed that, when the *E. coli* expression strain for EcDCR was grown with riboflavin

supplement in the medium, EcDCR only had FAD, but not FMN, suggesting an intrinsic weak binding for FMN in this family of proteins. Interestingly, with FAD bound to the CTD but no FMN bound to the NTD, this “incomplete EcDCR” served as a serendipitous negative control for the CTD of Dred_2421. Our results showed that this FMN-lacking EcDCR did not have significant iron-reducing activity, which suggested that iron-reduction is not a universal activity for the FAD-binding domain (CTD) in this family of proteins, and thus highlighted the significance of this activity of the CTD of Dred_2421.

Based on the structure of its homolog, EcDCR, the electron transfer pathway for the iron reduction activity of Dred_2421 could be hypothesized to be NADH-FAD-(4Fe-4S)-FMN-Fe, as shown in FigureS3A. However, our data described above suggest that this is actually not the case. Instead, the added electron acceptor (Fe^{3+}) can intercept the electrons from the FADH_2 before they are taken by the 4Fe-4S cluster (Figure S3B)

With only the CTD involved in iron-reduction, the additional NTD amino acids in Dred_2421 may be nothing more than structural if iron-reduction is the only function of the full-length protein. *In vivo*, Dred_2421 might have other functions in addition to the iron-reduction activity. For example, could it also be a 2,4-dienoyl-CoA reductase like its homolog in *E. coli*? Although we did not detect such activity with the Dred_2421 protein we purified (data not shown), other electron acceptors and donors are possible. Further studies are needed to further understand the *in vivo* function of Dred_2421.

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Figure Legends

Figure 1. Domain analysis of Dred_2421 using EcDCR crystal structure (PDB code: 1PS9) [26] as a model. **A)** Overall structure of full length EcDCR. The NTD is shown in purple, CTD in green. Substrates and co-factors are shown as sticks, with NADPH (left) and 2, 4-dienoyl-CoA (right) in gray, FAD (left) and FMN (right) in yellow, and 4Fe-4S cluster in brown. **B)** A simplified scheme of the EcDCR structure in the same orientation as shown in A. **C)** HPLC traces to show the flavin molecules in Dred_2421. **D)** UV-Vis scan of Dred_2421 to show the characteristic peak at 420 nm (red trace) attributed to the 4Fe-4S cluster. The purple and blue traces show the scan after reduction by 1 mM and 5 mM sodium dithionite, respectively. **E)** and **F)** Structures of NTD and Long-NTD. **G)** and **H)** Structures of CTD and Compact-CTD. The iron-sulfur cluster-binding structure in CTD is shown in gray. The “bump” structure in CTD is shown in blue. The four-glycine linker in Compact-CTD is shown as a red loop. Structure pictures were generated using PyMOL [30].

Figure 2. Analysis of NTD variants and their homologs. **A)** HPLC traces to show the FMN molecule in these proteins. **B)** Observation of the purple color formation from the ferrozine-based colorimetric assay in test tubes. GSU1371 reduces Fe (III) using NADH as the electron donor. GSU3330 does not reduce Fe (III) using either NADH or NADPH. See Methods for details.

Figure 3. Analysis of CTD and Compact-CTD. **A)** HPLC traces to show the FAD molecule in the CTD variants. **B)** Observation of the purple color formation from the ferrozine-based colorimetric assay in test tubes. Both CTD and Compact-CTD show similar iron-reducing activity as Dred_2421. **C)** Real-time monitoring of the reaction progress using the absorbance at 562 nm shows consistent result to the colorimetric assays.

Figure 4. Analysis of EcDCR. **A)** HPLC traces to show the FAD molecule in the EcDCR protein we purified. **B)** Observation of the purple color formation from the ferrozine-based colorimetric assay in test

381 tubes. The purified EcDCR does not show significant iron-reducing activity using either NADPH or
382 NADH. Dred_2421 was used as a control.

Figure 1
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Figure 2
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