

Solution structure for an *Encephalitozoon cuniculi* adrenodoxin-like protein in the oxidized state

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Abstract

Encephalitozoon cuniculi is a unicellular, obligate intracellular eukaryotic parasite in the Microsporidia family and one of the agents responsible for microsporidiosis infections in humans. Like most Microsporidia, the genome of *E. cuniculi* is markedly reduced and the organism contains mitochondria-like organelles called mitosomes instead of mitochondria. Here we report the solution NMR structure for a protein physically associated with mitosome-like organelles in *E. cuniculi*, the 128-residue, adrenodoxin-like protein *Ec*-Adx (UniProt ID Q8SV19) in the [2Fe-2S] ferredoxin superfamily. Oxidized *Ec*-Adx contains a mixed four-strand β -sheet, β 2- β 1- β 4- β 3 ($\downarrow \uparrow \uparrow \downarrow$), loosely encircled by three α -helices and two 3_{10} -helices. This fold is similar to the structure observed in other adrenodoxin and adrenodoxin-like proteins except for the absence of a fifth anti-parallel β -strand next to β 3 and the position of α 3. Cross peaks are missing or cannot be unambiguously assigned for 20 amide resonances in the ^1H - ^{15}N HSQC spectrum of *Ec*-Adx. These missing residues are clustered primarily in two regions, G48-V61 and L94-L98, containing the four cysteine residues predicted to ligate the paramagnetic [2Fe-2S] cluster. Missing amide resonances in ^1H - ^{15}N HSQC spectra are detrimental to NMR-based solution structure calculations because ^1H - ^1H NOE restraints are absent (glass half-empty) and this may account for the absent β -strand (β 5) and the position of α 3 in oxidized *Ec*-Adx. On the other hand, the missing amide

resonances unambiguously identify the presence, and immediate environment, of the paramagnetic [2Fe-2S] cluster in oxidized *Ec*-Adx (glass half-full).

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1 | INTRODUCTION

Microsporidia are an odd collection of obligate intracellular eukaryotic parasites that were only recently grouped into the Fungi kingdom.¹ They are odd because, in addition to an elaborate infection mechanism involving spores, these eukaryotes do not have mitochondria.² Most of the >1,300 formally described microsporidia species infect animals with 15 of these documented to infect humans.³ Symptoms of human infections (microsporidiosis) include neurological, renal, and/or gastrointestinal disorders with immunocompromised individuals at most risk of life threatening complications.^{4,5} Transmission can occur in multiple ways including fecal-fecal or oral-oral routes, the ingestion of food contaminated with infected fecal material, aerosol inhalation, or from infected organs received through transplantation.^{5,6} As detection methods improve human microsporidiosis may prove to be more prevalent than currently thought. Because there currently are no approved standardized treatments for microsporidiosis and there is some evidence that microsporidia are showing signs of resistance to the most commonly used antimicrobial (albendazole) for general treatment, new strategies targeting microsporidiosis are needed.⁷

One of the Microsporidia species known to infect humans is *Encephalitozoon cuniculi*. At 2.9 Mb, the 11 linear chromosomes of *E. cuniculi* comprise the smallest known eukaryotic genome with 1,997 potential protein-coding genes.^{8,9} Among the consequences of the compact genome is shorter putative proteins relative to their eukaryotic orthologues, reduced metabolic capacities that requires scavenging resources from the host, and the absence of mitochondria with all the typically associated mitochondrial proteins.⁸ In place of mitochondria, *E. cuniculi* contains a mitosome, a chromosome-less, mitochondrion-derived organelle,^{10,11} and the *E. cuniculi* genome encodes only

22 proteins with significant sequence similarity to the 423 proteins associated with yeast mitochondria. Among the 22 mitosome-associated genes identified in *E. cuniculi* are five genes involved with the synthesis and cytosolic export of Fe-S clusters, essential functions that appear to be conserved in mitochondria.^{9,12} One of these five *E. cuniculi* genes encodes for a 128-residue adrenodoxin-like protein, *Ec-Adx*, a subclass of [2Fe-2S]-type ferredoxins.^{13,14} A punctuate distribution of immunofluorescent tagged *Ec-Adx* in the cytoplasm of *E. cuniculi* suggests this protein may be associated with a mitosome-like organelle where it contributes to iron-sulfur cluster assembly and other ferredoxin-associated functions.¹⁵

Ferredoxins comprise a large superfamily of structurally diverse iron-sulfur proteins found in all kingdoms of life.¹⁶ They function as electron carriers and play essential roles in a plethora of metabolic reactions. Adrenodoxins refer to a subclass of ferredoxins that shuttle electrons from an NADPH-dependent adrenodoxin reductase to cytochrome P450 as part of steroid hormone biosynthesis and vitamin D metabolism.^{14,17} While the genome of *E. cuniculi* contains an annotated adrenodoxin reductase gene (ECU11_1310m.01) it does not appear to contain a cytochrome P450 carrier protein. This observation, together with the compact nature of the *E. cuniculi* genome plus the absence of mitochondria suggests the *Ec-Adx* may serve functional roles distinct from human and other higher eukaryotic adrenodoxins.⁸ As a first step towards identifying potentially unique functions for *Ec-Adx*, the solution structure for this protein in the oxidized state was solved using NMR-based methods.

2 | RESULTS AND DISCUSSION

2.1 | Solution structure of oxidized *Ec*-Adx

The color of the purified and concentrated protein (~ 1 mM), with or without the N-terminal tag, was brownish-red, a sign that iron (eg; the [2Fe-2S] complex) was associated with *Ec*-Adx.^{18,19} Before and after cleavage of the N-terminal tag, oxidized *Ec*-Adx eluted off a size exclusion column with a retention time expected for an approximately 14.4 kDa protein (data not shown), suggesting it was a monomer in solution as reported for other adrenodoxins at these concentrations (mM).^{13,20} This conclusion was corroborated by the overall rotational correlation time (τ_c) estimated from ¹⁵N spin relaxation rates at 20°C, 7.0 ± 0.3 ns, that is in the range expected for an ~14 kDa protein. Furthermore, there was good magnetization transfer in all the backbone NMR assignment experiments, an unlikely observation if *Ec*-Adx formed stable dimers or higher order oligomers. While *Ec*-Adx behaves as a monomer at mM protein concentrations, at lower protein concentrations (< 0.1 mM) adrenodoxins have been reported to assemble into dimers, a complex that may be physiological relevant at the levels found in mitochondria.²¹

The ¹H-¹⁵N HSQC spectrum of oxidized *Ec*-Adx shown in Figure S1 contains cross peaks of near uniform intensities and good dispersion in both dimensions, features characteristic of a folded protein.²² All the amide cross peaks were unambiguously assigned except for the two cross peaks labeled with a question mark. This left 20 residues, colored cyan in the primary amino acid sequence of *Ec*-Adx shown in Figure 1(b), for which amide resonances were not observed or could not be unambiguously assigned. Most of these residues were in two regions containing the four

cysteine residues (C50, C56, C59 and C96) whose side chains are predicted, on the basis of sequence alignment to adrenodoxins with known structures, to ligate the iron-sulfur complex. The absence of cross peaks for these four cysteine residues confirms that these residues are near the [2Fe-2S] cluster because in the oxidized state both irons are high-spin Fe(III) and paramagnetic.^{20,23} A major consequence of this paramagnetic moiety in a protein environment is that the line widths of nearby resonances broaden beyond detection and disappear.^{19,20}

While paramagnetic atoms may also perturb the strength of the NOEs near the paramagnetic site, the largest “damage” to the structure calculations are the elimination of observable chemical shifts near the paramagnetic site (eg: the 20 missing or unassigned amide resonance in Figure 1a and their associated side chain resonances). Consequently, structure calculations using the observable NOE (1099), torsion angle (138 Phi and Psi), and hydrogen bond (32) data were continued without adjustment for any distant paramagnetic effects except for the addition of 16 restraints between the side chain sulfur atom of the four missing cysteine residues and the [2Fe-2S] cluster. Analysis of the final structure ensemble with the PSVS validation-software package confirmed a quality set of final structures.²⁴ Ramachandran statistics for the Phi/Psi pairs of all the residues in the ensemble were in favored (94%) or allowed (6%) regions, all the structure-quality Z scores were acceptable (> -4), and the RMSD of the structured core of each ensemble from the mean structure was just under 0.9 Å for the backbone atoms (N-C $^{\alpha}$ -C=O) and 1.33 Å for all heavy atoms. A detailed summary of the restraints and the PSVS analyses are provided in Table 1.

Figure 1(b) is a backbone superposition of ordered regions in the final ensemble of 20 structures calculated for *Ec*-Adx. As illustrated more clearly in Figure 1(c), a cartoon representation of the structure closest to the average structure in the ensemble, the *Ec*-Adx structure consists of a mixed strand β -sheet, $\beta 2$ - $\beta 1$ - $\beta 4$ - $\beta 3$ ($\downarrow\uparrow\uparrow\downarrow$), loosely encircled by three helices, $\alpha 1$ (V35-N42), $\alpha 2$ (E65-L71) and $\alpha 3$ (D76-D83), and two 3_{10} helices, $\eta 1$ (G90-S92) and $\eta 2$ (K102-F104). Both termini are disordered and omitted from the ensemble presented in Figure

1b. While there is some disorder in the loops between the elements of secondary structure, the most disordered region is around the [2Fe-2S] cluster as expected due to the paramagnetism induced lack of restraints for this region. As shown in Figure 1(d), the missing or unassigned amide resonances all surround the paramagnetic [2Fe-2S] cluster.

A search for structures similar to *Ec*-Adx (5UJ5) using the DALI server²⁵ identified similarities to many proteins annotated as adrenodoxins, putidaredoxins, and ferredoxins with the highest Z-score (9.0) observed for the human ferredoxin Fdx2 (*Hs*-Fdx2). There are two human mitochondrial ferredoxins, *Hs*-Fdx1 (3PLM) and *Hs*-Fdx2 (2Y5C), that are very similar structurally (DALI generated backbone RMSD of 1.3 Å) despite 33% sequence identity.^{17,19} The primary amino acid sequences for both these human ferredoxins are shown in Figure 1a along with the elements of secondary structure for the structure closest to *Ec*-Adx, *Hs*-Fdx2. The sequence alignment with the secondary structure elements show the elements and position of secondary structure are very similar for the two proteins except for the extra β -strand ($\beta 4$) in *Hs*-Fdx2 and the position of one 3_{10} -helix ($\eta 1$). In *Ec*-Adx the equivalent amino acid sequence includes one

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residue (L94) that is missing or could not be unambiguously assigned in the ^1H - ^{15}N HSQC spectrum of *Ec*-Adx [highlight white in Fig. 1(a)] and this region is next to the sequence containing the fourth cysteine ligated to the [2Fe-2S] cluster that is also missing assigned ^1H - ^{15}N HSQC cross peaks (C96-L98). Consequently, it is possible that the β -sheet in *Ec*-Adx also contains five β -strands, but, one strand is not observed in our NMR solution structure due to the paramagnetic effect of the [2Fe-2S] cluster.

Figure 1(e) is a superposition of the structures of *Ec*-Adx (blue, 5UJ5) and *Hs*-Fdx2 (red, 2Y5C) generated by Chimera.²⁶ While only 38% of the primary amino acid sequence of *Ec*-Adx is identical to the sequence of *Hs*-Fdx2 [Fig. 1(a)], the two structures generally superimpose well upon each other, with a DALI generated backbone RMSD of 3.2 Å, except for one α -helix, α 3. In the crystal structure of *Hs*-Fdx2 α 3 sits over top of the [2Fe-2S] cluster. If α 3 sits too close over the [2Fe-2S] cluster in *Ec*-Adx it would not be possible to identify ^1H - ^1H NOEs between α 3 and the residues nearest the [2Fe-2S] cluster because of the paramagnetic effect. Hence, the position of α 3 in the solution structure of *Ec*-Adx may not represent its true position. Note that in the position of β 5 in the structure of *Hs*-Fdx2 there is an unstructured loop over top the β -sheet in *Ec*-Adx which, as discussed in the previous paragraph, cannot be defined as a β -strand due to the paramagnetic effect as well.

2.2 | Solution structure of *Ec*-Adx changes as a function of the [2Fe-2S] oxidation state

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It has been observed that the ^1H - ^{15}N HSQC spectrum of ferredoxins change in the oxidized and reduced states^{19,20} In the reduced state one iron is Fe(II) and the other Fe(III), and consequently paramagnetic effects will still be observed.^{20,23} While some of the chemical shift differences between the two redox states may be due the different paramagnetic effects of the two states, this is likely limited to 8 Å from the [2Fe-2S] cluster.²⁰

To determine if the ^1H - ^{15}N HSQC spectrum of *Ec*-Adx was dependent on its redox state the oxidized protein was reduced under anoxic conditions and an ^1H - ^{15}N HSQC spectrum collected. Conversion of oxidized *Ec*-Adx into the reduced state with sodium dithionite was corroborated by a visible color change in the protein solution from dark brown to pink.¹⁹ The ^1H - ^{15}N HSQC spectra of reduced (red) and oxidized (black) *Ec*-Adx, overlaid in Figure 2(a), show substantial differences, as reported for other ferredoxins, further supporting a change in oxidation state. The wide spread chemical shift perturbations are likely not due to self-association as the estimated overall rotational correlation time (τ_c) at 20°C, 7.3 ± 0.5 ns, was similar to that observed for oxidized *Ec*-Adx, 7.0 ± 0.3 ns. Only 44 amide resonances are not significantly perturbed between the two redox states and these are mapped onto a representation cartoon structure of *Ec*-Adx in Figure 2(b). The unperturbed chemical shifts cluster in regions of the primary amino acid sequence and are, for the most part, the most distant regions from the [2Fe-2S] cluster. However, these most distant regions are also intertwined with some perturbed regions, suggesting the redox state is influencing the overall structure to some degree. It should be noted that most of $\alpha 3$ is not included in an unperturbed region, supporting the previous suggestion that this helix may really be

near the [2Fe-2S] cluster in oxidized *Ec*-Adx and influenced by the different paramagnetic effect in the reduced state (or alternatively, the position of this helix changes in the reduced state).

3 | CONCLUSIONS

The solution structure of oxidized *Ec*-Adx is generally similar to the structure of other adrenodoxins with the closest similarity to a protein found in one of *E. cuniculi*'s hosts, human Fdx2. The solution structure for *Ec*-Adx may be even more similar to *Hs*-Fdx2 if the absent β -strand (β 5) and the position of α 3 are artifacts of the structure calculations due to the lack of distance restraints manifest by the paramagnetic nature of the [2Fe-2S] cluster. Differences in the ^1H - ^{15}N HSQC spectra of reduced and oxidized *Ec*-Adx are suggestive of different paramagnetic effects of the [2Fe-2S] cluster and structural differences, however, it is difficult to judge the magnitude of the structural differences without further extensive experimentation. While missing amide resonances in ^1H - ^{15}N HSQC spectra are detrimental to NMR-based solution structure calculations because they prohibit NOE observations or unambiguous NOE assignments,²⁷ they can also provide useful information such as the identification of regions with unusual backbone dynamics,²⁸ or, as is the case here, the presence of a paramagnetic species and its immediate environment.²⁹

In summary, solving the structure for *Ec*-Adx as a first step towards identifying potentially unique functions for this protein suggests that its core structure is similar to other adrenodoxins. This has generally been observed for the structure of other ferredoxins^{17,30} including the two

discussed here, *Hs-Fdx1* and *Hs-Fdx2*. These two human ferredoxins share only 33% sequence identity and while there is evidence they are promiscuous, capable of performing the same function but at different efficiencies,¹⁹ there is also conflicting evidence that they may have unique primary functions.¹⁷ Indirect evidence for the later conclusion is that a mutation to the *Hs-Fdx2* gene has been associated with a novel mitochondrial muscle myopathy.³¹ Hence, specific features of the function of *Hs-Fdx1* and *Hs-Fdx2* likely rest in the 67% of their sequence that are not identical, either in the regions between elements of secondary, or as suggested for *Hs-Fdx1* and *Hs-Fdx2*, less conserved regions of elements of secondary such $\alpha 1$, $\alpha 2$, and $\beta 2$ [Fig. 1(a)].¹⁹ The primary amino acid sequence between *Ec-Adx* and *Hs-Fdx2* are also largely non-identical (60%), and hence, these differences could be the basis for different biological functions, and perhaps more importantly, the basis for new antimicrobial drugs targeting *E. cuniculi*. While further biochemical studies are required to identify the biological function(s) of *Ec-Adx*, especially with regards to potential roles associated with human infections, the compact nature of the *E. cuniculi* genome, the absence of mitochondria in *E. cuniculi*, the association of *Ec-Adx* with mitosome-like organelles in the cytoplasm of *E. cuniculi*,¹⁵ and the solution structure of *Ec-Adx* presented here suggest *Ec-Adx* is a drug target still worth attention.⁸

4 | EXPERIMENTAL PROCEDURES

4.1 | Cloning, expression, and purification of *Ec-Adx*

The *Ec-Adx* gene (UniProt ID Q8SV19) was amplified from the genomic DNA of *E. cuniculi* strain GB-M1 using the oligonucleotide primers 5'-**GGGTCCTGGTTCGATGGACATGTTTCAGTGCTCCGG**-3' (forward) and 5'-**CTTGTTTCGTGCTGTTTATTAATGGGGCTTGGGCTTGAACCC**-3' (reverse) (Invitrogen, Carlsbad, CA) containing LIC primers (bold). Ligation independent cloning was then used to insert the amplified gene into the pET-14b-derived expression vector AVA0421 at a site (*NruI/PmeI*) that provided an 21-residue extension at the N-terminus (MA**HHHHHH**MGTLEAQTQGPS-) that contained a poly-histidine metal affinity tag (bold) and the human rhinovirus 3C protease cleavage sequence (underlined).³² Using a heat shock method, the recombinant plasmid was used to transform chemically competent *Escherichia coli* BL21(DE3)-R3-pRARE2 cells from which ~ 1 mL ~ 15% glycerol stocks were prepared from a single colony and frozen (-80°C) until required. To obtain uniformly ¹⁵N-, ¹³C-labeled *Ec-Adx*, 750 mL of minimal medium (Miller) containing ¹⁵NH₄Cl (1 mg/mL), D-[¹³C₆]glucose (2.0 mg/mL), NaCl (50 μg/mL), MgSO₄ (120 μg/mL), CaCl₂ (11 μg/mL), Fe₂Cl₃ (10 ng/mL), chloramphenicol (35 μg/mL), and ampicillin (100 μg/mL) was inoculated directly with 20 mL of LB culture grown to an OD₆₀₀ reading of approximately one from a frozen stock culture. Upon reaching an OD₆₀₀ reading of ~ 0.8 (37°C), the culture was transferred to a 25°C incubator shaker and protein expression induced with isopropyl β-D-1-thiogalactopyranoside (0.026 μg/mL). Approximately four hours later the cells were harvested by mild centrifugation and frozen (-80°C). *Ec-Adx* was purified from the thawed pellet using a conventional two-step protocol: metal chelate

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affinity chromatography on a 20 mL Ni-Agarose 6 FastFlow column (GE Healthcare, Piscataway, NJ) followed by gel-filtration chromatography on a Superdex75 HiLoad 26/60 column (GE Healthcare, Piscataway, NJ).²⁷ After the final step the protein (NMR buffer: 100 mM NaCl, 20 mM Tris, pH 7.0) was concentrated to ~ 15 mg/mL and diluted with an equal volume of 3C protease buffer (150 mM NaCl, 25 mM Tris, pH 7.5). The reaction was complete following overnight incubation at 5°C with 3C protease (1 µg protease/50 µg *Ec*-Adx) and the solution reapplied to the gel-filtration column to separate the cleaved protein from the N-terminal tag and 3C protease. The cleaved *Ec*-Adx fractions were pooled and concentrated to ~ 10 mg/mL for NMR data collection. Note that after N-terminal tag removal *Ec*-Adx contained four scar residues, GPGS-, before the first native residue.

4.2 | Preparation of reduced *Ec*-Adx

Reduced *Ec*-Adx was prepared following the protocol described by Cia *et al.*¹⁹ Oxidized *Ec*-Adx (~ 1 mM, 500 µL) was purged with nitrogen, sealed, and placed in a Vacuum Atmospheres-NEXUS glovebox (Hawthorne, CA) overnight ($O_2 < 0.5$ ppm) along with similarly purged NMR buffer. Sodium dithionite (~ 10 mM) was added to the protein solution and allowed to sit for approximately one hour. The sodium dithionite was removed by repetitive spin-filtrations (10 kDa molecular weight cut-off) from ~ 500 to ~100 µL with the final volume adjusted to 300 µL. While still in the anoxic chamber, the reduced *Ec*-Adx was placed into a Shigemi tube and sealed airtight.

4.3 | NMR data collection

All NMR data for oxidized *Ec*-Adx was collected at 20°C on a double-labeled (¹³C-, ¹⁵N-) sample (~ 1.0 mM) using Agilent spectrometers (500 to 800 MHz ¹H resonance frequency) equipped with an HCN-probe (room temperature or cyro) and pulse field gradients. The chemical shifts (¹H, ¹³C, and ¹⁵N) of the backbone and side chain resonances were assigned from the analysis of two-dimensional ¹H-¹⁵N HSQC, ¹H-¹³C HSQC, HBCBCGCDHD, and HBCBCGDCHE spectra and three-dimensional HNCACB, CBCA(CO)NH, HNCA, HCC-TOCSY-NNH, CC-TOCSY-NNH, HNCO, ¹⁵N-edited NOESY-HSQC, and ¹³C-edited NOESY-HSQC (aliphatic and aromatic) spectra. The NOE data, collected with a mixing time of 90 ms, were extensively employed to make the side chain ¹H assignments. Identification of backbone hydrogen bonds were made by lyophilizing a ¹⁵N-labeled NMR sample, re-dissolving in a similar volume of 99.8% D₂O, rapidly collecting a ¹H-¹⁵N HSQC spectrum (~ 10 minutes later), and assigning amide resonances that had not yet exchanged with D₂O. An overall rotational correlation time, τ_c , was estimated for oxidized and reduced *Ec*-Adx from the ratio of collective backbone amide ¹⁵N T₁ and T_{1ρ} measurements.³³ The raw NMR data were processed with Felix2007 (MSI, San Diego, CA) and then analyzed with Sparky (v3.115).³⁴ The ¹H, ¹³C, and ¹⁵N chemical shifts were deposited into the BioMagResBank database (www.bmrb.wisc.edu) with the BMRB number 30231.

4.4 | Structure calculations

The assigned ^1H , ^{13}C , and ^{15}N chemical shifts, the peak-picked NOESY data, and TALOS-derived backbone torsion angles served as the initial experimental inputs in iterative structure calculations using the program CYANA (v 2.1).³⁵ The dihedral Psi (Ψ) and Phi (Φ) torsion angle restraints were obtained by the input of the assigned chemical shifts into the program TALOS+ using the online webserver (<https://spin.niddk.nih.gov/bax/nmrserver/talos/>).³⁶ Hydrogen bond restraints (1.8–2.0 Å and 2.7–3.0 Å for the NH–O and N–O distances, respectively) were introduced next on the basis of proximity in early structure calculations and the observation of slowly exchanging amides in the deuterium exchange experiment. Near the end of the calculation process, 16 iron-sulfur restraints were introduced based on distances observed in the crystal structure of oxidized yeast adrenodoxin (2MJD): CysS $^{\gamma}$ -Fe = 2.1 - 2.2 Å, Fe-Fe = 2.6 - 2.7 Å, S $^{\gamma}$ -S $^{\gamma}$ = 3.4 - 3.5 Å.³⁷ Structural quality was assessed using the online Protein Structure Validation Suite (PSVS, v1.5)²⁴ with pertinent values included in the structure statistics summary (Table 1). The atomic coordinates for the final ensemble of 20 *Ec*-Adx structures have been deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) with the PDB ID code 5UJ5.

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REFERENCES

1. Van de Peer Y, Ben Ali A, Meyer A (2000) Microsporidia: Accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. *Gene* 246:1-8.
2. Keeling P (2009) Five questions about microsporidia. *PLoS Pathog* 5:e1000489.
3. Wittner M, Weiss LM The microsporidia and microsporidiosis. Washington (D.C.): ASM Press.
4. Didier ES, Maddry JA, Brindley PJ, Stovall ME, Didier PJ (2005) Therapeutic strategies for human microsporidia infections. *Expert Rev Anti Infect Ther* 3:419-434.
5. Mathis A, Weber R, Deplazes P (2005) Zoonotic potential of the microsporidia. *Clin Microbiol Rev* 18:423-445.
6. Hocevar SN, Paddock CD, Spak CW, Rosenblatt R, Diaz-Luna H, Castillo I, Luna S, Friedman GC, Antony S, Stoddard RA, Tiller RV, Peterson T, Blau DM, Sriram RR, da Silva A, de Almeida M, Benedict T, Goldsmith CS, Zaki SR, Visvesvara GS, Kuehnert

MJ (2014) Microsporidiosis acquired through solid organ transplantation: A public health investigation. *Ann Intern Med* 160:213-220.

7. Santiana M, Pau C, Takvorian PM, Cali A (2015) Analysis of the beta-tubulin gene and morphological changes of the microsporidium *annaliia algerae* both suggest albendazole sensitivity. *J Eukaryot Microbiol* 62:60-68.
8. Katinka MD, Duprat S, Cornillot E, Metenier G, Thomarat F, Prensier G, Barbe V, Peyretailade E, Brottier P, Wincker P, Delbac F, El Alaoui H, Peyret P, Saurin W, Gouy M, Weissenbach J, Vivarès CP (2001) Genome sequence and gene compaction of the eukaryote parasite *encephalitozoon cuniculi*. *Nature* 414:450-453.
9. Burri L, Keeling PJ (2007) Protein targeting in parasites with cryptic mitochondria. *Intl J Parasitol* 37:265-272.
10. Burri L, Williams BA, Bursac D, Lithgow T, Keeling PJ (2006) Microsporidian mitosomes retain elements of the general mitochondrial targeting system. *Proc Natl Acad Sci USA* 103:15916-15920.
11. Makiuchi T, Nozaki T (2014) Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa. *Biochimie* 100:3-17.
12. Lill R (2009) Function and biogenesis of iron-sulphur proteins. *Nature* 460:831-838.
13. Grinberg AV, Hannemann F, Schiffler B, Muller J, Heinemann U, Bernhardt R (2000) Adrenodoxin: Structure, stability, and electron transfer properties. *Proteins* 40:590-612.

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14. Ewen KM, Ringle M, Bernhardt R (2012) Adrenodoxin--a versatile ferredoxin. *IUBMB Life* 64:506-512.
 15. Williams BA, Cali A, Takvorian PM, Keeling PJ (2008) Distinct localization patterns of two putative mitochondrial proteins in the microsporidian *Encephalitozoon cuniculi*. *J Eukaryot Microbiol* 55:131-133.
 16. Atkinson JT, Campbell I, Bennett GN, Silberg JJ (2016) Cellular assays for ferredoxins: A strategy for understanding electron flow through protein carriers that link metabolic pathways. *Biochemistry* 55:7047-7064.
 17. Sheftel AD, Stehling O, Pierik AJ, Elsasser HP, Muhlenhoff U, Webert H, Hobler A, Hannemann F, Bernhardt R, Lill R (2010) Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2, with distinct roles in steroidogenesis, heme, and Fe/S cluster biosynthesis. *Proc Natl Acad Sci USA* 107:11775-11780.
 18. Buchko GW, Hewitt SN, Napuli AJ, Van Voorhis WC, Myler PJ (2011) Solution-state NMR structure and biophysical characterization of zinc-substituted rubredoxin B (Rv3250c) from *Mycobacterium tuberculosis*. *Acta Cryst F* 67:1148-1153.
 19. Cai K, Liu G, Frederick RO, Xiao R, Montelione GT, Markley JL (2016) Structural/functional properties of human Nfu1, an intermediate [4Fe-4S] carrier in human mitochondrial iron-sulfur cluster biogenesis. *Structure* 24:2080-2091.

20. Beilke D, Weiss R, Lohr F, Pristovsek P, Hannemann F, Bernhardt R, Ruterjans H (2002) A new electron transport mechanism in mitochondrial steroid hydroxylase systems based on structural changes upon the reduction of adrenodoxin. *Biochemistry* 41:7969-7978.
21. Pikuleva IA, Tesh K, Waterman MR, Kim Y (2000) The tertiary structure of full-length bovine adrenodoxin suggests functional dimers. *Arch Biochem Biophys* 373:44-55.
22. Yee A, Chang X, Pineda-Lucena A, Wu B, Semesi A, Le B, Ramelot T, Lee GM, Bhattacharyya S, Gutierrez P, Denisov A, Lee C-H, Cort JR, Kozlov G, Liao J, Finak G, Chen L, Wishart D, Lee W, McIntosh LP, Gehring K, Kennedy MA, Edwards AM, Arrowsmith CH (2002) An NMR approach to structural proteomics. *Proc Natl Acad Sci USA* 99:1825-1830.
23. Beinert H, Holm RH, Munck E (1997) Iron-sulfur clusters: Nature's modular, multipurpose structures. *Science* 277:653-659.
24. Bhattacharya A, Tejero R, Montelione G (2007) Evaluating protein structures determined by structural genomics consortia. *Proteins* 66:778-795.
25. Holm L (2019) Benchmarking fold detection by DaliLite v.5. *Bioinformatics* <https://doi.org/10.1093/bioinformatics/btz536>.
26. Meng EC, Pettersen EF, Couch GS, Huang CC, Ferrin TE (2006) Tools for integrated sequence-structure analysis with ucsf chimera. *BMC Bioinform* 7:339.

27. Buchko GW, Hewitt SN, Van Voorhis WC, Myler PJ (2018) Solution NMR structures of oxidized and reduced *Ehrlichia chaffeensis* thioredoxin: NMR-invisible structure owing to backbone dynamics. *Acta Cryst F*74:46-56.
28. Buchko GW, Perkins A, Parsonage D, Poole LB, Karplus PA (2016) Backbone chemical shift assignments for *Xanthomonas campestris* peroxiredoxin Q in the reduced and oxidized states: A dramatic change in backbone dynamics. *Biomol NMR Assign* 10:57-61.
29. Buchko GW, Daughdrill GW, de Lorimier R, Rao BK, Isern NG, Lingbeck JM, Taylor JS, Wold MS, Gochin M, Spicer LD, Lowry DF, Kennedy MA (1999) Interactions of human nucleotide excision repair protein XPA with DNA and RPA70 Δ C327: Chemical shift mapping and ^{15}N NMR relaxation studies. *Biochemistry* 38:15116-15128.
30. Morales R, Charon MH, Hudry-Clergeon G, Petillot Y, Norager S, Medina M, Frey M (1999) Refined X-ray structures of the oxidized, at 1.3 Å, and reduced, at 1.17 Å, [2Fe-2S] ferredoxin from the cyanobacterium *Anabaena PCC7119* show redox-linked conformational changes. *Biochemistry* 38:15764-15773.
31. Spiegel R, Saada A, Halvardson J, Soiferman D, Shaag A, Edvardson S, Horovitz Y, Khayat M, Shalev SA, Feuk L, Elpeleg O (2014) Deleterious mutation in *Fdx11* gene is associated with a novel mitochondrial muscle myopathy. *Eur J Hum Genet* 22:902-906.

32. Choi R, Kelly A, Hewitt SN, Napuli AJ, Van Voorhis WC (2011) Immobilized metal-affinity chromatography protein-recovery screening is predictive of crystallographic structure success. *Acta Cryst F* 67:998-1005.
33. Szyperski T, Yeh D, Sukumaran D, Moseley H, Montelione G (2002) Reduced-dimensionality NMR spectroscopy for high-throughput protein resonance assignment. *Proc Natl Acad Sci USA* 99:8009-8014.
34. Goddard TD, Kneller DG. Sparky 3. In. Sparky 3; San Francisco: University of California.
35. Güntert P (2004) Automated NMR structure calculation with CYANA. *Meth Mol Biol* 278:353-378.
36. Shen Y, Delaglio F, Cornilescu G, Bax A (2009) Talos+: A hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J Biomol NMR* 44:213-223.
37. Webert H, Freibert SA, Gallo A, Heidenreich T, Linne U, Amlacher S, Hurt E, Muhlenhoff U, Banci L, Lill R (2014) Functional reconstitution of mitochondrial Fe/S cluster synthesis on Isu1 reveals the involvement of ferredoxin. *Nat Commun* 5:5013.

FIGURE LEGENDS

FIGURE 1. Primary amino acid sequence and solution structure of *Ec*-Adx. (a) Clustal Omega sequence alignment of *Ec*-Adx and the related mitochondria human proteins, *Hs*-Fdx1 and *Hs*-Fdx2. Identical residues are shaded peach and conserved residues blue. Amide residues with amide resonances not unambiguously identified or missing in the ^1H - ^{15}N HSQC spectrum of *Ec*-

Adx are highlighted in white. Above and below the alignments are the elements of secondary structure observed in *Ec*-Adx (5UJ5) and *Hs*-Fdx2 (2Y5C), respectively. The α -helices, 3_{10} -helices, and β -strands are colored blue, purple, and magenta, respectively. The four conserved cysteine residues that ligate the [2Fe-2S] cluster are indicated with a red arrow. (b) Backbone superposition of the ordered regions in the final ensemble of 20 structures calculated for *Ec*-Adx (2UJ5). The α -helices, 3_{10} -helices, and β -strands are colored blue, purple, and magenta, respectively. Parts of the N- and C-termini are removed for clarity and the [2Fe-2S] cluster is not shown. (c) Cartoon representation of the structure closest to the ensemble's average structure following the same color scheme as in (b) with the side chains of the four cysteine residues holding the [2Fe-2S] cluster (spheres) shown in stick representation. (d) Solvent accessible surface rendition of the structure shown in (c) with the residues with missing or unassigned amide resonances colored cyan and the cysteine residues holding the [2Fe-2S] cluster (spheres) colored orange. (e) Overlay of the cartoon representations of the structure of *Ec*-Adx (blue, 5UJ5) and *Hs*-Fdx2 (red, 2Y5C) generated by Chimera. For clarity only the iron-sulfur cluster for *Ec*-Adx is shown. The curved black arrow indicates the most significant difference between the two structures, the position of $\alpha 3$.

FIGURE 2. Perturbations to the *Ec*-Adx structure as a function of the oxidation state of the [2Fe-2S] cluster. (a) Overlay of the ^1H - ^{15}N HSQC spectra of oxidized (black) and reduced (red) *Ec*-Adx collected under similar conditions (~ 1.0 mM, 20°C , in 100 mM NaCl, 20 mM Tris, pH 7.0).

The 44 unperturbed amide cross peaks in the spectra of *Ec*-Adx in both redox states are labelled (defined as amide cross peaks that still “touch” each other). (b) Cartoon representation of the *Ec*-Adx structure closest to the average structure in the calculated ensemble. Residues with unperturbed amide chemical shifts after reduction, identified in Figure 1(a), are colored red. Amide cross peaks that were missing or not unambiguously assigned in the oxidized state of *Ec*-Adx are colored cyan and the remaining perturbed amide resonances are colored grey.

Table 1. Summary of the structural statistics for *Ech*-Trx in the reduced and oxidized state^a

| | Ec-Adx |
|---|----------------------|
| PDB ID | 5UJ5 |
| BMRB ID | 30231 |
| SSGCID ID | EncuA.00705.a |
| Restraints for Structure Calculations | |
| Total NOEs | 1099 |
| Intraresidue NOEs | 298 |
| Sequential (i, i + 1) NOEs | 347 |
| Medium-range (i, i + j; 1 < j ≤ 4) NOEs | 118 |
| Long-range (i, i + j; j > 4) NOEs | 335 |
| Phi (Φ) angles | 69 |
| Psi (Ψ) angles | 69 |
| Hydrogen bonds | 32 |
| [2Fe-2S] complex | 16 |
| Structure Calculations | |
| Number of structures calculated | 100 |
| Number of structures used in ensemble | 20 |
| RMSD to Mean (Å) (all residues) | |
| Backbone N-C ^α -C=O Atoms | 7.05 ± 1.00 Å |
| All Heavy Atoms | 7.13 ± 1.10 Å |
| RMSD to Mean (Å) (PSVS ordered residues)^b | |
| Backbone N-C ^α -C=O Atoms | 0.89 ± 0.20 Å |
| All Heavy Atoms | 1.33 ± 0.19 Å |
| Ramachandran Plots Summary^b | |
| Most favored regions | 93.9 % |
| Allowed regions | 6.1 % |
| Disallowed regions | 0 % |
| Global Quality Scores | |
| | Z-score (Raw) |
| Procheck (all) | -3.90 (-0.66) |
| Procheck (Φ, Ψ) | -1.49 (-0.46) |
| MolProbity clash score | -0.11 (9.45) |

^aAll statistics are for the 20-structure ensemble deposited in the Protein Data Bank.

^bRichardson Lab's Molprobity using ordered regions P11-E47,V61-R93, R99-V111.





