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Reduction potential and heme-pocket polarity in low potential cytochrome b_5 of *Giardia intestinalis*

Zhen (Alice) Yang [a], Robert Pazdzior [b], Janet Yee [a,b], Steven Rafferty [a,b,*]

[a] *Environmental and Life Sciences Graduate Program, Trent University, Canada*
[b] *Biochemistry & Molecular Biology Program, Trent University, Canada*

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ABSTRACT

Although it lacks mitochondria and the ability to synthesize heme, the protozoan parasite *Giardia intestinalis* encodes several heme proteins. This includes four members of the cytochrome b_5 family, three of which are of similar size to mammalian cytochromes b_5 but with reduction potentials that are 140 to 180 mV lower. While no structures have yet been determined for any of these proteins, homology modeling points to an increase in heme pocket polarity as a reason for their low potentials. To test this we measured the reduction potentials of four mutants of Giardia cytochrome b_5 isotype-I (gCYTB5-I) in which polar residues at two candidate positions (C84, Y51) in the heme pocket were changed to nonpolar ones (C84A, C84F; Y51L, Y51F). All mutants were expressed with comparable levels of heme incorporation and had UV–visible spectra consistent with low spin bis-histidyl coordination. These mutations increased the reduction potential by 18 to 57 mV and highlight the influence of C84, which is a residue unique to gCYTB5-I and whose mutation to alanine caused the largest increase. The influence of these two residues plus that of Y61 reported previously accounts for much of the reduction potential difference between gCYTB5-I and microsomal cytochrome b_5. A complementary triple mutant of the latter with the hydrophilic residues found in gCYTB5-I bound heme less effectively but nonetheless had a reduction potential that was 135 mV lower than wild type.

1. Introduction

Giardia intestinalis, a microaerotolerant anaerobic protozoan parasite that adheres to the epithelial cells in the upper small intestine of mammals, birds and reptiles is one of the most common causes of infectious diarrhea worldwide [1]. Although it is a eukaryote, Giardia lacks a mitochondrial respiratory chain and the capacity for oxidative phosphorylation, and instead relies on substrate-level phosphorylation and pyrophosphate for energy production [2–4]. Besides lacking respiratory cytochromes, other common heme proteins such as catalase, peroxidases, oxidases, and monooxygenases are also absent. Moreover the Giardia genome encodes none of the genes for heme biosynthesis, a pathway that is nearly universal among eukaryotes and prokaryotes. Surprisingly, its genome does encode several heme proteins, including one flavohemoglobin and four members of the cytochrome b_5 family of electron transfer proteins [5,6].

The heme-binding domain of cytochrome b_5 is approximately 80 amino acid residues with two conserved axial histidine ligands to the heme iron [7]. While such proteins are found in all kingdoms of life, the most well-studied members of this class are microsomal (CYTB5A), and mitochondrial cytochromes b_5 (CYTB5B) [8,9], each of which is anchored to its respective organelle by a carboxyl-terminal transmembrane alpha helix. CYTB5A has roles in fatty acid desaturation, sterol biosynthesis, and cytochrome P450-catalyzed monooxygenations and it supports the activity of indolamine 2,3-dioxygenase [10–12]. A soluble splice variant of CYTB5A is expressed in erythrocytes where it acts as an electron shuttle to reduce methemoglobin and restore it to a functional state [13]. The mitochondria CYTB5B can direct electrons into the respiratory chain through donation to cytochrome *c* [14]. Cytochrome b_5 folding domains also occur in larger redox enzymes, including sulfite oxidase, nitrate reductases, yeast lactate dehydrogenase, and NADH cytochrome b_5 oxidoreductase [15,16]. Significantly, none of these biochemical processes have been demonstrated in Giardia, nor are these enzymes encoded in its genome; the presence of four cytochrome b_5 genes remains a puzzle.

All four Giardia cytochromes b_5 (gCYTb5s) have been expressed recombinantly as soluble heme-binding proteins [17–19]. Three isotypes (gCYTB5-I, II, III) are of similar size to CYTB5A and CYTB5B and lack a carboxyl terminal transmembrane anchor. They also possess the invariant histidine ligands and have 25 to 40% sequence identity to other members of this protein family; among the gCYTB5s the level of amino acid identity is 36–41%. No Giardia cytochrome b_5 structures have been determined experimentally to date, however structures have been obtained by homology modeling using the cytochrome b_5 domain of human NADH cytochrome b_5 oxidoreductase 4 (Ncb5or,

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[*] Corresponding author at: Biochemistry and Molecular Biology Program, Trent University, Peterborough, ON K9L 0G2, Canada.
E-mail address: srafferty@trentu.ca (S. Rafferty).

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3lf5.pdb) as a template, which has ~34% sequence identity to each of the Giardia proteins (Fig. 1). The template and the gCYTB5s share the following: the pair of axial histidines (H65, H88; numbered according to gCYTB5-I); additional heme pocket residues (Y61, P66, V91); and residues of the β-sheet platform on which the heme-binding α-helices are supported (W48, G53, V55, Y56, and G103). However, the heme-binding pockets of gCYTB5s are more hydrophilic compared to other members of the cytochrome b_5 family, such as CYTB5A and CYTB5B, and are predicted to have fewer acidic residues on the face of the protein containing the exposed heme edge [19].

While reduction potentials for other known cytochromes b_5 range from +78 mV to −100 mV *vs* SHE, the reduction potential of gCYTB5-I, II and III are −171 mV, −140 mV and −157 mV respectively compared to +7 mV for bovine CYTB5A [17]. As suggested by their homology models, gCYTB5s have more polar heme-binding pockets than CYTB5A and CYTB5B (Fig. 2). We have shown that a tyrosine residue corresponding to position-61 of gCYTB5-I is a major determinant, as its mutation to phenylalanine raises the reduction potential by nearly 100 mV [17]. This tyrosine residue is common to all of the gCYTB5s and is likewise responsible in part for their low reduction potentials. However, there are two other sites within the heme-binding pocket that also may help to stabilize the oxidized state of the heme; in gCYTB5-I these are a cysteine residue at position 84 and a tyrosine residue at position-51. To study their influence we made mutations at these positions that introduced residues found in bovine CYTB5A (C84F; Y51L) or were isosteric but less polar (C84A, Y51F) and measured their reduction potentials by spectroelectrochemistry. We also investigated the properties of a bovine CYTB5A triple mutant (L25Y/F35Y/F58C) which introduces the three key hydrophilic heme pocket residues of gCYTB5-I. To facilitate comparison between analogous positions among the gCYTB5s and CYTB5A, which differ in sequence length hence numbering, please refer to Table 1, where they are cross-referenced.

2. Procedure

Bacterial expression vectors for bovine CYTB5A, Giardia gCYTB5-I, II, III, and gCYTB5-I single mutants were prepared using the services provided by DNA 2.0 (Menlo Park CA). The coding sequences were codon-optimized for expression in *Escherichia coli* and also encoded *N*-terminal, tobacco-etch virus (TEV) protease cleavable hexahistidine-tags. These sequences were placed in a pJ401Express vector background, which contains an IPTG-inducible T5 promoter and confers resistance to kanamycin. Vectors were transformed into chemically competent BL21 cells by calcium chloride transformation. Transformants were grown under kanamycin selection (30 μg/mL) in Terrific Broth, and protein expression was induced with IPTG (1 mM) and the heme precursor δ-aminolevulinic acid (0.1 mM). Cells were grown with shaking at 30 °C and were harvested 18–24 h post-induction. Cells were lysed with 1% octylthioglucoside in $\mu = 0.1$ M sodium phosphate buffer, pH 7.0 supplemented with 1 mM TCEP and 1 mM of the protease inhibitors PMSF and captopril. The cell suspension was incubated at room temperature for 30 min, after which time 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 0.01 mg/mL of DNase I were added. The cell suspension was centrifuged at 15,000 *g* for 20 min. Recombinant cytochromes b_5 were purified from the supernatant by immobilized metal affinity chromatography on cobalt-charged HisPur resin (Thermo-Fisher Scientific, Oakville ON).

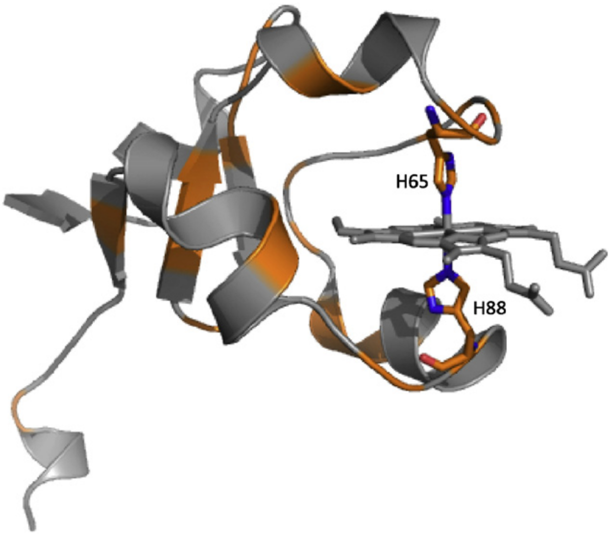

Fig. 1. Ribbon structure of the homology model of gCYTB5-I based on the template structure of the cytochrome b_5 domain of human NADH-cytochrome b_5 oxidoreductase, 3lf5.pdb). In orange are the positions at which model and template have identical residues.

Spectroelectrochemistry was performed with a combination of gold honeycomb electrode/auxiliary electrode in a specialized quartz cuvette, a silver-silver chloride reference electrode, and a WaveNow potentiostat, with all components provided by Pine Research Instrumentation (Durham NC). The cell contained 0.4 to 0.6 mL of solution. To exclude oxygen the system was flushed with a gentle stream of argon gas throughout the experiment; a thin layer of silicon oil on top of the aqueous solution prevented its evaporation over the course of the experiments, which usually took 8 to 10 h. Solution conditions include 0.2–0.5 mM cytochrome, $\mu = 0.1$ M sodium phosphate, and pH 7.0, 22 °C. $Ru(NH_3)6Cl_3$, 2-hydroxy-1, 4-naphthoquinone, and methyl viologen (Sigma-Aldrich Canada, Oakville ON) were used as mediators. Spectroscopic changes as a function of applied potential were recorded with a Cary 400 Bio UV–visible spectrophotometer, with absorbance changes at the heme alpha band (554–559 nm) being used for measurements.

3. Results and discussion

G. intestinalis encodes four members of the cytochrome b_5 family, three of which are of similar size (~15 kDa), consisting of a central cytochrome b_5 folding domain of ~80 residues flanked by 12–24 residues at each end. Unlike mammalian CYTB5A and CYTB5B these proteins do not have hydrophobic membrane anchors and are expressed as soluble proteins. A fourth isotype, gCYTB5-IV, is also soluble, but is also larger (29 kDa) and contains an additional folding domain of unknown function [18]. No role has yet been assigned to any of these proteins.

In the absence of crystallographic or NMR structures for the gCYTB5s we use homology models [19] based on a template of the cytochrome b_5 domain of human NADH-cytochrome b_5 oxidoreductase (Ncb5or) [15] to provide information on the likely structural features of these proteins. Ncb5or has 34% sequence identity to gCYTB5-I, with identical spacing of the coordinating histidine residues (22 intervening residues, compared to 23 for bovine CYTB5A), and similar EPR spectra consistent with nearly perpendicular imidazole planes for the coordinating histidine residues [17,21], compared to a coplanar geometry as observed in CYTB5A. Ncb5or also has a tyrosine residue at a position analogous to gCYTB5-I Y61, which is common to all four Giardia cytochrome b_5 isotypes but not to CYTB5A, where phenylalanine occurs at this position.

The heme-binding sites of CYTB5A and gCYTB5-I are presented in Fig. 2. We had previously identified gCYTB5 Y61 as a major contributor to the low reduction potentials of the Giardia cytochromes, as its mutation to phenylalanine increases its potential by nearly 100 mV [17]. The strong influence of this residue is also supported by previous spectroelectrochemical studies on CYTB5A in which mutation of F35 to tyrosine lowers the reduction potential from +2 mV to −64 mV [22]. However, several other residues within the heme pockets of the gCYTB5s may also contribute to their lower reduction potentials, and we chose to study a set of four single-site mutants in gCYTB5-I at two such positions, C84 and Y51. At each of these we made mutations to