The Crystal Structure of Six Transmembrane Antigen of the Prostate 4 (Steap4), a Ferri/Cuprireductase, Suggests a Novel Inter-Domain Flavin Binding Site

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Background: In-depth biochemical and structural analyses of Steap4 are lacking.

Results: Crystallographic and kinetic characterization of Steap4 metalloreductase and flavin dependent NADPH oxidase activities are reported.

Conclusion: Physiologically relevant ferric/cupric reductase and flavin dependent NADPH oxidase activities are observed at neutral and endosomal pH; the N-terminal tail is dispensable for these activities.

Significance: Metalloreductase activity should be considered in Steap4 associated insulin resistance.

SUMMARY

Steap4 is a cell surface metalloreductase linked to obesity associated insulin resistance. Initial characterization of its cell surface metalloreductase activity has been reported, but thorough biochemical characterization of this activity is lacking. Here we report detailed kinetic analysis of the Steap4 cell surface metalloreductase activities. Steap4 shows physiologically relevant \( K_m \) values for both \( Fe^{3+} \) and \( Cu^{2+} \) and retains activity at acidic pH, suggesting it may also function within intracellular organelles to reduce these metals. Flavin dependent NADPH oxidase activity that was much greater than the equivalent Steap3 construct was observed for the isolated N-terminal oxidoreductase domain. The crystal structure of the Steap4 oxidoreductase domain was determined, providing a structural explanation for these differing activities. Structure-function work also suggested Steap4 utilizes an inter-domain flavin binding site to shuttle electrons between the oxidoreductase and transmembrane domains, and showed that the disordered N-terminal residues do not contribute to enzymatic activity.

The first member of the Six Transmembrane Epithelial Antigen of the Prostate (Steap) family of proteins, was identified as a prostate specific gene highly expressed in advanced prostate cancer (1). Three additional members of the family were subsequently identified and designated Steap2, Steap3 and Steap4 (Steap2-4). Steap2-4 differ significantly from Steap1 by the addition of a cytosolic N-terminal oxidoreductase domain. This N-terminal domain works in conjunction with the C-terminal transmembrane domain to confer cell surface ferric and cupric reductase activity to these larger Steap2-4 family members (2,3).

Steap1 and Steap2 are highly overexpressed in a number of different human cancers (prostate, bladder, colon, pancreas, ovary, testis, breast, cervix, Ewing’s sarcoma) (4-6), however, their roles in both normal and cancer cells are
not well understood (7). In contrast, Steap3 is the only family member for which a clear physiological role has been demonstrated. It serves as the major ferric reductase of the transferrin cycle, where it reduces endosomal Fe$^{3+}$ to Fe$^{2+}$, which is then transported across the endosomal membrane by DMT-1 (divalent metal transporter-1) (2,8-10). Accordingly, the Steap3$^{-/-}$ mouse exhibits microcytic hypochromic anemia (2), and a similar phenotype has been linked to low STEAP3 expression in humans (11).

Like Steap1 and Steap2, functions for Steap4 (also known as Stamp2 and TIARP) are also less clearly defined. In addition to its cell surface metalloreductase activity, it has also been suggested to facilitate trafficking in endocytic and exocytic pathways, and to play a role in macrophage inflammation, where it protects against atherosclerosis (6,12). In addition, the literature also frequently identifies Steap4 as an obesity related protein associated with insulin sensitivity, with knockdown or loss of Steap4 leading to insulin resistance (6,13-17). Obesity associated insulin resistance is a complex metabolic disorder that currently defies explanation by a single etiological pathway, but three recurring themes are found (18). These are: (I) Accumulation of ectopic lipid metabolites; (II) Activation of the unfolded protein response pathway; (III) Activity of innate immune pathways.

With regard to (III), Steap4 expression in adipocytes is normally induced by nutritional stress, leptin, and proinflammatory cytokines including tumor necrosis factor alpha (TNF$\alpha$), interleukin-1$\beta$ and interleukin-6, where it promotes insulin sensitivity (13,15,17,19-27). Similarly, in hepatocytes and HeLa cells, CCAAT/Enhancer-binding proteins $\alpha$ (CEBPA) directly induce transcription of Steap4 in response to feeding, and both CEBPA and the proinflammatory transcription factor STAT3 positively regulate Steap4 in response to IL-6 (17,23).

Conversely, knock-down or loss of Steap4 expression in adipocytes inhibits translocation of GLUT4 to the plasma membrane, inhibiting insulin stimulated glucose transport, rendering the cells insulin insensitive (13,28). Lack of Steap4 also leads to increased production of IL-6 in these cells. Consistent with these observations, Steap4$^{-/-}$ mice are reported to exhibit aberrant inflammatory responses to nutrients and acute inflammatory stimuli, and their adipose tissue exhibits overt inflammation. Further, the mice are also reported to develop insulin resistance (17). Finally, recent studies have also found correlations between STEAP4 and insulin resistance in humans (19,29-33).

A central question, then, is whether the metalloreductase activity of Steap4 in adipocytes and hepatocytes is related to its association with insulin resistance. In this regard, connections between iron metabolism and the unfolded protein response (see II above) are well established (34). So too are connections between iron metabolism and innate immunity (34), including the Steap4 specific connections to IL-6, TNF$\alpha$ and STAT3 discussed above (III). From this perspective then, a role in iron homeostasis seems consistent with its role in insulin sensitivity, and for this reason, biochemical understanding of the Steap4 metalloredox activity is of significant interest.

Our current understanding of the structural, functional and mechanistic aspects of Steap4 metalloreductase activity are largely inferred from Steap3, where the N-terminal oxidoreductase domain is believed to utilize NADPH to reduce a currently undefined flavin (2,35). The electrons in the reduced flavin are thought to flow, one at a time, into a single heme group housed within the C-terminal transmembrane domain. The heme then facilitates translocation of the electrons across the lipid bilayer to the lumen of the endosome, where Fe$^{3+}$ is reduced to Fe$^{2+}$ (2,35). This heme containing C-terminal domain shows distant homology to the transmembrane domains of the eukaryotic Fre and Nox family proteins, and to YedZ in bacteria (2,36), each of which are involved in transmembrane electron transfer.

Outside the Steap family, the closest structural homologs for the N-terminal oxidoreductase domain is the prokaryotic $F_{420}$ NADPH oxidoreductase, or FNO family of proteins. These enzymes utilize a unique deazaflavin (8-hydroxy-5-deazaflavin, or $F_{420}$) that is not found in mammals (2,35,37). Accordingly, as in FNO, the structure of the
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Steap3 oxidoreductase domain revealed a dinucleotide binding fold that recognizes NADPH, followed by a smaller, approximately 60 residue subdomain presumably involved in flavin recognition, either FAD or FMN (38). The biochemical and structural analysis of Steap3 also suggested that the full-length protein may function as a dimer in vivo (38).

Based on sequence homology and mutagenesis studies (2), Steap2 and Steap4 are also hypothesized to utilize NADPH. However, to date, NADPH oxidase activity has not been demonstrated for any member of the Steap protein family, and there is precedence for the use of other electron donors in cell surface metalloreductases. For example duodenal cytochrome b (Dcytb) utilizes ascorbate (39,40). Similarly, while Steap proteins are thought to utilize a flavin as cosubstrate or cofactor (2,38), direct experimental evidence for a reduced flavin intermediate is also lacking. Indeed, other than an initial demonstration of cell surface metalloreductase activity in whole cells, an in-depth kinetic analysis of these activities for any member of the Steap family is lacking.

Here we report the first detailed kinetic analysis of cell surface metalloreductase activity for the full-length Steap4 protein, including pH profiles and metal specificities. We also report the crystal structure of the Steap4 N-terminal oxidoreductase domain, and kinetic characterization of its NADPH oxidase and flavin reductase activities. This represents the first structural study of Steap4, and the first detailed kinetic analysis of the NADPH oxidase, flavin reductase and metalloreductase activities for any Steap protein.

EXPERIMENTAL PROCEDURES

Cell Surface Ferric and Cupric Reductase Assays - A Rattus norvegicus Steap4 full-length cDNA clone (Open Biosystems) was PCR-amplified and ligated into pCDNA3.1 (Invitrogen) with an N-terminal Strep-II tag using HindIII and XbaI restriction sites. Steap4 mutants were made by PCR with appropriate primers and were cloned into the same sites or were made from the wild-type Steap4 vector using a QuikChange mutagenesis kit (Stratagene). All constructs were sequence verified. HEK-293F cells (Invitrogen) were maintained in Freestyle 293 Expression Medium (Invitrogen) in spinner flasks at 37 °C and 5% v/v CO\(_2\). Transfections utilized linear 25 kDa polyethylenimine (PEI; Polysciences) as described (41). Briefly, plasmid and PEI (26 µg and 52 µg, respectively) were added to 2.6 ml phosphate buffered saline (PBS), pH 7.2. The solution was incubated at room temperature for 15 minutes before adding to a spinner flask containing 23.4 ml of cells resuspended in fresh medium at 1.11 x 10\(^{6}\) cells/ml (1 µg DNA per 10\(^{5}\) cells).

Twenty-four hours post transfection, cells were assayed for metalloreductase activity as with a colorimetric endpoint assay. Cells were pelleted (1,000 x g, 5 min), washed three times with PBS, pH 7.2, and resuspended in 25 mM MES, 25 mM MOPS, 140 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.8 mM CaCl\(_2\), 800 µM MgCl\(_2\), pH 7.2 and aliquoted into 24-well plates (2.5 x 10\(^{5}\) cells/well). The buffer was supplemented with 25 mM Tris for pH dependent experiments. Metal-chelate and chromogenic indicator (ferrozine or bathocuproine disulfonate [BCS]) were added to each well, and the cells were incubated in the dark at 37 °C. The absorbance of the supernatant was measured after 35 minutes to determine the amount of reduced metal. Iron(II)-ferrozine complexes were assayed at 562 nm and copper(I)-BCS complexes at 482 nm. Standard curves for each metal-indicator complex were used to determine picomoles of reduced metal. Activities were calculated as picomoles of reduced metal per minute divided by total cellular protein per well. Total cell protein content was determined by the Bradford method (42) (Bio-Rad) using cells lysed by thirty minute incubation in 1 % v/v Triton X-100 at 4 °C. Fe\(^{3+}\)-NTA was prepared by addition of NTA to an FeCl\(_3\) solution dissolved in 0.1 M HCl at a 1:1 Fe:NTA molar ratio. Fe\(^{3+}\)-citrate was prepared by dissolving Fe\(^{3+}\)-citrate in H\(_2\)O. Cu\(^{2+}\)-NTA was prepared by addition of NTA to a CuSO\(_4\) solution (1:4 Cu:NTA ratio). Cu\(^{2+}\)-histidine solution was prepared by addition of L-histidine to a CuSO\(_4\) solution (1:2 Cu:histidine ratio). All metal solutions were adjusted to pH 7.0. To correct for endogenous ferric or cupric reductase activities, the cell surface activity of empty vector-transfected cells was subtracted in
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Each experiment. Each experiment utilized a common culture of transfected cells to allow direct comparison between the metalloreductase activities. Three activity measurements were made at each substrate concentration and the rates obtained were fit by nonlinear least squares analysis to the Michaelis-Menten equation (Eq. 1) using GraphPad Prism 5.0.

\[ v_o = \frac{V_{max}[S]}{K_m+[S]} \]  
(Eq. 1)

The mean and standard deviation for the kinetic parameters in Table 1 were derived from at least three independent transfections.

Immunofluorescence Microscopy – HEK-293F cells were maintained in DMEM supplemented with 10 % v/v FBS in T-75 flasks at 37 °C and 5 % v/v CO₂. Twenty-four hours prior to transfection, cells were plated onto glass cover slips in a 6-well dish. Transfections were performed with LipoFectamine 2000 (Invitrogen) according to the manufacturer’s protocol using the same Steap4 expression constructs detailed above. Twenty-four hours post transfection, cells were washed three times with PBS and fixed in 4 % v/v formaldehyde-PBS for 15 minutes. Cells were then washed three times with PBS and permeabilized by incubation with 0.2 % v/v Triton X-100-PBS for 20 minutes. Cells were washed three times with PBS and subsequently incubated in blocking buffer (1 % v/v BSA-PBS) for 30 minutes. Cells were then incubated with primary antibody diluted in blocking buffer (anti-StrepMAB [IBA Lifesciences] at 1:250 and anti-CD71 [FITC-conjugated; BD Biosciences] at 1:100) for one hour. Cells were then washed three times with PBS for five minutes and incubated 60 min with 5 µg/ml anti-mouse IgG₁, Alexa Fluor 633-conjugated secondary antibody (Invitrogen) in blocking buffer. Cells were subsequently washed three times with PBS, stained with DAPI for 5 min, and mounted on glass cover slides with Vectashield mounting medium (Vector Laboratories). Samples were visualized with a Leica SP5 confocal microscope. Images were processed with Imaris software (Bitplane).

Expression and Purification of the Steap4 N-terminal oxidoreductase domain - The open reading frame of the rat Steap4 oxidoreductase domain was amplified from cDNA (Open Biosystems) using primers that added an N- or C-terminal His₆-tag and attB sites for Gateway cloning into pDEST14 (Invitrogen). Mutants were made using a QuickChange kit (Stratagene). All clones were sequence verified. The human STEAP3 oxidoreductase fragment (1-215 C-terminal His₆) was expressed and purified as described (38). Neither the human nor the rat Steap fragments copurified with a flavin. Rat oxidoreductase domain constructs were overexpressed by autoinduction (43) (24 h at 25 °C) in E.coli BL21(DE3)RIL (Stratagene), and cells were pelleted and stored at -80 °C. Cells were lysed by microfluidizer (Microfluidics) in lysis buffer [20 mM Tris, 0.5 M NaCl, 5 mM imidazole, 10 mM 2-mercaptoethanol, pH 8.0] plus 100 µM phenylmethylsulfonyl fluoride. Bacterial debris was pelleted (30,000 g x 20 min, 4 °C). The supernatant was bound to 2 ml of Ni-NTA resin at 22 °C and washed with 10 column volumes of lysis buffer before eluting with 20 mM Tris, 0.1 M NaCl, 0.3 M imidazole, 10 mM 2-mercaptoethanol and 20 % v/v glycerol, pH 8.0. The eluant was analyzed by 12 % Tris-tricine SDS-PAGE. If necessary, contaminants were removed with a 5 ml HiTrap Q column (GE Healthcare). Typically, fractions with the oxidoreductase domain were pooled, diluted 1:10 into buffer A [20 mM Tris, 20 % v/v glycerol, 1 mM TCEP (tris (2-carboxylethyl) phosphine), pH 8.0] and bound to the Q column at 22 °C. The Q column was washed to baseline with buffer A, then protein was eluted with a linear gradient of 20 mM Tris, 1.0 M NaCl, 10 % v/v glycerol, 1 mM TCEP at pH 8.0. The oxidoreductase domain eluted at ~150 mM NaCl. Fractions were analyzed by SDS-PAGE, and those with oxidoreductase domain were pooled, concentrated to ~10 mg/ml by ultrafiltration (Amicon Ultra-4) and applied to a Superdex-75 HR 10/30 column (GE Healthcare) equilibrated with 10 mM Tris, 150 mM NaCl, 10 % v/v glycerol, 1 mM TCEP, pH 8.0 at 22 °C. Fractions with oxidoreductase domain were pooled, concentrated to ~10 mg/ml by ultrafiltration and stored at 4 °C. Protein concentrations were determined by Bradford assay (42) with BSA standard. For analytical ultracentrifugation, protein was exchanged into 10 mM Tris, 50 mM NaCl, 2 % v/v glycerol, 2 mM TCEP, pH 8.0, and sent to the University of
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Texas Health Sciences Center for Macromolecular Interactions for analysis.

**NADPH Oxidase Assay** – The oxidation of NADPH was monitored spectrophotometrically at 340 nm using the WinUV Kinetics module in a Varian Cary 50 Bio spectrophotometer. Typical reactions (350 μL) were performed in 0.1 M citric acid, 0.15 M NaCl, pH 5.5 at 22 °C. The initial pH optimization was done with 200 μM NADPH and 200 μM FAD using 1 cm cuvettes. Due to the need to work with higher flavin concentrations to measure flavin K\(_m\) values, subsequent work utilized 0.2 cm cuvettes with NADPH held constant at 100 μM. Three hundred nanomolar protein was used for the more active 1-203 fragment and 3-10 μM protein for the less active 1-195 construct. Components were mixed and the reaction was initiated by addition of NADPH. The auto-oxidation of NADPH in reaction buffer with substrates but without protein was subtracted to yield reaction velocity. Within each experiment, three velocity measurements were made at each substrate concentration. The initial rates obtained from these assays were fit by nonlinear least squares analysis to the Michaelis-Menten equation (Eq. 1) using GraphPad Prism. A representative experiment for each flavin is shown in Fig. 3. Kinetic parameters in Table 2 are the mean and standard deviation from 3 or more such experiments, each using an independent preparation of protein. Substrate stock solutions were made in water and stored at -20 °C in single-use aliquots. The concentration of stock solutions was determined in 0.1 M sodium phosphate, pH 7.0, using the extinction coefficients, NADPH \(\varepsilon_{340\text{nm}} = 6,220 \text{ M}^{-1}\text{cm}^{-1}\); FAD \(\varepsilon_{450\text{nm}} = 11,300 \text{ M}^{-1}\text{cm}^{-1}\); FMN \(\varepsilon_{446\text{nm}} = 12,200 \text{ M}^{-1}\text{cm}^{-1}\); riboflavin \(\varepsilon_{445\text{nm}} = 12,500 \text{ M}^{-1}\text{cm}^{-1}\) (44-46).

**Crystallization, Data Collection and Model Refinement** - Crystals of the rat Steap4 N-terminal domain, residues 1-195 with a C-terminal His\(_6\)-tag, were grown by hanging drop vapor diffusion. Drops contained 2 μL of protein at 7 mg/ml, 1 mM NADPH and 2 μL of well solution (0.1 M bis-Tris HCl pH 5.5, 1.9-2.2 M (NH\(_4\))\(_2\)SO\(_4\)). Cryoprotectant was introduced by sequential soaks (2 - 5 min) in well solution supplemented with 1 mM NADPH and 10 %, 12.5 % and 15 % v/v glycerol. A dataset to 2.2 Å was collected at 100 K at SSRL beamline 9-2. Data were processed with HKL2000 (47) (Table 3). The structure was solved by molecular replacement with MRBUMP (48) using a search model derived from the human Steap3 structure [2VQ3, (35)]. Model building and refinement using COOT and REFMAC5 (48) yielded a model with R\(_{\text{work}}\) = 20.3 % and R\(_{\text{free}}\) = 23.8 % (Table 3). The model was deposited under accession 2YJZ. Figures were prepared using PyMOL (49) and LIGPLOT (50).

**RESULTS**

**Cell Surface Ferric and Cupric Reductase Activities for Rat Steap4** – While the initial demonstration of Steap4 metalloreductase activity was done with the mouse protein (3), our efforts to express and crystallize the mouse (and human) Steap4 N-terminal oxidoreductase domain were unsuccessful. In contrast, we found that the *Rattus norvegicus* (rat) Steap4 oxidoreductase domain was amenable to structural studies (below), and for this reason we chose rat Steap4 for our functional work.

**Does Rat Steap4 Exhibit a Preference for Fe\(^{3+}\) or Cu\(^{2+}\)?** – Steap2-4 have been shown to reduce both Fe\(^{3+}\) and Cu\(^{2+}\) in cell surface metalloreductase assays when transfected into HEK293T cells (3). While it is clear that Fe\(^{3+}\) is a physiologically relevant substrate for Steap3 in the transferrin cycle, it is not clear whether Fe\(^{3+}\) or Cu\(^{2+}\) are physiologically relevant substrates for Steap4. To address the relative specificity of Steap4 for iron and copper, kinetic parameters were derived from cell surface metalloreductase data fit to the Michaelis-Menten equation for each of four different substrates: Fe\(^{3+}\)-NTA, Fe\(^{3+}\)-citrate, Cu\(^{2+}\)-NTA and Cu\(^{2+}\)-histidine. For each independent experiment, cells from the same transfection were used to measure each of these 4 activities, allowing direct comparison of the iron and copper data within each experiment (Fig. 1). The experiments were repeated three times (different transfections) and the kinetic constants averaged to give the values in Table 1. For each substrate, the mean V\(_{\text{max}}\) values were quite similar, ranging from 0.35 to 0.54 pmol μg\(^{-1}\) min\(^{-1}\). More relevant to the question of specificity, the K\(_m\) values for Fe\(^{3+}\)-NTA and
Fe$^{3+}$-citrate were $4.8 \pm 0.7$ μM and $6.8 \pm 1.9$ μM, respectively. Similarly, K_m values for Cu$^{2+}$-NTA and Cu$^{2+}$-histidine were $11.2 \pm 4.4$ μM and $9.9 \pm 2.2$ μM. While the Cu$^{2+}$-NTA data were less reproducible between experiments than the other substrates, it was quite reproducible between replicates in any one experiment (Fig. 1).

To place these data within the context of physiologically relevant metalloreductases, first consider duodenal cytochrome b (Dcytb), a ferric/cupric reductase implicated in intestinal iron absorption. Reported K_m values for Dcytb are 74 and 23 μM for iron and copper NTA chelates, respectively (51). Steap4 thus shows higher affinity than DcytB for each of these metals. In the same vein, Steap4 K_m values for Fe$^{3+}$-NTA are comparable to those for mouse Steap3 (Kleven and Lawrence, unpublished), and thus consistent with a potential functional overlap with Steap3 (2,3,35).

Potential Steap4 Activity in Acidic Organelles - While nontransferrin bound iron, or NTBI, can undergo reduction and transport at the cell surface, plasma iron is generally present in iron-loaded transferrin or ferritin, which is endocytosed and concentrated within endosomal and lysosomal compartments, respectively, where the iron is then unloaded and reduced prior to transport across these organelar membranes (2,52). Because the lumen of these compartments is mildly acidic, endosomal and lysosomal ferric reductases would be expected to function at acidic pH (pH 5-6.5). Accordingly, we examined the pH dependence of Steap4 metalloreductase activity. We found significant ferric and cupric reductase activity between pH 5.0 and pH 7.0 (Fig. 2). However, at basic pH, the ferric reductase activity quickly declined, while the cupric reductase activity was retained. As for Dcytb, the loss of ferric reductase activity may reflect the relative insolubility of ferric ion at basic pH (51).

Regardless, the pH curves clearly demonstrate the potential for Steap4 activity in endosomes, lysosomes and other organelles with acidic lumenal pH.

N-terminal NADPH Oxidase Activity - With the goal of finding soluble fragments suitable for kinetic and structural analysis of the putative Steap4 NADPH oxidase activity, we purified two oxidoreductase domain fragments that lacked the C-terminal transmembrane domain. These two fragments differed by 8 residues at the C-terminus. The C-terminus of the longer construct, residues 1-203, was equivalent to the human Steap3 construct previously utilized for crystallographic studies (35). The second, shorter construct spanned residues 1-195. Both constructs had C-terminal His_6-tags.

For each construct, the purified protein was colorless and absorbance spectroscopy suggested these fragments did not copurify with a flavin. Initial work with the shorter construct at pH 7 (~ cytosolic pH) demonstrated low, but measurable NADPH oxidase activity in the presence of exogenous flavin. When the longer construct was utilized, a substantial increase in activity was seen (Fig. 3). We found that both constructs displayed similar pH dependence, with maximal activity at pH 5.5. In addition to FAD, both constructs were also clearly able to utilize FMN and riboflavin.

Using the longer construct and titrating flavin concentrations up 400 μM, it was possible to measure K_m values for each of these flavins (Table 2). The K_m values for FAD (114 ± 22 μM), FMN (128 ± 20 μM) and riboflavin (183 ± 61 μM) were largely similar, differing by less than a factor of two, as were the k_cat/K_m values, demonstrating that the longer Steap4 fragment does not strongly differentiate between these flavins. This, in turn, suggests recognition is primarily through the isoalloxazine and/or ribitol moieties common to these three flavins.

We also tested the activity of the equivalent human Steap3 oxidoreductase domain fragment, residues 1-215. The extra length of the human protein is due to additional N-terminal residues, such that residue 203 in the rat Steap4 corresponds to residue 215 in human Steap3 (Suppl. Fig. 1). However, the activity of the Steap3 1-215 construct was only barely detectable, and in contrast to the equivalent rat Steap4 construct, millimolar flavin concentrations were required to observe activity (Fig. 3).

**Oligomeric State of the Oxidoreductase Domain in Solution**– Both the long and short oxidoreductase constructs appeared to be monomeric in solution, as assayed by size exclusion chromatography. At protein
concentrations up to 13 mg/ml (570 μM) in 150 mM NaCl at pH 8.0 (standard purification conditions) the apparent molar mass of Steap4 1-203 was 24 kDa, in good agreement with the theoretical mass of a 23 kDa monomer. This was confirmed by analytical ultracentrifugation for the Steap4 1-203 construct (data not shown).

**Structure of the Steap4 oxidoreductase domain** – While we were unable to grow crystals of the long Steap4 oxidoreductase domain suitable for structure determination, single crystals of the short construct grown in the presence of NADPH diffracted to 2.2 Å. With this data (Table 3) we determined the structure of the shorter construct by molecular replacement.

**Structural Similarity to Steap3** - Although there are several critical differences (discussed below), the structure of the Steap4 oxidoreductase domain is largely similar to Steap3, with a root mean square deviation (RMSD) of 1.0 Å over 169 structurally equivalent Cα positions. Like Steap3, it folds into a mixed 8-stranded β-sheet with accompanying α-helices that is formed of two subdomains (Fig. 4A). The first 6 strands (β1-β6) run parallel and form a classic dinucleotide binding element. The second subdomain is composed of an extended reverse turn containing a short bit of alpha helix (α5), followed by a βαβ supersecondary structural element (β5-α6-β8) that contributes two C-terminal β-strands. These strands run parallel to each other, but anti-parallel to the first 6 strands. This is followed by a terminal α-helix (α7) that extends back to the edge of the active site cleft. This cleft accommodates the nicotinamide moiety of NADPH, and presumably the isoalloxazine ring of a flavin.

In the archaeal FNO structure, the first subdomain functions to bind NADPH. Accordingly, we find that the crystallized Steap4 nucleotide binding subdomain does indeed bind NADPH, and the conformation is nearly identical to that seen for Steap3 (35). A multiple sequence alignment identifies 20 strictly conserved residues within the oxidoreductase domain (Suppl. Fig. 1). Notably, 8 of these (40 %) make direct contact with the bound NADPH, as illustrated in Fig 4B. Clearly then, the multiple sequence alignment is able to discriminate residues critical to Steap family metalloreductase activity, many of which are involved in substrate recognition.

We lack a flavin bound structure for any Steap. Attempts to co-crystallize or soak flavin into the Steap4 crystals were unsuccessful. However, superpositional docking of the FNO ternary complex (with NADPH and F420) on Steap4 predicts the binding of NADPH to Steap4 relatively well. This suggests the FNO ternary complex could also serve as a starting point to model potential flavin interactions with Steap4. Thus, when the nicotinamide ring of FNO is positioned on the bound Steap4 nicotinamide ring, and an isoalloxazine ring is substituted for the tricyclic ring of F420, we find that the isoalloxazine ring is nicely accommodated by Steap4. Potential hydrogen bond interactions between Asn106 and O4 of the isoalloxazine ring, and between the 2’-OH of the nicotinamide ribose and isoalloxazine O4 are suggested (Fig.4C). So too are hydrophobic interactions with the Thr136 and Ser138 side chains. Notably, Asn106 and Ser138 are strictly conserved, while Thr136 tolerates only the isosteric Val substitution (Suppl. Fig. 1). Similarly, the H-bond interaction with the ribose ring should also be conserved. Overall, however, much of the docked flavin remains solvent exposed. This docking exercise thus highlights the uncharacteristically open nature of the flavin binding site. Importantly, however, the predicted open nature of the site is entirely consistent with the relatively high Km values for FMN and FAD seen for the soluble Steap4 oxidoreductase domain constructs (Table 2).

**Structural Similarity to the Steap3 Dimer** – While the rat Steap4 oxidoreductase domain appears monomeric by size exclusion and analytical ultracentrifugation (above), the four subunits within the crystallographic asymmetric unit are packed as two very similar “dimers”. These dimers are not only nearly identical to each other, but also to the Steap3 crystal dimer. Importantly, each of these 3 crystallographically independent dimers utilize the same set of secondary structural elements, which include residues that are highly conserved in the mammalian Steap3 and Steap4 sequences (35). They also bury the same approximate surface
area (~700 Å² per subunit) and orient the truncated C-termini on the same side of the dimer. In the context of the full-length protein, the C-termini of the N-terminal domain should mark the membrane proximal face of the dimer, as inspection of the multiple sequence alignment (Suppl. Fig.1) suggests a short connecting loop (~ 7 residues) between the last residue of the oxidoreductase domain and the first transmembrane helix in the C-terminal domain. Thus, in concert with the solution state homodimer observed for the Steap3 oxidoreductase domain, the crystallographic Steap4 dimers reinforce the idea that full-length Steap3 and Steap4 are likely to function as homodimers in vivo (38).

The Disordered N-Terminal Residues are Unnecessary for Enzymatic Activity - Two portions of the Steap4 oxidoreductase domain are not visible in the electron density and are likely disordered in the crystal. These are, first, a short loop between α5 and β7 (residues 147-149) which is also poorly ordered in Steap3 (residues 157-162). Second, the N-terminal 18 residues are also disordered, as are the first 28 residues at the N-terminus of the Steap3 oxidoreductase domain (35). Interestingly, these extreme N-terminal residues are the most poorly conserved region among Steap family oxidoreductase domains (Suppl. Fig. 1). This suggests these N-terminal residues may be unnecessary for enzymatic activity.

To test this hypothesis, we constructed two variants, (I) a 19 residue N-terminal deletion in the full length Steap4 protein to give the Steap4 20-470 variant, and (II), the equivalent N-terminal deletion within the stand-alone oxidoreductase domain to give the Steap4 20-203 variant. We then analyzed the respective cell surface metallooxidoreductase and NADPH oxidase activities of these variants. Within experimental error, the $K_m$ and $V_{max}$ values for the metallooxidoreductase activity of the Steap4 20-470 variant were essentially unchanged (Table 1). Similarly the N-terminal truncation to the isolated oxidoreductase domain (Steap4 20-203) showed no significant change in the $V_{max}$ and $K_m$ values for NADPH and FAD relative to the Steap4 oxidoreductase domain (Steap4 1-203, Table 2). It thus appears that these N-terminal residues are not directly involved in the enzymatic activity of the Steap proteins, and instead function in an alternative role (discussed below).

Structural Basis for Steap4 Oxidoreductase Activity - The NADPH oxidase activity of the Steap3 and Steap4 oxidoreductase domains (Fig. 3) revealed a striking difference between the activities of seemingly equivalent constructs. While Steap4 1-203 shows significant flavin dependent NADPH oxidase activity, Steap3 1-215 shows only minimal activity (Fig. 3). A structural explanation for the increased activity of Steap4 is of obvious interest.

A closer examination of the Steap3 and Steap4 oxidoreductase crystal structures reveals a number of minor differences, but two seem particularly significant with regard to the enhanced activity of Steap4. The first is the connection between strands β4 and β5, which is present as a short α-helix in Steap3 (α5), but as extended “random coil” in Steap4 (Fig. 5A). The second is found at the C-terminal tail. In Steap3, the C-terminal helix (α9) is broken at the edge of the active site pocket by Pro205, with the Leu206 side chain dropping into the putative binding site for the isoalloxazine moiety of the flavin (Fig. 5A). In contrast, while an equivalent proline in Steap4 (Pro193) also disrupts the C-terminal helix (α7), the helix is merely kinked and the corresponding leucine (Leu194) does not descend into the putative substrate binding site, but instead tucks up against the side chain of Met110. Notably, Met110 lies within the extended β4-β5 loop, i.e., it is found in the same “random coil” segment that substitutes for the α-5 helix of Steap3 (described above). Interestingly, structural superposition of Steap3 on Steap4 shows that the Leu206 side chain of Steap3 is excluded from adopting a similar position due to the presence of the Steap3 α5 helix (Fig 5A). Thus, helix loss in the β4-β5 loop may correlate with the “active” conformation of the C-terminal tail.

The multiple sequence alignment provides additional insight in this regard. First, all mammalian Steap2 and Steap4 sequences contain a hydrophobic residue equivalent to Met110. In contrast, the mammalian Steap3 sequences contain a hydrophilic side chain at this position along with a two residue...
hydrophilic insertion that, collectively, appear to support helix formation (Suppl. Fig. 1). Helix formation in the β4-β5 connection may thus be conserved in mammalian Steap3 structures, but absent in Steap2 and Steap4.

While the structural differences between Steap3 and Steap4 correlate nicely with the increased NADPH oxidase activity of Steap4, they do not explain the difference between the short and long Steap4 constructs (Fig. 1). However, further work at higher FAD concentrations using 0.2 cm cuvettes allowed $K_m$ values to be determined for the shorter construct as well, revealing a striking difference. Specifically, truncation of residues 196-203 increased the $K_m$ for FAD from 114 +/- 22 μM to 825 +/- 293 μM (Table 2), suggesting these residues participate in FAD recognition, perhaps by extending over the top of the flavin binding site, as they do in FNO, to sandwich the isoalloxazine rings between these C-terminal residues and the nicotinamide of NADPH (Fig. 5B). Of these residues, Leu196 and Trp200 are strictly conserved and might therefore play a particularly significant role in isoalloxazine recognition.

Steap4 Variants: Leu194Ala, Leu196Ala and Trp200Ala – To test this idea, we characterized the Leu196Ala and Trp200Ala variants, not only in the isolated oxidoreductase domain using the NADPH oxidase assay, but also in full-length Steap4 where we measured cell surface metalloreductase activity. At the same time, we also characterized the Leu194Ala variants, since this residue is also strictly conserved and might therefore play a particularly significant role in flavin recognition. However, both wild-type and variant Steap4 showing partial colocalization with transferrin receptor in the transfected HEK293 cells (Fig. 6). Overall, within the context of full-length protein and the cell surface metalloreductase assay, we concluded that these variant proteins do not exhibit any significant differences. Thus, if these residues contribute to flavin recognition, they do not significantly alter the flavin $K_m$ relative to endogenous flavin concentrations within the intact cell.

Oxidoreductase Domain Variants: Leu194Ala, Leu196Ala and Trp200Ala – When we examined the effects of these substitutions on the FAD dependent NADPH oxidase activity of the isolated oxidoreductase domain, changes in NADPH $K_m$ values were not expected, and indeed, not seen for any of the 3 variants (Table 2). Similarly, significant changes in $V_{max}$ were also not observed. With regard to FAD, the $K_m$ for the Trp200Ala mutant was identical to wild type, which suggests Trp200 does not participate in flavin recognition. Instead, Trp200 may function in the linker between the N- and C-terminal domains or within the C-terminal domain itself. The latter possibility is suggested by conservation of Trp200 in Steap1, which lacks the N-terminal oxidoreductase domain (Suppl. Fig. 1). The picture was less clear with the Leu194Ala and Leu196Ala variants. While the mean FAD $K_m$ values for Leu194Ala (186 +/- 44 μM) and Leu196Ala (253 +/- 104 μM) are higher than wild type, they are arguably within experimental error. Thus, while we are unable to conclude whether the conformation of Steap4 Leu194 may play a role in positioning Leu196 over the isoalloxazine, we can certainly conclude that neither variant fully recapitulates the drop in NADPH oxidase activity and increased $K_m$ for FAD seen in the shorter construct. Thus, rather than a single conserved residue, it instead appears that the ensemble of C-terminal residues (196-203) is collectively responsible for the increased activity.

Because the cell surface $V_{max}$ values are dependent upon the amount of Steap4 present on the cell surface, we also monitored the subcellular localization of wild-type (3) and variant Steap4 proteins by immunofluorescence. The subcellular localization did not appear significantly altered in any of the mutants; with both wild-type and variant Steap4 showing positional colocalization with transferrin receptor in the transfected HEK293 cells (Fig. 6). Overall, consistent with the full-length protein and the cell surface metalloreductase assay, we concluded that these variant proteins do not exhibit any significant differences. Thus, if these residues contribute to flavin recognition, they do not significantly alter the flavin $K_m$ relative to endogenous flavin concentrations within the intact cell.
DISCUSSION

Physiologically Relevant Metalloreductase Activities - There has been significant work on the potential role of Steap4 in obesity associated insulin resistance. In contrast, biochemical characterization of its metalloreductase activity is limited, consisting of only an initial, though critically important demonstration of cell surface ferric and cupric reductase activities (3). Here we have presented the first in-depth analysis of the Steap4 ferric and cupric reductase activities. We found that the affinity of Steap4 for ferric chelates is equal to or greater than those of other characterized ferric reductases implicated in iron homeostasis, thus demonstrating the potential for physiologically relevant Steap4 ferric reductase activity. Further, Steap4 also shows significant cell surface metalloreductase activity at acidic pH (pH 5-6.5), suggesting Steap4 has the capacity to function in endosomal or lysosomal compartments that concentrate iron, and other cellular locations that experience acidic pH. Steap4 also shows significant activity at pH 7.5, near the physiological pH of blood plasma. Thus, Steap4 might also participate in reduction of non-transferrin bound iron (NTBI), enabling iron acquisition during iron overload, perhaps in collaboration with Zip8 or Zip14 (53-55).

The potential for physiologically relevant cupric reductase activity is less clear. Reduction of Cu$^{2+}$ to Cu$^{1+}$ is required prior to transport by human CTR1, the primary copper transporter involved in cellular uptake in mammals (56,57). While the relevant copper reductase has not yet been identified, several recent reviews consider the Steap family members and/or Dcytb for this role (57,58). In this regard, it is interesting that Steap4 shows greater affinity for Cu$^{2+}$-NTA and Cu$^{2+}$-histidine than Dcytb. Perhaps even more relevant is the K_m value for copper transport by human Ctrl (2.5 μM), which is also active at acidic pH (5.5 – 6.5) (59). While the K_m for reduction of Cu$^{2+}$-histidine (~10 μM) by Steap4 is somewhat higher than the K_m for copper transport by Ctrl, these K_m values are at least on the same order of magnitude, suggesting the cupric reductase activity might also be physiologically relevant.

Oxidoreductase Domain – While Steap2-4 have been presumed to be NADPH and flavin dependent metalloreductases, our work here with the isolated oxidoreductase domain represents the first demonstration of flavin dependent NADPH oxidase activity for any member of the Steap family. Experimental confirmation of this predicted activity is germane to the function of Steap2-4 in general. However, it will also greatly facilitate future structure-function studies of Steap4. As opposed to the full length protein, dissection of the NADPH oxidase activity from the metalloreductase activity allows readout on the initial stages of electron transfer.

N-terminal Oxidoreductase Residues - It is noteworthy that truncation of the non-conserved residues at the N-terminus of Steap4 had no significant effect on the metalloreductase and NADPH oxidase activities of the full-length protein and the truncated oxidoreductase domain, respectively. At the same time, deletion of the N-terminus in the full-length protein did not significantly alter Steap4 colocalization with the transferrin receptor, suggesting that loss of these N-terminal residues does not alter the subcellular localization (Fig. 6). Together, these observations suggest that the disordered N-terminal residues may instead serve a regulatory role, or alternatively, in protein-protein interactions specific to the individual Steap2-4 proteins (60,61). In this context, it is interesting that phosphoproteomic studies have consistently found the N-termini of human and mouse Steap3 to be phosphorylated at Ser17 and Ser20 (62-71), and that these residues are well conserved in the mammalian Steap3 sequences (Suppl. Fig. 1). Proteomic studies have also demonstrated ubiquitylation of mouse Steap4 at Lys3 and Lys18 (72), which are similarly well conserved in other mammalian Steap4 proteins (Suppl. Fig. 1).

An Incomplete Flavin Binding Site in the Oxidorecutase Domain - Several lines of evidence suggest that the N-terminal oxidoreductase domain contains only a portion of the flavin binding site present in the full-length Steap4 protein. First, the inability of the oxidoreductase domain to discriminate between
flavins suggests that the N-terminal domain interacts primarily with the isoalloxazine ring and/or ribitol moieties. However, the full-length protein would presumably display a flavin preference in vivo. Thus, portions of the full-length protein not present in our crystal structure would likely be responsible for flavin discrimination, and contribute residues important for flavin binding.

Second, unlike the N-terminal fragment studied here, FMN and FAD reductases generally have $K_m$ values for flavins in the high nanomolar to low micromolar range (73). Specific examples with potential relevance to the Steap proteins include archaeal FNO, whose $K_m$ for F420 is 10 μM (74), E. coli ferric reductase (Fre) with a $K_m$ value of 0.6-2.5 μM (75,76) and human NADPH Oxidase (Nox) with a $K_m$ of 60 nm (77,78). These values are approximately 10 to 1000-fold lower than the $K_m$ values measured for truncated Steap4. We also note that flavin $K_m$ values $> 100$ μM are not physiologically relevant, as cells containing such high flavin concentrations would be intensely yellow (OD$_{450} > 1$). In this light, it becomes clear that the isolated N-terminal domain of Steap4 does not contain a high affinity flavin binding site.

Third, our structural data also support the partial flavin site hypothesis. The crystal structures of the Steap4 oxidoreductase domain reveal a putative flavin binding site that is distinctly different from the F420 binding site in FNO. In FNO, the C-terminal helix is significantly longer, and extends over the top of the active site, sandwiching the isoalloxazine ring between the nicotinamide of NADPH on one side, and hydrophobic residues of the C-terminal helix on the other (Fig. 5). In contrast, the equivalent residues in Steap4 contain two helix breaking proline residues, such that the C-terminal helix is truncated at the edge of the active site, giving rise to an uncharacteristically solvent exposed active site structure.

Importantly, these structural results are consistent with the kinetic analysis. Specifically, in contrast to Steap3, docking studies show that the Steap4 active site could nicely accommodate the isoalloxazine ring, but that in accordance with the observed $K_m$ values, additional interactions that might discriminate between the various flavins are not predicted. Similarly, while the predicted interactions with the isoalloxazine are relatively well conserved, the open, solvent exposed active site would also predict an uncharacteristically high $K_m$ value.

In this context, it is important to remember that the structural and kinetic data on flavin binding and specificity are derived from the truncated oxidoreductase domain, and that while FNO is a soluble single domain protein, Steap4 is instead a two domain integral membrane protein in which the flavin is proposed to serve as an intermediate in electron transfer, shuttling electrons between NADPH, bound in the oxidoreductase domain, and the heme group in the transmembrane domain. In addition, access to the enlarged entrance of the Steap oxidoreductase active site (relative to FNO) is primarily from the presumed membrane proximal face of the oxidoreductase domain, which would be expected to abut the transmembrane domain. For these reasons, it seems logical that the transmembrane domain might participate in the construction of the flavin binding site.

Further support for this idea comes from YedZ, a single domain membrane protein with homology to the Steap transmembrane domain (79,80). Importantly, YedZ has been observed to copurify with non-covalently bound FMN, demonstrating the ability of a Steap4-like transmembrane domain to participate in flavin recognition (81). This is in contrast to Fre and Nox where the flavin binding sites are instead found in a cytoplasmic domain. We hypothesize that unlike Fre and Nox, Steap proteins instead utilize the transmembrane domain to complete a high affinity flavin binding site, with the flavin bound at the interface between the N- and C-terminal domains, giving rise to physiologically relevant affinity ($K_m$ value) for FMN or FAD. Correspondingly, loss of the C-terminal YedZ-like domain in the truncated Steap4 oxidoreductase domain should lead to an aberrantly high, non-physiological $K_m$ for flavin, as observed (Table 2).

This hypothesis is also consistent with a recent evolutionary study on the origin of the ferric reductase domain superfamily. This study suggests that Steap proteins are indeed more closely related to the YedZ-like proteins than to
Fre and Nox (80). Unlike Fre and Nox, which contain two heme groups (surface proximal and cytoplasm proximal), Steap and YedZ are thought to contain only a single, surface proximal heme (2,3,80). It will be interesting to determine whether loss of the cytoplasm proximal heme in the Steap transmembrane domain is due to substitution of a cytoplasm proximal flavin binding site.

If the Steap4 transmembrane domain is involved in flavin recognition, the high incidence of strictly conserved residues in the NADPH binding site of the oxidoreductase domain suggests that residues involved in flavin recognition would be similarly conserved. In this light, strictly conserved residues in the transmembrane domain occur most frequently within the first and second cytoplasmic loops (Suppl. Fig 1). However, kinetic work to assess the potential role of these residues in flavin recognition will require the ability to measure NADPH and flavin dependent metalloreductase activity, preferably with purified full length protein.

Additional work is thus needed to understand the mechanism of Steap4 iron and copper reduction in greater detail, and this includes the need for structural and functional analysis of purified full length protein. At the same time, however, the biochemical analysis presented here suggests that Steap4 possesses physiologically relevant ferric and cupric reductase activities. For this reason, attention should also turn to specific investigations on the role of Steap4 ferric and cupric reductase activities in obesity associated insulin resistance and atherosclerosis.
REFERENCES


Interdomain Flavin Binding in Steap4, a \( \text{Fe}^{3+}, \text{Cu}^{2+} \) Reductase


40. McKie, A. T., Barrow, D., Latunde-Dada, G. O., Rolfs, A., Sager, G., Mudaly, E.,
Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T. J., Raja, K. B., Shirali,
reductase associated with the absorption of dietary iron. Science 291, 1755-1759
41. Raymond, C., Tom, R., Perret, S., Moussouami, P., L'Abbe, D., St-Laurent, G., and
Durocher, Y. (2011) A simplified polyethylenimine-mediated transfection process for
large-scale and high-throughput applications. Methods 55, 44-51
Quantities of Protein Utilizing Principle of Protein-Dye Binding. Analytical Biochemistry
72, 248-254
43. Studier, F. W. (2005) Protein production by auto-induction in high density shaking
cultures. Protein Expr. Purif. 41, 207-234
Totowa, NJ
45. Fischer, M., Haase, I., Feicht, R., Richter, G., Gerhardt, S., Changeux, J. P., Huber, R.,
and Bacher, A. (2002) Biosynthesis of riboflavin - 6,7-dimethyl-8-ribityllumazine
synthase of Schizosaccharomyces pombe. Eur. J. Biochem. 269, 519-526
of the apoprotein. Biochemistry 7, 2666-2672
760-763
generate schematic diagrams of protein-ligand interactions. Protein Eng. 8, 127-134
functions as both a ferric and a cupric reductase in vitro. FEBS Lett. 582, 1901-1906
uptake of H-ferritin are mediated by human transferrin receptor-1. Proc Natl Acad Sci U
S A 107, 3505-3510
53. Wang, C. Y., Jenkitkasemwong, S., Duarte, S., Sparkman, B. K., Shawki, A., Mackenzie,
B., and Knutson, M. D. (2012) ZIP8 is an iron and zinc transporter whose cell-surface
expression is up-regulated by cellular iron loading. J Biol Chem 287, 34032-34043
(ZIP14) promotes the cellular assimilation of iron from transferrin. J Biol Chem 285,
32141-32150
55. Wang, C. Y., and Knutson, M. D. Hepatocyte divalent metal-ion transporter-1 is
dispensable for hepatic iron accumulation and non-transferrin-bound iron uptake in mice.
Hepatology
Uptake of copper from plasma proteins in cells where expression of CTR1 has been
modulated. Biometals 25, 697-709
for a metal with special needs. J. Biol. Chem. 284, 25461-25465


Interdomain Flavin Binding in Steap4, a Fe$^{3+}$, Cu$^{2+}$ Reductase

FOOTNOTES

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3The abbreviations used are: BCS, bathocuproine disulfonate; CEBPA, CCAAT/Enhancer-binding proteins α; Dcytb, duodenal cytochrome b; DMT-1, divalent metal transporter-1; F$_{420}$, 8-hydroxy-5-deazaflavin; FNO, F$_{420}$$^\text{H2}$: NADP$^+$ oxidoreductase; Fre, ferric reductase; GLUT4, glucose transporter 4; IL-6, interleukin-6; Nox, NADPH oxidase; NTA, nitrilotriacetic acid; RMSD, root mean square deviation; Stamp2, six-transmembrane protein of the prostate2; STAT3, signal transducer and activator of transcription 3; Steap, six transmembrane antigen of the prostate; TIARP, TNFα-induced adipose-related protein; TCEP, tris(2-carboxylethyl)phosphine; TNFα, tumor necrosis factor alpha;

FIGURE LEGENDS

FIGURE 1. Cell surface metalloreductase activity. The cell surface metalloreductase activity of various Steap4 constructs was measured using 4 different substrates. Shown here are representative data for the Steap4 1-470 construct. A typical experiment is shown for each substrate, (A) Fe$^{3+}$-NTA, (B) Fe$^{3+}$-citrate, (C) Cu$^{2+}$-NTA, and (D) Cu$^{2+}$-histidine. V$_{\text{max}}$ and K$_{m}$ values were derived by fitting the Michaelis-Menten equation to the data with GraphPad Prism 5.0. The kinetic parameters reported in Table 1 represent the mean of at least three such independent experiments.

FIGURE 2. Cell surface metalloreductase activity of full-length Rat Steap4 (Steap4 1-470) was measured as a function of pH in the presence of 50 µM Fe$^{3+}$-NTA or 50 µM Cu$^{2+}$-NTA. Substantial ferric and cupric reductase activity was seen at acidic pH, suggesting that in addition to cell surface activity, Steap4 may also function within the acidic lumen of intracellular compartments.

FIGURE 3. Flavin dependent NADPH oxidase activity of the purified N-terminal cytoplasmic domain. (A) Activity as a function of pH. Two constructs of the Rat Steap4 N-terminal oxidoreductase domain were compared with the Human Steap3 1-215 fragment. The longer Steap4 construct (1-203, filled circles) had significantly higher activity than the shorter construct (1-195, open circles); both constructs were significantly more active than the Human Steap3 fragment (open triangles). (B, C and D) The activity of Steap4 1-203 as function of flavin concentration. A typical experiment is shown for each flavin, along with the V$_{\text{max}}$ and K$_{m}$ values derived by fitting the Michaelis-Menten equation to the data. The maximum riboflavin concentration (D) was limited to due to its low solubility in assay buffer. The kinetic parameters reported in Table 2 represent the mean of at least three such independent experiments.

FIGURE 4. Rat Steap4 structure. (A) Stereo view of the Steap4 N-terminal domain with bound NADPH. The N-terminal domain (red, residues 19-132) is formed by a classic dinucleotide binding fold [6 stranded parallel β-sheet (3-2-1-4-5-6) with β-strands connected by right handed α-helical crossovers]. This is followed by an additional 63-residue subdomain (blue, residues 133-195) formed of 2 additional strands running antiparallel to the first 6 strands, and 3 connecting α-helices. The C-terminal helix, α7, terminates near the nicotinamide ring of the NADPH (green). The disordered residues Leu147-Asp148-
Ala149 are indicated by dashed line between α5 and β7. N- and C-termini are denoted by N and C, respectively. (B) NADPH Recognition. Rat Steap4 residues that make hydrogen bonds (green dashed lines) with NADPH are shown in a 2-dimensional LigPlot (50) ball and stick representation. Residues that make van der Waals interactions with NADPH are shown surrounded by red arcs. Strictly conserved residues are underlined. Carbon atoms are black, nitrogen is blue, oxygen is red, and phosphorus is orange. (C) Putative flavin interactions. Flavin was docked onto the Steap4 protein by superposition of the nicotinamide ring in the Steap4 structure with the nicotinamide ring of the flavin containing FNO structure (1JAY). Potential FMN interactions were highlighted after substituting an isoalloxazine ring for the tricyclic ring of F420, and truncating the tail of F420 to replicate the ribitol-phosphate of FMN. Conserved residues are highlighted as in (B). For clarity, only the nicotinamide ribosyl moiety of NADPH is shown.

FIGURE 5. Fig. 5. Structural Differences between Steap3 and Steap4. (A) Stereo Figure. Superposition of Rat Steap4 (red) on human Steap3 (blue) highlights structural differences near the putative flavin binding site. In Steap3 Pro205 breaks the C-terminal helix allowing Leu206 to drop into the active site pocket where it clashes with the docked FMN (gray). In contrast, the Steap4 C-terminal helix includes an extra turn, repositioning the corresponding Leu194 against the β5-α4 loop where it makes hydrophobic contact with Met110. A similar interaction in Steap3 may be prevented by insertion of the hydrophilic α-5 helix at this position (See text for details). (B) Superposition of the archaeal FNO on Rat Steap4. In Steap4 (red), the C-terminal helix (α7) terminates near the flavin binding site. In FNO, the corresponding helix (gray) extends over the tricyclic ring of F420 allowing the FNO Leu196 side chain to interact with F420 (gray). For clarity, only the corresponding helix of FNO is shown.

FIGURE 6. Co-localization of rat Steap4 and transferrin receptor. HEK-293F cells were transfected with various Steap4 constructs and visualized with immunofluorescence. Steap4 (green) and endogenous transferrin receptor-1 (TFR; red) were visualized by confocal microscopy after staining with anti-Strep-II and anti-TfR antibodies, respectively. Channels were merged to show colocalization (yellow), along with the addition of DAPI to identify nuclei (blue). No significant change in expression pattern was observed for any of the mutants. White bars indicate 10 µm.
### Table 1. Full-length Steap4 cell surface metalloreductase activity.

<table>
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<tr>
<th>Construct</th>
<th>Fe-NTA</th>
<th>Fe-Citrate</th>
<th>Cu-NTA</th>
<th>Cu-Histidine</th>
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<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol/µg cell lysate-min)</td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol/µg cell lysate-min)</td>
</tr>
<tr>
<td>Steap4 (1-470)</td>
<td>4.8 ± 0.7</td>
<td>0.39 ± 0.04</td>
<td>6.8 ± 1.9</td>
<td>0.35 ± 0.05</td>
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<tr>
<td>Steap4(20-470)</td>
<td>4.6 ± 2.9</td>
<td>0.30 ± 0.05</td>
<td>3.9 ± 0.4</td>
<td>0.27 ± 0.03</td>
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<td>Steap4 (1-470)-L194A</td>
<td>7.9 ± 1.0</td>
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<td>8.9 ± 4.8</td>
<td>0.93 ± 0.38</td>
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<tr>
<td>Steap4 (1-470)-L196A</td>
<td>5.9 ± 1.9</td>
<td>0.36 ± 0.12</td>
<td>9.4 ± 3.4</td>
<td>0.33 ± 0.10</td>
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<tr>
<td>Steap4 (1-470)-W200A</td>
<td>1.2 ± 0.5</td>
<td>0.32 ± 0.13</td>
<td>4.1 ± 1.3</td>
<td>0.22 ± 0.22</td>
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</tbody>
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$k_{cat}/K_m$:

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<tr>
<th>Construct</th>
<th>Fe-NTA</th>
<th>Fe-Citrate</th>
<th>Cu-NTA</th>
<th>Cu-Histidine</th>
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<tr>
<td>Steap4 (1-470)</td>
<td>0.08</td>
<td>0.05</td>
<td>0.05</td>
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Data are mean ± standard deviation from least three completely independent experiments, i.e., different transfections.

### Table 2. N-terminal oxidoreductase domain NADPH oxidase activity.

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<tr>
<th>Construct</th>
<th>$V_{max}$ (µmole/min-mg)</th>
<th>NADPH</th>
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<th>FMN</th>
<th>riboflavin</th>
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<td>$K_m$ (µM)</td>
<td>$K_m$ (µM)</td>
<td>$K_m$ (µM)</td>
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<td>Steap4(1-203)</td>
<td>2.2 ± 0.3</td>
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<td>128 ± 20</td>
<td>183 ± 61</td>
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<td>Steap4(1-195)</td>
<td>0.6 ± 0.2</td>
<td>8.5 ± 0.9</td>
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<td>Steap4(20-203)</td>
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<td>128 ± 31</td>
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<td>Steap4(1-203)L194A</td>
<td>1.7 ± 0.5</td>
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<td>Steap4(1-203)L196A</td>
<td>2.3 ± 0.8</td>
<td>7.0 ± 3.8</td>
<td>253 ± 104</td>
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<tr>
<td>Steap4(1-203)W200A</td>
<td>2.2 ± 0.5</td>
<td>7.0 ± 1.4</td>
<td>104 ± 28</td>
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$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$):

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<th>FAD</th>
<th>FMN</th>
<th>riboflavin</th>
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</tbody>
</table>

Data are the mean ± standard deviation from at least three independent experiments, i.e., different enzyme preparations. $V_{max}$ was measured at constant NADPH (100 µM) by varying the FAD concentration, typically 25 - 400 µM, although concentrations up to 1000 µM were used for some constructs. Kinetic data were fit to the Michaelis-Menten equation with GraphPad Prism.
Table 3. X-ray data collection and refinement statistics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>69.228, 85.111, 128.035</td>
</tr>
<tr>
<td>α = β = γ (°)</td>
<td>90.00°</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.99984</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50.00 - 2.20 (2.28-2.20)</td>
</tr>
<tr>
<td>Total reflections observed</td>
<td>303,911</td>
</tr>
<tr>
<td>Unique reflections</td>
<td></td>
</tr>
<tr>
<td>Observed</td>
<td>39,044</td>
</tr>
<tr>
<td>Total possible</td>
<td>39,398</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.8 (6.4)</td>
</tr>
<tr>
<td>Data coverage (%)</td>
<td>99.1 (94.8)</td>
</tr>
<tr>
<td>Mean (I)/sigma (I)</td>
<td>7.9 (2.8)</td>
</tr>
<tr>
<td>Rsym (%)</td>
<td>10.1 (45.9)</td>
</tr>
<tr>
<td>Refinement resolution range (Å)</td>
<td>37.00 - 2.20</td>
</tr>
<tr>
<td>Rcryst (%) / Rfree (%)</td>
<td>20.3 / 23.8</td>
</tr>
<tr>
<td>Coordinate error (Å)</td>
<td>0.151</td>
</tr>
<tr>
<td>Real space correlation coefficient</td>
<td>0.957</td>
</tr>
<tr>
<td>RMSD from ideality: bonds (Å) / angles (deg)</td>
<td>0.021 / 1.861</td>
</tr>
<tr>
<td>Ramachandran plot: favored / outliers (%)</td>
<td>98.2 / 0.0</td>
</tr>
<tr>
<td>Average residual B values (Å²)</td>
<td>34.747</td>
</tr>
<tr>
<td>PDB ID</td>
<td>2YJZ</td>
</tr>
</tbody>
</table>

a Numbers in parentheses refer to the highest resolution shell.

b \( R_{\text{sym}} = \frac{\sum h \sum_i |I_i(h) - \langle I(h)\rangle|}{\sum h I(h)} \), where \( I_i(h) \) is the \( i \)th measurement of the \( h \)th reflection, and \( \langle I(h)\rangle \) is the average value of the reflection intensity.

c \( R_{\text{cryst}} = \frac{\sum ||F_o|| - |F_c||}{\sum |F_o|} \), where \( F_o \) and \( F_c \) are the structure factor amplitudes from the data and the model, respectively. \( R_{\text{free}} \) is calculated similarly, using 5% of the structure factors held back as a test set.

d Based on maximum likelihood.

e Correlation coefficient is between the model and the 2mFo - DFc electron density map.

f Calculated using MOLPROBITY.
Figure 1

A. Ferric Reductase Activity (pmol/min-µg cell protein)

Fe$^{3+}$-NTA
$V_{max} = 0.38$ pmol/min-µg
$K_M = 5.0$ µM

B. Ferric Reductase Activity (pmol/min-µg cell protein)

Fe$^{3+}$-Citrate
$V_{max} = 0.35$ pmol/min-µg
$K_M = 8.8$ µM

C. Cupric Reductase Activity (pmol/min-µg cell protein)

Cu$^{2+}$-NTA
$V_{max} = 0.65$ pmol/min-µg
$K_M = 11.0$ µM

D. Cupric Reductase Activity (pmol/min-µg cell protein)

Cu$^{2+}$-Histidine
$V_{max} = 0.71$ pmol/min-µg
$K_M = 12.3$ µM
Figure 3

A. Rat Steap4 1-203
   Rat Steap4 1-195
   Human Steap3 1-215

NADPH Oxidase Activity
(μmol/min-mg)

pH

B. $V_{max} = 2.4 \mu\text{mol/min-mg}$
   $K_m = 139 \mu\text{M}$

μmol/min-mg

[FAD] (μM)

C. $V_{max} = 2.1 \mu\text{mol/min-mg}$
   $K_m = 144 \mu\text{M}$

μmol/min-mg

[FMN] (μM)

D. $V_{max} = 2.9 \mu\text{mol/min-mg}$
   $K_m = 238 \mu\text{M}$

μmol/min-mg

[Riboflavin] (μM)
Figure 5

A.

B.
Figure 6
The Crystal Structure of Six Transmembrane Antigen of the Prostate 4 (Steap4), a Ferri/Cuprireductase, Suggests a Novel Inter-Domain Flavin Binding Site
George H. Gauss, Mark D. Kleven, Anoop K. Sendamarai, Mark D. Fleming and C. Martin Lawrence

J. Biol. Chem. published online June 3, 2013

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