The apicomplexan *Cryptosporidium parvum* possesses a single mitochondrial-type ferredoxin and ferredoxin:NADP⁺ reductase system

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Abstract: We have successfully expressed recombinant mitochondrial-type ferredoxin (mtFd) and ferredoxin:NADP⁺ reductase (mtFNR) from *Cryptosporidium parvum* and characterized their biochemical features for the first time for an apicomplexan. Both *C. parvum* mtFd (CpmtFd) and FNR (CpmtFNR) were obtained and purified as holo-proteins, in which the correct assembly of [2Fe–2S] cluster in Fd and that of FAD in FNR were confirmed and characterized by UV/vis and electron paramagnetic resonance. These proteins were fully functional and CpmtFNR was capable of transferring electrons from NADPH to CpmtFd in a cytochrome c-coupled assay that followed a typical Michaelis-Menten kinetics. Apicomplexan mtFd and mtFNR proteins were evolutionarily divergent from their counterparts in humans and animals and could be explored as potential drug targets in *Cryptosporidium* and other apicomplexans.

Keywords: apicomplexan; *Cryptosporidium*; ferredoxin; ferredoxin:NADP⁺ reductase; electron transfer

Additional Supporting Information may be found in the online version of this article.

Abbreviations: Adx, adrenodoxin; BI, Bayesian inference; BP, bootstrap proportion; EPR, electron paramagnetic resonance; Fd, ferredoxin; FNR, ferredoxin:NADP⁺ oxidoreductase; HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; ICP-MS, inductively coupled plasma mass spectroscopy; IPTG, Isopropyl-β-D-thio-galactoside; LB, Luria-Bertani; ML, maximum likelihood; mtFd, mitochondrial-type Fd; mtFNR, mitochondrial FNR; NTA, nitrilotriacetic acid; ptFd, plant-type Fd; ptFNR, plant-type FNR; PP, posterior probability; Tdx, thioredoxin; WAG, Whelan and Goldman amino acid substitution model.

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Figure 1. The electron transfer system conducted by ferredoxin (Fd) and FAD-containing FNR. The use of cytochrome c (cyt c) and an artificial electron acceptor in the assay is also illustrated.

Introduction

Ferredoxin (Fd) and ferredoxin:NADP⁺ oxidoreductase (FNR) [EC 1.18.1.2] are widely spread in all three domains of life (Eukaryota, Bacteria, and Archaea).¹ Fd-/FNR-coupled electron transfer systems participate in various vital or critical metabolic processes, ranging from photosynthesis and energy metabolism to various biosynthetic reactions. In the well-studied plant photosystem I, an oxidized Fd molecule (Fd_{ox}) first receives an electron driven by light energy to form a reduced Fd (Fd_{red}). The FAD-containing FNR then catalyzes the transfer of the electron to NADP⁺, which recycles Fd_{red} back to Fd_{ox} (Fig. 1). On the other hand, FNR mainly catalyzes the conversion from Fd_{ox} to Fd_{red} in reactions other than photosynthesis.¹ Fd can be roughly classified into plant (plastid) type, mitochondrial (adrenodoxin) type, and thioredoxin type; while FNR can be classified into plant (plastid)- and mitochondrial-type enzymes.¹ Although prokaryotes do not possess plastids and mitochondria, their Fd and FNR systems are also grouped into either plant- or mitochondrial-like proteins based on their sequence and structural features. The plant- and mitochondrial-type FNRs (mtFNRs) are in fact two phylogenetically and structurally unrelated enzymes, although they perform essentially the same functions.¹

Among protists, apicomplexans are a Phylum of parasites that include many important human and animal pathogens such as *Plasmodium*, *Babesia*, *Toxoplasma*, *Eimeria*, and *Cryptosporidium* species. With the exception for *Cryptosporidium*, and prob-

ably *Gregarina*, apicomplexans possess a chloroplast-derived apicoplast that is nonphotosynthetic but may be involved in many essential functions such as the biosynthesis of fatty acids, isoprenoids, and heme.^{2–6} Plastid-containing apicomplexans and a related dinoflagellate (*Perkinsus marinus*) possess plant-type Fd (ptFd) and ptFNR that are targeted to the apicoplast.^{7–10} These ptFd and ptFNR proteins from *Plasmodium* and *Toxoplasma* have been reported and their biological and biochemical features are being extensively studied, which has started to provide many new insights into their biochemical features and biological roles in the apicomplexans.^{7–16}

Additionally, most apicomplexans also possess mitochondria that are largely classic in both structure and functions (such as the presence of Krebs cycle and cytochrome-based respiratory chain).¹⁷ In contrast, the mitochondrion of *Cryptosporidium* is highly reduced in size and function.^{17–21} Mitochondrial-type Fd (mtFd) and mtFNR genes have been observed in virtually all apicomplexans, including *Cryptosporidium parvum*, as well as all other members of the Alveolata for which genome sequences are available (Table I). However, in contrast to the plant-type system, virtually nothing is known about the biochemistry and function of the mitochondrial-type electron transfer system in the Apicomplexa.

The genus of *Cryptosporidium* represents an early branch at the base of the Apicomplexa.²² Different *Cryptosporidium* species may infect different animals from fishes, reptiles, and birds to mammals with varied degrees of host specificities.²³ For example, *C. parvum* and *C. hominis* are the two major species that infect humans and are considered as important opportunistic pathogens in AIDS patients and a potential bioterrorism threat. The recently completed genome-sequencing projects revealed that both *C. parvum* and *C. hominis* do not encode any apicoplast-specific pathways including ptFd and ptFNR but they do possess single-copy mtFd and mtFNR genes.^{20,21}

Here, we report the molecular and biochemical analysis of the *C. parvum* mtFd (CpmtFd) and FNR (CpmtFNR) for the first time for an apicomplexan.

Table I. Type of Ferredoxin (Fd) and Ferredoxin:NADP⁺ Reductase (FNR) Present in the Alveolata (Apicomplexa, Dinoflagellata, and Ciliophora)

Group	Representative species	mtFd-I	mtFd-II	ptFd	mtFNR	ptFNR
Apicomplexa	<i>Cryptosporidium parvum</i>	–	+	–	+	–
	<i>Toxoplasma gondii</i>	+	+	+	+	+
	<i>Plasmodium falciparum</i>	–	+	+	+	+
	<i>Theileria parva</i>	–	+	+	+	+
	<i>Babesia bovis</i>	–	+	+	+	+
Dinoflagellata	<i>Perkinsus marinus</i>	+	+	+	+	+
Ciliophora	<i>Paramecium tetraurelia</i>	–	+	–	+	–
	<i>Tetrahymena thermophila</i>	+	+	–	+	–

mtFd and mtFNR are also referred to as adrenodoxin (Ad or Adx) and adrenodoxin:NADP⁺ reductase (AdR).

Our study reveals that apicomplexan mtFd and mtFNR are highly divergent from their counterparts in humans and animals, suggesting that, like ptFd and ptFNR in *Plasmodium* and *Toxoplasma*, mtFd and mtFNR could also serve as rational drug targets in *Cryptosporidium* and other apicomplexans.

Results

Cryptosporidium possesses a single mtFd/mtFNR system that is highly divergent from humans and animals

Eukaryotic Fd/FNR electron transfer systems can be compartmentalized in either mitochondria or plastids. Like plants, apicomplexans may possess both plastid and mitochondrial Fd/FNR systems. Genes encoding ptFd and ptFNR have been reported in *Plasmodium* and *Toxoplasma* and their biochemical features are being thoroughly studied (e.g., Refs. 7,8,11–14,16). By data mining all available genome sequences, we have found that genes encoding ptFd and ptFNR are present in almost all apicomplexans analyzed here with the exception of *Cryptosporidium* species (Table I). This appears to be correlated well with the absence of an apicoplast in this parasite. We have also noticed that genes encoding ptFd and ptFNR are absent in the two available ciliates genomes (i.e., *Paramecium* and *Tetrahymena*; Table I) but it is unclear whether this is also true for other ciliates. On the other hand, mtFd and mtFNR genes are present in all members within the Superphylum Alveolata for which genome sequences are available including most apicomplexans and the dinoflagellate *P. marinus*.

Cryptosporidium lacks ptFd/ptFNR but possesses only an mtFd/mtFNR system. Among the three sequenced *Cryptosporidium* genomes, single-copy Fd genes are found in *C. parvum* (*CpmtFd*) and *C. muris* (*CmmtFd*) but not in *C. hominis* (*ChmtFd* if present). It is likely that the *ChmtFd* locus was simply missed by the genome sequencing (as the *C. hominis* genome is not completely sequenced). Sequence and domain analyses confirm that *CpmtFd* and *CmmtFd* genes encode mitochondrial-type proteins (mtFd). Both *CpmtFd* and *CmmtFd* proteins contain an N-terminal mitochondrial targeting signal peptide as determined using both nonplant and plant networks by TargetP and MitoPred algorithms (confidence values ranging from 80 to 95%). Therefore, although detailed subcellular localization of *CpmtFd* and *CmmtFd* proteins remains to be determined, they are predicted to be targeted into the remnant mitochondria in *Cryptosporidium*.

CpmtFd and *CmmtFd* proteins contain four Cys residues that are conserved in all Fds for chelating the [2Fe–2S] cluster [Fig. 3(A); Supporting Information Fig. S1]. Recently, a Type II mtFd (mtFd-II) featured with a highly conserved C-terminal VDGxxPxPH motif has been found in the majority of

eukaryotic groups with the exception of fungi.⁹ In comparison, Type I mtFd (mtFd-I) proteins lack the unique motif but have varied C-terminal sequences. Our analysis with an increased number of genome sequences further extends the conclusion by finding that mtFd-II genes are actually present in the other members of alveolata (i.e., dinoflagellates and ciliates; Table I). In fact, almost all alveolates possess only mtFd-II genes, except for *Toxoplasma gondii* and *P. marinus* that also possess mtFd-I genes. These observations implied that both mtFd-I and mtFd-II genes might be present in the ancestral alveolates, but mtFd-I genes were lost among the majority of lineages in alveolates.

In our phylogenetic reconstructions on mtFd-II proteins using a Bayesian inference (BI) method, animals and plants/algae formed individual clusters, while apicomplexans, *Perkinsus* (dinoflagellate) and ciliates were placed between the animal and plant clusters [Fig. 4(A)]. The BI tree was supported moderately to highly by posterior probability (PP) values. The best maximum likelihood (ML) tree inferred from the mtFd-II proteins produced a similar topology. However, the relationships between the three major clusters were not well resolved by ML bootstrapping analysis, as many nodes were not supported by bootstrap proportion (BP) values under the 50% majority law [Fig. 4(A)]. The poor resolution in ML bootstrapping analysis is likely a result of an insufficient number of amino acid (aa) positions available for phylogeny since Fds are a group of very small proteins. Nonetheless, it is conclusive that apicomplexan Fds are likely a group of “intermediate” form of proteins as they are clearly not grouped together with either the plant or animal clusters.

On the other hand, single-copy mtFNR genes could be identified in all *C. parvum*, *C. hominis*, and *C. muris* genomes. Among them, *CmFNR* has a well-defined N-terminal mitochondrial targeting signal as determined by both TargetP and MitoPred, whereas those in *CpmtFNR* and *ChmtFNR* are undetermined, as their putative initial methionine residues are positioned shortly before the first FAD motif of the enzymatic region [Fig. 2(B)]. There is a region upstream to the putative start codon of *CpmtFNR* and *ChFNR* genes that can be conceptually translated in-frame into a stretch of aa [see the presentation of sequence in Fig. 2(B)]. However, a putative start codon could not be identified in the intergenic region further upstream to that stretch of aa and the location of the N-terminus remains to be resolved.

The enzymatic region of *CpmtFNR* proteins contain at least six highly conserved domains or motifs, including three FAD-pyrophosphate binding and one NADP-pyrophosphate binding motifs that are previously described as important to the function of this group of enzymes [Fig. 3(B); Supporting Information Fig. S1].¹ The N-terminal half of the apicomplexan

Figure 2. Strategy of subcloning of CpmtFd and CpmtFNR into pMAL-c2X-derived MBPHT-Cherry2 and pET21a expression vectors, respectively. The N-terminal sequences for CpmtFd and CpmtFNR excluded from expression are highlighted in red. Arrows indicate the cleavage sites by TEV protease and factor Xa, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FNR is generally more conserved while the C-terminal region is highly divergent in comparison with other eukaryotic and prokaryotic sequences. There is also a ~75 aa insertion in *Cryptosporidium* mtFNR sequences [marked as insertion in Fig. 3(B)]. Considering that mRNA species isolated from sporozoites might contain incompletely spliced introns,²⁴ we decided to test whether this was also a cryptic intron as previously observed in the *C. parvum* phosphopantetheinyl transferase (*CpPPTase*) gene.²⁵ Nonetheless, our RT-PCR analysis using RNA isolated from parasite early intracellular stage confirmed that this insertion was fully transcribed, rather than a cryptic intron (data not shown).

Orthologs of mtFNR are present in all major taxonomic groups, including animals, plants, fungi, bacteria, and alveolates. Phylogenetic trees inferred from a large dataset using BI and ML methods clearly separated eukaryotes from prokaryotes, but relationships within eukaryotes were not well resolved. We therefore rebuilt a smaller dataset containing only 60 representative eukaryotic sequences, which greatly increased the number of alignable aa positions to 245. The resulting trees were better resolved and separated animals, fungi, and plants into clearly defined individual clusters. Apicomplexan FNRs formed two clades, in which *Toxoplasma* was grouped together with *Perkinsus* at the base of plants, and the remaining apicomplexans including *Cryptosporidium* and hematozoa were united with *Polysphondylium* (a diatom) and ciliates as a sister to the plant FNRs [Fig. 4(B)]. The alveolate sequences were placed as a sister to the plants,

which was then followed by the fungal and animal clades. The PP supporting values in BI analysis were moderate to high at all nodes, while BP values in ML analysis were moderate to high at many internal nodes within various clusters, although the best ML tree has a similar topology as the BI tree.

Collectively, our comprehensive phylogenetic analysis indicates that the apicomplexan mtFd-II and mtFNR proteins formed phylogenetic groups either as an intermediate group between plants and animals (for mtFd-II) or as a group with apparent plant affinity (for mtFNR). These data support the notion that the unique apicomplexan mtFd/mtFNR system is divergent from those in their hosts, that is, humans and animals.

CpmtFd and CpmtFNR genes are actively transcribed during the complex parasite life cycle

Our real-time quantitative RT-PCR (qRT-PCR) analysis showed that the transcripts of both *CpmtFd* and *CpmtFNR* genes were detectable in all parasite life cycle stages and their expression profiles followed a similar pattern (Fig. 5). The expression levels in oocysts were the lowest for both genes, which is correlated with the relatively low metabolic status in the virtually “hibernating” environmental stage. Their expression levels were relatively consistent during the intracellular stages from 6 to 36 h postinfection, representing the first and second generations of asexual developmental merogony. However, the expression of both *CpmtFd* and *CpmtFNR* genes were highly elevated in the later stages cultured for 48 and 72 h. The exact biological significance on the

Figure 3. Structures of mtFd-II-type CpmtFd (A) and mtFNR-type CpmtFNR (B) and sequence comparison of their conserved domains and motifs with representative species. In CpmtFd, the conserved C-terminal VD_{GxxPx}PH motif characteristic to Type II mitochondrial-type ferredoxin (mtFd-II) is boxed in gray. The four Cys residues involved in the binding of [2Fe–2S] cluster is marked by stars. In CpmtFNR, six conserved motifs are identified, including four motifs contain highly conserved residues (marked by stars) that are previously known to be involved in FAD-pyrophosphate binding and NADP-pyrophosphate binding. The N-terminal sequence without a defined upstream start Met residue but predicted as a secretory signal (if translated) is question marked. In both proteins, regions expressed as recombinant proteins are shown. A sequence log display for those regions can be found in Supporting Information Figure S1.

elevated expression remains to be determined, but it signifies a much more active metabolic process involving the Fd/FNR-based electron transfer system during their sexual development and oocysts production. Additionally, the expression of *CpmtFd* and *CpmtFNR* followed a similar pattern, indicating that both genes likely share the same or similar transcriptional regulatory mechanisms.

Expression conditions needed to be manipulated for heterogeneous expression of functional holo-CpmtFd and holo-CpmtFNR proteins

CpmtFd and *CpmtFNR* genes are defined by 504 nt and 1608 nt open reading frames (ORFs) that predict proteins with molecular masses at 19.3 and 62.4 kDa, respectively. Both defined (CpmtFd) and unde-

fined (CpmtFNR) N-terminal signal peptides are excluded from expression of fusion proteins as discussed in Materials and Methods Section. The major challenge to study the function of Fd/FNR using recombinant proteins is the production of holo-form proteins, in which the [2Fe–2S] cluster and flavin need to be correctly assembled into Fd and FNR proteins, respectively. Our earlier attempts to express full-length CpmtFd protein could not produce sufficient proteins, in which the presence of highly hydrophobic signal peptide was likely the cause of problem as previously noticed by us and other investigators.^{26,27} We hence removed the region encoding the first 40 aa from the signal peptide in subsequent subcloning into pET21a vector for expression. Despite the improvement in expression, the recombinant protein was mainly an apoprotein even with

Figure 4. Phylogenetic reconstructions reveal apicomplexan mtFd-II and mtFNR are unique groups of proteins that are highly divergent from their counterparts in humans and animals. A: Phylogenetic tree inferred from mtFd-II proteins with 38 taxa and 112 amino acid (aa) positions by Bayesian inference (BI) and ML bootstrapping methods. B: Phylogenetic tree inferred from mtFNR proteins with 60 taxa and 245 aa positions. Both BI and ML analyses used a WAG amino acid substitution model with the consideration of fraction of invariance (F_{inv}) and 12-rate of gamma distribution [$\Gamma_{(12)}$]. PP values in BI analysis were summarized after 25% first trees (resulted from 106 generation of runs) were discarded. ML bootstrap proportion (BP) support values were summarized from 100 replicated sequences under a majority ruling law. In both trees, only nodes supported by $\geq 50\%$ PP or BP values are labeled. Nodes highlighted with solid circles (or solid diamonds) are supported by PP at 1.0 and BP values $\geq 95.0\%$ (or $90.0\text{--}94.9\%$), respectively.

Figure 5. Expression profiles of CpmtFd and CpmtFNR genes in parasite oocysts and intracellular developmental stages as determined by real-time quantitative RT-PCR. Fold changes are plotted in relative to that in the oocyst stage.

the addition of an iron source in the medium. Therefore, we switched to MBPHT-Cherry2 vector, which greatly improved the expression of soluble holo-CpmtFd as an maltose-binding protein (MBP)-fused protein using an autoinduction protocol (final yield at ~20 mg/L of cell culture). The MBP tag could be effectively removed by digestion with tobacco etch virus (TEV) protease to produce soluble CpmtFd protein (Fig. 6).

The purified holo-CpmtFd protein appeared in dark brown, which is a characteristic of iron-containing proteins. The Fe/protein ratio was ~1.9 as determined by ICP-MS (inductively coupled plasma mass spectroscopy) metal analysis. The UV/vis spectra of CpmtFd under oxidized conditions displayed multiple absorbance peaks at or near 460, 416, 343, and 276 nm that were comparable to the Fd proteins from *Escherichia coli* and other species [Table II, Fig. 7(A)].^{14,16,28–31} The A_{416}/A_{276} ratio was 0.57, indicating that the ~75% protein contained the [2Fe–2S] cluster.^{14,28–31} Furthermore, the electron paramagnetic resonance (EPR) spectrum of reduced CpmtFd also clearly exhibited a stoichiometric incorporation of an [2Fe–2S] as a mixed-valence ($\text{Fe}^{3+}/\text{Fe}^{2+}$) metal center [Table II, Fig. 7(B)], in which the g values were comparable to those reported for the holo-Fd proteins from other organisms.³²

Table II. Comparison of Optical Absorption Maxima and Electron Paramagnetic Resonance (EPR) g Values of holo-Fd Proteins from *Cryptosporidium parvum*, Humans, and Other Major Groups

	Optimal absorption maxima (nm) ^a				EPR spectroscopy ^b			
					g_x	g_y	g_z	g_{av}^c
CpmtFd	278	322, 343	416	460	1.94	1.94	2.02	1.97
HsFd	276	322	414	455	1.94	1.94	2.02	1.97
Vertebrates		342	415	458	1.94	1.94	2.02	1.96
Plant type	282	332	422	466	1.88	1.96	2.05	1.96
Bacterial type	276	331	423	463	1.92	1.95	2.00	1.96

^a UV/vis spectral features for human and other types of holo-Fd proteins were cited from Refs. 28–31.

^b EPR g values for human and other types of holo-Fd proteins were cited from Ref. 32.

^c Calculated from $g_{av} = (g_x^2 + g_y^2 + g_z^2)^{1/2}$.

Figure 6. SDS-PAGE fractionation of recombinant CpmtFd and CpmtFNR proteins in which the MBP or His tags were removed. Tobacco etch virus (TEV) protease was also loaded for comparison.

The His tag-fused CpmtFNR was also successfully expressed as holo-protein and purified into homogeneity (Fig. 6). The majority of purified CpmtFNR protein formed dimers as observed by FPLC analysis. The UV/vis spectrum of the holo-CpmtFNR revealed a wide absorbance peak at 350–450 nm, while the apo-CpmtFNR displayed no peak in the area (Supporting Information Fig. S2), indicating that the FAD was assembled into the recombinant CpmtFNR protein.^{14,16} The cofactor flavin in holo-CpmtFNR was further detected and confirmed by an isocratic chromatography-based method. Greater than 50% of the recombinant CpmtFNR protein contained the cofactor FAD as determined by comparing the sample peak intensity to a standard curve prepared from authentic FAD as previously reported.³⁴

CpmtFNR was capable of transferring electrons from NADPH to CpmtFd

The cytochrome *c* (cyt *c*) reductase (Fd-dependent) assay demonstrated that both CpmtFd and CpmtFNR

Figure 7. A: UV/vis spectrum of recombinant CpmtFd displayed multiple peaks of absorbance at 460, 416, 343, and 278 nm, which are characteristic to the [2Fe–2S]-containing proteins such as that for *Escherichia coli* ferredoxin (superimposed here based on Ref. 33). B: Electron paramagnetic resonance (EPR) spectrum of CpmtFd in reduced form at 4 K.

were functional, and CpmtFNR was able to transfer electrons from NADPH to CpmtFd using cyt *c* as an end electron acceptor. The activity of CpmtFNR toward NADPH under various pH conditions followed typical Michaelis-Menten kinetics (Table III). The maximum velocity values ($V_{\max} = 0.434$, 0.0047 , and $0.0121 \mu\text{M s}^{-1}$ at pH 6.8, 7.4, and 8.2, respectively), the Michaelis constant values ($K_m = 24.16$, 3.66 , and $9.42 \mu\text{M}$ at pH 6.8, 7.4, and 8.2, respectively), as well as the turnover rates (k_{cat}) were comparable to the ptFNR from *T. gondii* and *Plasmodium falciparum*, indicating that both CpmtFNR and CpmtFd could function effectively in this assay system (Table III). Similar to the PfFNR, the activity of CpmtFNR could be greatly affected by pH values. It could apparently function at pH 6.8 more efficiently than at pH 7.4 and 8.2 as indicated by the $V_{\max}$ and k_{cat}/K_m values (Table III). However, the affinity and efficiency of CpmtFNR were generally lower than those of other apicomplexans or higher eukaryotes including humans. As mentioned above, recombinant CpmtFNR protein was mostly present as dimers. It has been reported that the NADP-dependent disulfide-linked homodimeriza-

tion process of PfFNR could result in enzyme inactivation.⁷ Therefore, it is possible that the dimerization of CpmtFNR might also contribute to the reduced affinity toward NADPH that was observed in this study.

Discussion

Among apicomplexans, *C. parvum* and *C. hominis* lack a mitochondrial genome, Krebs cycle, and cytochrome-based respiratory chain. However, *Cryptosporidium* species possesses a number of mitochondrial proteins such as some heat-shock proteins, an alternative oxidase, mtFd and mtFNR, and several other [Fe-S] cluster assembling and metabolic enzymes.^{20,21,23,36} The presence of mtFd and mtFNR in *Cryptosporidium* indicates that Fd-based electron transfer is one of the indispensable functions in all types of mitochondria or equivalent organelles among eukaryotes. In the *Cryptosporidium* mitochondrion relict, mtFd/mtFNR system and the [Fe-S] cluster biosynthetic pathway are mutually dependent on each other, as the former participates in the vital electron transfer during the [Fe-S] cluster assembly, while the latter provide the metal center

Table III. Comparison of Kinetic Parameters of CpmtFNR/CpmtFd Toward NADPH with Those from Other Apicomplexans, Humans, and Representative Plants

Species ^a	Type	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)	pH	e^- Acceptor	References
CpmtFNR	Mito	21.71	24.16	0.90	6.8	cyt <i>c</i>	This study
CpmtFNR	Mito	0.24	3.66	0.064	7.4	cyt <i>c</i>	This study
CpmtFNR	Mito	0.60	9.42	0.064	8.2	cyt <i>c</i>	This study
PfFNR	Plant	13.0	5.5	2.4	7.0	cyt <i>c</i>	11
PfFNR	Plant	23	61	0.37	8.2	cyt <i>c</i>	11
TgFNR	Plant	115	1.9	61	8.2	cyt <i>c</i>	16
SoFNR	Plant	180	5	36	8.2	cyt <i>c</i>	16
ZmFNR	Plant	227	7	32	8.2	cyt <i>c</i>	16
HsFNR	Mito	11.8	0.8	14.75	7.2	cyt <i>c</i>	35

^a Species initials: Cp, *Cryptosporidium parvum*; Pf, *Plasmodium falciparum*; Tg, *Toxoplasma gondii*; So, *Spinacia oleracea*; Zm: *Zea Mays* (root-type FNR); Hs, homo sapiens.

to mtFd. Another Fd-/FNR-dependent enzyme could be the oxygen-sensitive pyruvate:NADP⁺ oxidoreductase (PNO) that catalyzes the formation of acetyl-CoA from pyruvate. CpPNO protein is composed of an N-terminal pyruvate:ferredoxin oxidoreductase (PFO) domain and a C-terminal domain homologous to NADPH-cytochrome P450 reductase (CPR). This PFO-CPR fused architecture is extremely unique as it has so far only been found in another distant protist *Euglena gracilis*.³⁷

Our phylogenetic reconstructions reveal that the mtFd and mtFNR proteins from *Cryptosporidium* and other apicomplexans are highly divergent from their counterparts in humans and animals. This notion is in fact more apparent at the protein sequence level. For example, after excluding the N-terminal targeting signal peptides, and based on the well-aligned protein sequences, CpmtFd displays significantly higher identity scores with those from plants and fungi than humans (e.g., 50.8% with *Arabidopsis thaliana* and 42.9% with *Saccharomyces cerevisiae* vs. 22.8% with humans). CpFNR is even more divergent at aa level. In addition to the much less conserved C-terminal region and the presence of a large aa insertion, CpmtFNR only displays ~16–17% identity scores to those representative fungal, plant, and human orthologs. These observations indicate that the single mtFd/mtFNR system may be explored as a novel drug target in *Cryptosporidium* and other apicomplexans.

Materials and Methods

Data mining and phylogenetic reconstructions

CpmtFd and *CpmtFNR* genes have been annotated by the *C. parvum* genome-sequencing project and deposited into the GenBank with accession numbers of XM_001388296 and XM_627171, respectively. Additional searches using various Fd and FNR protein sequences as queries confirmed that all three available *Cryptosporidium* genomes (i.e., *C. parvum*, *C. hominis*, and *C. muris*) encode only a single copy of mtFd and mtFNR genes but not any ptFd and ptFNR genes. We also extended our searches to all available genome and protein databases of alveolates including more than 10 apicomplexans, one dinoflagellate and two ciliates, and identified both mitochondrial- and plant-type ptFd and ptFNR genes from almost all species with the exception of ciliates and *Cryptosporidium* (Table I). Since *C. parvum* (the organism of interest in this study) lacks any ptFd and ptFNR, our subsequent sequence analysis and phylogenetic reconstructions were only performed on mtFd and mtFNR proteins.

To identify the conserved domains and motifs, various mtFd and mtFNR sequences were retrieved from NCBI databases after repeated BLAST searches using CpmtFd and CpmtFNR protein

sequences as queries. Multiple sequence alignments were performed using the MacVector program (v.11.0.4), MUSCLE program (v.3.6), or a structure-based algorithm [EXPRESSO(3DCoffee)] at the T-COFFEE server (<http://www.tcoffee.org>). The identified conserved domains and motifs were displayed as sequence logos using Weblogo 3.1 at its web server (<http://weblogo.threeplusone.com/manual.html>).

For the phylogenetic analysis, we first constructed large Fd and FNR datasets that contained most unique sequences available from the databases. After multiple sequence alignments performed as described above, quick neighbor-joining (NJ) trees were first inferred from protein sequences with Poisson-corrected distances using MacVector. Based on the topologies of these NJ trees, representative sequences from various clades or taxonomic groups were reselected to build smaller datasets for more detailed phylogenetic reconstructions using ML and BI methods as previously described.^{2,25} ML bootstrapping analysis of 100 sequence replicates utilized the TreeFinder program (version October 2008) (<http://www.treefinder.de/>), in which a WAG aa substitution model was used and the rate heterogeneity considered the fraction of invariance (F_{inv}) and 12-rate gamma distribution [$\Gamma_{(12)}$]. BI analysis also used the same model (i.e., WAG + F_{inv} + Γ_{12}) in which two independent runs (each containing four chains running simultaneously) were performed for 10⁶ generations. The current trees were saved every 1000 generations, and the PP values were calculated from the saved BI trees obtained after the runs converged.

Expression profiles of CpmtFd and CpmtFNR genes at various *C. parvum* life cycle stages

The expression patterns of *CpmtFd* and *CpmtFNR* genes during the complex parasite life cycle were assayed by qRT-PCR method as previously described.^{25,38} Briefly, total RNA was isolated from various life cycle stages of *C. parvum* (IOWA-1 strain) using an RNeasy isolation kit (Qiagen), which include the environmental stage of oocysts and intracellular developmental stages obtained by infecting human HCT-8 cells 6–72 h (that roughly cover the parasites development from first and second generations of merogony, to gametogenesis and oocyst production). A SYBR-green based one-step RT-PCR kit (Qiagen) and an iCycler iQ Real-Time PCR Detection System (Bio-Rad) were used to detect *CpmtFd* and *CpmtFNR* transcripts. The following primer pairs were used: 5' AAG CTG CTC AGC ATG AAG AA 3' and 5' TTC GTC GAC TTT GAT TTG ACA 3' for *CpmtFd*; 5' GCG GTT ATC ATT GGA AAT GG 3' and 5' CCA ACC TCT CCG TCC AAT AA 3' for *CpmtFNR*. Parasite 18S rRNA levels were also detected using primers Cp18S-1011F (5' TTG TTC CTT ACT CCT TCA GCA C 3') and Cp18S-1185R (5'

TCC TTC CTA TGT CTG GAC CTG 3'), which were used as controls for normalization. Reactions containing 20 ng of total RNA and 0.2 μ M of specified primers were first incubated at 48°C for 30 min to synthesize cDNA, heated at 95°C for 15 min to inactivate reverse transcriptase, and then subjected to 40 thermal cycles (95°C 20 s, 50°C 30 s, and 72°C 30 s) of PCR amplification. Because RNA isolated from intracellular parasites was mixed with host cell RNA, human 18S rRNA was also detected as controls. The levels of *CpmtFd* and *CpmtFNR* transcripts in all samples were normalized by calculating the ΔC_T between the transcripts and 18S RNA (i.e., $C_{T[Fd \text{ or } FNR]} - C_{T[18S]}$), and their relative levels of expression using an empirical formula $2^{-\Delta C_T}$. Their fold changes in each developmental stage were plotted relative to that in the oocysts stage.

Cloning and expression of *C. parvum* ferredoxin and ferredoxin reductase

To clone *CpmtFd* and *CpmtFNR* genes, their entire ORFs (i.e., 504 nt for *CpmtFd* and 1608 nt for *CpmtFdR*) were first amplified by RT-PCR from total RNA isolated from *C. parvum* oocysts (IOWA-1 strain) with a high-fidelity *Pfu* DNA polymerase and cloned into a pCR2.1-TOPO vector (Invitrogen). These cloned genes (cDNA) were sequenced to confirm their identities and used for subsequent gene manipulations. For the expression of recombinant proteins, *CpmtFd* gene was subcloned into an MBPHT-mCherry2 vector after excluding the 5' region encoding the first 40 aa within the N-terminal mitochondrial targeting signal domain [Fig. 2(A)]. MBPHT-mCherry2 vector was derived from pMAL-c2x by inserting a His(6) tag and a TEV protease cleavage site between MBP tag and multiple cloning site, so that the fusion proteins could be isolated by either amylose- or nickel resin-based affinity chromatography, and the MBP-His tags could be cleaved by TEV protease that is more specific than factor Xa.³⁹ For the *CpmtFNR* gene, there was a region (36 aa) upstream to the putative initial methionine residue that appears to be correlated to the N-terminal signal sequence present in *C. muris* [Fig. 2(B)] (also see the annotation for the locus cgd8-2710 at GenBank or <http://www.CryptoDB.org>). However, whether this region truly encodes a signal peptide cannot be firmly determined as no additional methionine residue could be identified in this region. We also excluded this region and subcloned the remaining sequence into a pET21a vector at the *Bam*HI/*Xho*I restriction sites [Fig. 2(B)].

Both recombinant proteins were expressed in *E. coli* Rosetta (DE3)pLysS cells. The expression of *CpmtFd* protein followed an autoinduction protocol as previously described.⁴⁰ Briefly, a single colony of bacterial transformants was transferred into a 5-mL Luria-Bertani (LB) broth containing 100 μ g/mL ampicillin

and allowed to grow overnight at 37°C. The next morning, bacterial suspension was transferred into 250 mL autoinduction medium and incubated at 37°C until OD₆₀₀ reached to 0.5–0.6, followed by additional growth at 25°C for overnight. For the expression of the *CpmtFNR* protein, a modified terrific broth containing 20 mM glucose, 2 g/L NaNO₃, and 50 μ g/mL ampicillin was used. After growing bacteria at 37°C until OD₆₀₀ reached 0.5–0.6, riboflavin (0.01 mM) and isopropyl- β -D-thio-galactoside (IPTG) (0.1 mM) were added into the medium as previously reported.³⁴ The subsequent overexpression was allowed to continue overnight at 16°C. In both the cases, bacterial pellets were collected by centrifugation at 6000g for 15 min and preserved at –80°C.

To isolate recombinant proteins, frozen bacterial pellets were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol; ~2 mL/g of bacteria) and sonicated on ice. Lysates were centrifuged at 11,000g at 4°C for 30 min, and supernatants were loaded on a nickel nitrilotriacetic acid (Ni-NTA) column (Qiagen) pre-equilibrated with buffer A. After extensive washes with buffer A containing 10 mM imidazole, fusion proteins were eluted with a 20–500 mM gradient imidazole. Fractions containing significant amounts of proteins were pooled together, concentrated with a YM-10 concentrator (GE Healthcare), and then desalted by gel filtration with a PD10 column (GE Healthcare). When MBP and His tags needed to be removed, TEV protease digestion was performed on Ni-NTA resin-immobilized fusion proteins before elution. Proteins were assayed freshly or stored at –80°C in a Tris-HCl buffer (50 mM, pH 7.4) containing 10% glycerol and 1 mM dithiothreitol. The quality of each purified protein was determined by SDS-PAGE analysis, and the concentrations were determined by a bicinchoninic acid method using BSA as a standard. The molecular weight of FPLC-purified *CpmtFd* was determined by ESI-MS with SCIEX API III plus electrospray mass spectrometer.

Biochemical characterization of holo-*CpmtFd* and holo-*CpmtFNR* proteins

Functional Fd and FNR require specific post-translational incorporation into proteins of [2Fe–2S] cluster or flavin, respectively. Whether [2Fe–2S] cluster was correctly assembled into the recombinant *CpmtFd* protein was determined by its characteristic spectrophotometric pattern in buffer C (50 mM Tris-HCl, pH 8.0) with a HP8453 UV/vis light spectrophotometer (Hewlett-Packard). EPR spectra of *CpmtFd* were recorded at 4 K on a Bruker EMX EPR spectrometer under the conditions of microwave frequency at 9.487 GHz, microwave power at 2.01 mW, modulation frequency at 100 kHz, modulation amplitude at 11.80 G, and time constant at 20 ms. The reduced form of *CpmtFd* with mixed-valence (Fe³⁺/Fe²⁺)

metal center was prepared by addition of sodium dithionite and the EPR sample was then quickly frozen in liquid nitrogen. To determine the metal content, recombinant CpmF_d was digested overnight in 1M HNO₃ (Trace Metal Grade) and then diluted into 0.2M HNO₃ solution for analysis with an X7 ICP-MS (Thermo Electron Corporation.). The Fe/protein ratio was calculated based on the ICP-MS metal analysis. Meanwhile, the UV/vis spectra of CpmF_d were used to estimate the ratio of holo-protein with typical multi-peaks, especially A_{416}/A_{276} .

The assembly of cofactor flavin in CpmF_{NR} was determined by UV/vis spectrometry. The flavin content was further confirmed by isocratic chromatography.³⁴ Briefly, flavin was released from recombinant CpmF_{NR} protein by heating samples at 95°C for 5 min in buffer A and isolated by isocratic chromatography in a methanol/water mixture (ratio, 30:70) containing 5 mM ammonium acetate (pH 6.0). In this separation, FAD and riboflavin had retention times of 4.5 and 10.2 min, respectively.

The CpmF_{NR}-catalyzed electron transfer from NADPH to CpmF_d_{ox} was analyzed by a cyt *c*-coupled assay, in which cyt *c* was used as an artificial terminal electron acceptor (see illustration in Fig. 1).¹⁶ Recombinant proteins with MBP or His tag removed by TEV digestion were used in electron transfer assays. Typical reactions were performed in Tris-HCl buffer (100 mM, pH 7.4, and pH 8.2) or 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffer (100 mM, pH 6.8) containing 0.02 μM CpmF_{NR}, 2.0 μM CpmF_d, 12 μM cyt *c*, and NADPH at concentrations varying from 0 to 100 μM. Glucose-6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (2 μg/mL) were added into the reaction to regenerate and maintain a constant level of NADPH. The reduction of cyt *c* ($\epsilon_{550\text{ nm}} = 19.6\text{ mM}^{-1}\text{ cm}^{-1}$) were monitored on an HP8453 spectrophotometer at 25°C and 550 nm. All assays were performed at least in triplicate.

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