Characterization of a Single b-type Heme, FAD, and Metal Binding Sites in the Transmembrane Domain of Six-transmembrane Epithelial Antigen of the Prostate (STEAP) Family Proteins*§

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Background: Steap metalloreductases are critical to metal homeostasis and linked to multiple diseases.
Results: Single b-type heme, FAD, and iron binding sites are identified in the Steap3 transmembrane domain and kinetically characterized.
Conclusion: Steap transmembrane domains contain a single b-type heme and a high affinity FAD binding site that coordinates intrasubunit, transmembrane electron transfer.
Significance: The findings extend to Steap family proteins in general, including Steap1.

Six-transmembrane epithelial antigen of the prostate 3 (Steap3) is the major ferric reductase in developing erythrocytes. Steap family proteins are defined by a shared transmembrane domain that in Steap3 has been shown to function as a transmembrane electron shuttle, moving cytoplasmic electrons derived from NADPH across the lipid bilayer to the extracellular face where they are used to reduce Fe3+ to Fe2+ and potentially Cu2+ to Cu1+. Although the cytoplasmic N-terminal oxidoreductase domain of Steap3 and Steap4 are relatively well characterized, little work has been done to characterize the transmembrane domain of any member of the Steap family. Here we identify high affinity FAD and iron binding sites and characterize a single b-type heme binding site in the Steap3 transmembrane domain. Furthermore, we show that Steap3 is functional as a homodimer and that it utilizes an intrasubunit electron transfer pathway through the single heme moiety rather than an intersubunit electron pathway through a potential domain-swapped dimer. Importantly, the sequence motifs in the transmembrane domain that are associated with the FAD and metal binding sites are not only present in Steap2 and Steap4 but also in Steap1, which lacks the N-terminal oxidoreductase domain. This strongly suggests that Steap1 harbors latent oxidoreductase activity.

The daily production of 200 billion erythrocytes accounts for nearly 80% of the total iron demand in humans (1). To meet this need, developing erythrocytes utilize the transferrin cycle to import iron into the cell. In this process, iron-loaded transferrin is bound at the cell surface by the transferrin receptor. This is followed by endocytosis and acidification of the endosomal compartment, which promotes release of Fe3+. The Fe3+ is then reduced to Fe2+ by six-transmembrane epithelial antigen of the prostate 3 (Steap3),2 the major ferric reductase of the erythroid transferrin cycle (2, 3). Finally, Fe2+ is transported across the endosomal membrane by divalent metal transporter 1 where it supports the synthesis of hemoglobin and other cellular needs or in iron-replete cells is sequestered within the iron storage protein ferritin.

Among proteins comprising the transferrin cycle, Steap3 is the most recent to be identified (2–4). The work by Ohgami et al. (2) included protein homology analyses that provided initial clues to its function and mechanism. Steap3 and its homologs Steap2 and Steap4 were predicted to be composed of two distinct domains: an N-terminal cytoplasmic domain and a C-terminal transmembrane domain (2, 5–7). The closest homolog for the N-terminal cytoplasmic domain is the prokaryotic F420H2:NADP+ oxidoreductase. F420H2:NADP+ oxidoreductase utilizes an elaborated Rossman or dinucleotide binding domain to bind NADPH and the flavin-derivative F420 (8). In methanogens, F420H2:NADP+ oxidoreductase reduces F420 to F420H2, which is subsequently used to reduce CO2 to methane. Because the flavin analog F420 is not known in mammals, the homology suggested that the N-terminal domain of Steap3 would instead bind NAD(P)H and a flavin, such as FAD or FMN.

The C-terminal domain was predicted to contain six transmembrane α-helices with distant homology to yeast ferric reductases and to mammalian NADPH oxidase among others (2). Thus, collectively, the transmembrane domains of the Steap, ferric reductase, and NADPH oxidase protein families place each of these within the greater “ferric reductase domain” (FRD) superfamily (9). In this light, members of the ferric

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§ This article contains supplemental Fig. 1 and a list showing Steap family alignment species.

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2 The abbreviations used are: Steap, six-transmembrane antigen of the prostate; ALA, α-aminolevulinic acid; BIFC, bimolecular fluorescence complementation; F420, 8-hydroxy-5-deazaflavin; FRD, ferric reductase domain; NTA, nitrilotriacetic acid.
FAD and Fe^{3+} Binding Sites in the STEAP Transmembrane Domain

TABLE 1

| Primers used in Steap3 cloning |
|-------------------------------|
| Cloning primers               |
| HindIII/StrepII               | 5'-AGG CTT GCC GCC ATG TGG AGC CAC CGC CAG TTG QA AAA TCG GCC-3' |
| Steap3/KpnI                   | 5'-GCT CTC GGT ACC TCA TAC GGT GGT GCT TCT CTC-3' |
| attB1/Steap3                  | 5'-GTA CAA AAA AGC AGG CTC GCC AGA AGA GAT GGA AAA GC-3' |
| hStrep3/attB2                 | 5'-GTA CAA GAA AGC TGG GTC CTA GAT GCC TCG TTC CTG-3' |
| Universal/attB1               | 5'-GGG GAC AAG AGG TGT AAA AAG AGC AGG CTC C-3' |
| Universal/attB2               | 5'-GGG GAC CAC TTT GTA CAA GAC AGC TGG GCT C-3' |
| Nhel/StrepII                  | 5'-GTP CTC GCT AGC AGG TGG AGC CAC CCG CAG CCG CTT-3' |
| Stepll/Strep3                 | 5'-ACC CCC AGT TCG AAA ACT CGG CAG AAG AGA TGG ACA AGC-3' |
| Steap3-EcoRI                  | 5'-GCC CAC GAA TTC CTA CTC GAT GTT GTG GCG GAT CAT-3' |
| Sall-His_tag                  | 5'-GCT CTC GTC GAC AGT CAT CAT CAT CAC CAT CAT CAC CAT CAC CAT CAC GAA-3' |
| His_tag/Steap3                | 5'-GAT CAT CAC CAT CAC CAT CAC AGT CAT CAC GAA GAG AGT AGG AGG CAG C-3' |
| His_tag/Steap3Not1            | 5'-GAG ACC GCC GCC GTT CAC TAC TGC TGC TGC TGC TGC CCG CAC-3' |

Thus, although biochemical and structural studies have provided valuable insights into Steap structure-function relationships in the N-terminal oxidoreductase domain, many questions remain regarding the C-terminal transmembrane domain and the full-length protein. Specifically, although sequence analysis identifies one pair of heme-coordinating residues, does the transmembrane domain indeed contain only a single heme cofactor? Or is there a second, cryptic heme present, and if so, how is it bound, and what type of heme is utilized (heme a, b, or c)? Does the full-length protein form a homodimer, and if so, what is the functional impact of dimerization? Does the full-length protein bind a specific flavin with high affinity? Does the transmembrane domain play a pivotal role in flavin recognition, and if so, which elements in the transmembrane domain are responsible for high affinity flavin recognition? Finally, where is the iron binding site? Does the iron bind adjacent to the heme, or are electrons transferred from the heme to an iron bound at a more distant site?

Experimental Procedures

Cloning and Mutagenesis of Steap3 Expression Constructs—
Homo sapiens STEAP3 cDNA was PCR-amplified with primers that generate a HindIII restriction site on the 5'-end of the transcript and a KpnI restriction site on the 3'-end (HindIII. Steap3 and Steap3.KpnI, respectively; Table 1). The gene was cloned into the pcDNA3.1 (Invitrogen) expression plasmid utilizing HindIII and KpnI restriction sites in the multiple cloning site. To generate fluorescent protein-tagged gene products, the Invitrogen Gateway cloning system (12) was used. Briefly, pDEST vectors for this work were made by cloning the Gateway recombination cassette (12) into Venus and Venus fragment plasmids (kindly provided by Dr. Chang-Deng Hu; Ref. 13). H. sapiens STEAP3 cDNA was PCR-amplified to add complete attB sites at each termini in two subsequent reactions, the first with attB1.Steap3 and Steap3.attB2 primers and the second with Universal.attB1 and Universal.attB2 primers. The product was cloned into pDONR201 using BP Clonase II (Invitrogen). An expression clone was then generated by LR Clonase II enzyme mixture (Invitrogen)-mediated recombination between the Steap3 entry clone and the destination vector pDEST.Venus. The resulting expression clone, Steap3.Venus, encodes a Steap3 fusion with an N-terminal FLAG tag and C-terminal Venus fluorescent protein. Subsequent mutations were introduced into the Steap3 gene using the QuikChange Lightning site-directed mutagenesis kit (Stratagene) with prim-
ers designed according to the manufacturer’s protocols. All mutations were verified by sequencing prior to use.

For bimolecular fluorescence complementation (BiFC) microscopy studies, Steap3 was cloned into two variants of the pDEST.Venus vector using the Gateway system. The variant pDEST.VN173 encodes a truncation of Venus containing residues 1–173. The variant pDEST.VC155 encodes a truncation of Venus containing residues 155–238 (13). The resulting expression plasmids were designated Steap3.VN173 and Steap3.VC155, respectively.

For BiFC activity studies, Steap3.VN173 was PCR-amplified with primers adding a Nhel restriction site and StrepII tag on the 5’-end and an EcoRI restriction site on the 3’-end of the Steap3.VN173 product (Nhel.StrepII, StrepII.Steap3, and Steap3.EcoRI, respectively). Steap3.VC155 was PCR-amplified with primers adding a SalI restriction site and hexahistidine tag on the 5’-end and NotI restriction site on the 3’-end of the Steap3.VC155 product (Sal-His_tag, His_tag-Steap3, and Steap3-NotI, respectively). Each amplified PCR product was cloned into the pIREs bicistronic vector (Clontech) into the first (Steap3.VN173) and second (Steap3.VC155) multiple cloning sites to yield pIRES.Steap3VN.Steap3VC.

Expression and Solubilization of Steap3—HEK-293F cells (Invitrogen) were maintained in Freestyle 293 Expression Medium (Invitrogen) in spinner flasks at 37 °C and 5% CO2. For transfection, cells were resuspended in fresh media at a density of 2.5 × 10^6 cells/ml. The expression plasmid was added to cells at a concentration of 3 μg/ml of culture volume and allowed to mix for 5 min. Linear polyethyleneimine (molecular weight, 25,000; Polyscience, Inc.) was then added to the cultures at a concentration of 9 μg/ml of culture. After 8 h, the expression cultures were supplemented with 2.2 mM valproic acid and 0.5 mM 5-aminolevulinic acid, and the culture volume was doubled by addition of fresh medium. Cells were harvested 48 h post-transfection by centrifugation (1,000 × g for 10 min) and resuspended in hypotonic buffer (20 mM HEPES, pH 7.5, 4°C) at a density of 4 ml of buffer/g of cell pellet. All subsequent steps were performed at 4°C. Cells were lysed by Dounce homogenization followed by passage through a 24-gauge needle three times and sonication for 10 s. The lysate was centrifuged for 20 min at 1,000 × g to remove unlysed cells and debris. The supernatant was recovered followed by isolation of membranes by ultracentrifugation (125,000 × g for 1 h). The pelleted membranes were resuspended by Dounce homogenization in resuspension buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) at a concentration of 2–3 mg/ml. Detergent-activated membranes were then prepared by addition of 1% Triton X-100 followed by rotation for 1 h.

Steap3 Ferric Reductase Assay—Triton X-100-activated membranes were assayed for ferric reductase activity using the following standard conditions. Membranes were added to reaction buffer (25 mM MES, 25 mM MOPS, 140 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.8 mM CaCl2, 800 μM MgCl2, pH 7.0, 37 °C) containing 200 μM Fe3+ -NTA, 400 μM ferrozine, and 5 μM FAD. The reaction was initiated by addition of 100 μM NADPH. Ferric ion reduction at 37 °C and formation of the Fe2+ -ferrozine complex was monitored at 562 nm using a thermostated Varian Cary 50 spectrophotometer using an extinction coefficient for the complex of 27,886 M^-1 cm^-1 (11). In each case, the reported activities are the differences between Steap3-transfected cells and empty vector controls. Titration data were subsequently fit to a Michaelis-Menten model with GraphPad Prism 5.0 software to determine the Michaelis constant (Km) and maximum reaction velocity (Vmax). Reported kinetic constants are the result of at least three completely independent experiments.

Confocal Microscopy—HEK-293F cells were maintained in DMEM supplemented with 10% FBS in T-75 flasks at 37 °C and 5% CO2. Twenty-four hours prior to transfection, cells were plated onto glass coverslips in a 6-well dish. Co-transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol with Steap3.VN173 and Steap3.VC155 expression plasmids. Twenty-four hours post-transfection, cells were washed three times with PBS, and then plasma membranes were stained by incubation with wheat germ agglutinin-Alexa Fluor 633 conjugate (Invitrogen) for 15 min, washed three times with PBS, and fixed in 4% formaldehyde in PBS for 15 min. Cells were then washed three times with PBS, stained with DAPI for 5 min, and mounted on glass coverslides with Vectashield mounting medium (Vector Laboratories). Samples were visualized with a Leica SP5 confocal microscope. Fluorophores were visualized at the following excitation and emission wavelengths: Venus, excitation, 514 nm; emission, 525–560 nm; DAPI, excitation, 405 nm; emission, 450–470 nm; wheat germ agglutinin, excitation, 633 nm; emission, 650–00 nm. Images were processed with Imaris software (Bitplane).

Heme Absorbance Analysis—Triton X-100-activated membranes were analyzed for heme content by absorbance spectroscopy on a Varian Cary 50 spectrophotometer. To obtain oxidized spectra, air-oxidized membranes (1 mg/ml) had their absorbance spectra recorded from 300 to 700 nm. To obtain reduced spectra, a few grains of sodium dithionite were added to the same sample used for the air-oxidized spectrum followed by an immediate spectral analysis from 300 to 700 nm. Finally, difference spectra were obtained by subtracting the oxidized from the reduced spectra.

Results

Ferric Reductase Activity of Steap3—Expression of exogenous human Steap3 (STEAP3) was performed in the Freestyle 293F system, an HEK-293-derived cell line that is easily transfectable and can grow to high densities in suspension cultures. To characterize the enzymatic activity of full-length Steap3, we first developed a ferric reductase activity assay for the intact protein in isolated membranes (see “Experimental Procedures”). Previous enzymatic assays monitored either cell surface ferric reductase activity (2, 3, 11) or flavin-dependent NADPH oxidase activity in a truncated protein (11). In contrast, this assay follows the formation of the Fe2+ -ferrozine complex (λmax = 562 nm) and allows the determination of kinetic constants for the full-length protein upon the addition of cytoplasmic (NADPH and FAD) and extracellular substrates (Fe3+).

We then attempted to purify Steap3 to homogeneity. To this end, we screened a variety of commonly used detergents from
which Triton X-100 was selected for its ability to solubilize Steap3 ferric reductase activity. However, although Steap3 in detergent-activated membranes retained activity for an extended period of time (days), all attempts to affinity-purify Steap3 activity from the detergent-activated membranes, including the use of anti-FLAG, Strep-tactin, and nickel-NTA resins, resulted in near total loss of enzyme activity. It is not clear whether some critical lipid or cofactor is lost during the on-column immobilization or whether the solubilized protein is simply unstable, but add-back experiments, reconstitution of eluted Steap3 into lipid vesicles, and the use of alternative detergents each failed to preserve or restore activity.

However, during the course of this work, it became clear that His6, FLAG, and Strep-II fusions at the C terminus of Steap3 did not adversely impact enzymatic activity in detergent-activated membranes. This suggested that we could potentially quantitate the amount of Steap3 present in the detergent-activated membranes by fusing Venus fluorescent protein to the C terminus of Steap3 (Steap3-Venus). Indeed, the fusion did not impact activity (Fig. 1A) and thus allowed the measurement of specific activity based upon the fluorescent content of the sample (Table 2). For these reasons, the Triton X-100-activated fraction (“crude membranes + Triton X-100”) of the Steap3-Venus fusion expressed in the presence of δ-aminolevulinic acid (see below) was used for subsequent enzymatic analysis because it contained the highest level of Steap3 specific activity (Table 2). Furthermore, we found that Steap3 expression was enhanced when valproic acid was added to the transfection medium. Valproic acid is a histone deacetylase inhibitor that has been shown previously to enhance transient gene expression in HEK-293 cells (14). The presence of valproic acid in the 293F transfection medium resulted in a ~1.6-fold increase in expression of Steap3-Venus over unsupplemented expression.

**FAD Is the Preferred Flavin for Steap3**—As described above, Steap3 detergent-activated membranes exhibit NADPH- and FAD-dependent ferric reductase activity. Importantly, however, the addition of other exogenous flavins, specifically FMN and riboflavin, did not contribute to increased ferric reductase activity above that seen for detergent-activated membranes in the absence of exogenous flavin (Fig. 1). This strongly suggests that full-length Steap3 does not utilize FMN or riboflavin as a substrate and that FAD is the physiologically relevant flavin.

**Steap3 Is a b-type Cytochrome—δ-Aminolevulinic acid (ALA) is a protoporphyrin precursor that is generated in vivo by δ-aminolevulinic acid synthase, the rate-limiting reaction in heme biosynthesis. ALA supplementation has thus been utilized in a variety of expression systems to improve heme incorporation in exogenously expressed hemoproteins (15, 16).** We found that supplementation with 0.5 μM ALA resulted in greater than a 2-fold increase in the difference spectrum Soret band at 429 nm in Steap3-containing membranes and an ~80% increase in membrane ferric reductase activity. Furthermore, the reduced-minus-oxidized difference spectra obtained for Triton X-100-treated membranes of wild-type Steap3-expressing cells indicate α, β, and γ Soret peaks at 560, 531, and 428 nm, respectively (Fig. 2A), indicative of the binding of a b-type heme cofactor.

**Steap3 Contains a Single b-type Heme Cofactor**—Sequence homology predicted that Steap3 utilizes bishistidine coordination via His-316 and His-409 to bind a heme cofactor in the transmembrane domain (2). However, other model systems for transmembrane ferric reduction, including yeast Fre1, mammalian duodenal cytochrome b, and mammalian NADPH oxidase, all utilize two heme groups to move electrons across the membrane. Thus, it has not been clear whether a second, cryptic heme binding site is present in Steap family proteins or whether Steap family metalloredoxases differ significantly from these other systems in utilizing a single heme cofactor. For this reason, we utilized site-directed mutagenesis to reexamine

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**TABLE 2**

| Fraction                        | Specific activity | Total fluorescence | Total activity |
|---------------------------------|-------------------|--------------------|---------------|
|                                 | pmol Fe3⁺ reduced/min | fluor unit         | pmol Fe3⁺ reduced/min |
| Lysate                          | 2.8               | 2,460              | 9,600         |
| Ultracentrifugation supernatant | 0                 | 0                  | 0             |
| Crude membranes                 | 2.3               | 2,080              | 4,780         |
| Crude membranes + TX-100        | 4.6               | 2,060              | 9,470         |
| Insoluble membranes             | 1.4               | 1,510              | 2,110         |
| Solubilized membranes           | 1.8               | 660                | 2,440         |

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**FIGURE 1.** Triton X-100-activated Steap3 is an NADPH- and FAD-dependent ferric reductase. A, Venus fluorescent protein fused to the C terminus of Steap3 does not alter ferric reductase activity and was thus utilized for activity studies. B, Steap3-containing Triton X-100-activated membranes exhibit maximal ferric reductase activity in the presence of both NADPH and FAD. The increase in activity observed with NADPH alone suggests that low levels of endogenous flavin are present in the membranes. Because the addition of FMN and riboflavin does not increase ferric reductase activity above that seen in the absence of exogenous flavin, full-length Steap3 shows a distinct preference for FAD. In each case, the reported activities are the differences between Steap3-transfected cells and empty vector controls.
This strongly suggests that Steap family metalloreductases utilize only a single heme cofactor to shuttle electrons across the transmembrane domain and that mechanistically they are fundamentally different from yeast Fre1 and mammalian proteins duodenal cytochrome b and NADPH oxidase.

**Steap3 Forms an Active Homodimer**—The isolated Steap3 N-terminal oxidoreductase domain has been reported to dimerize at low millimolar concentrations in solution and in the crystal, suggesting that full-length Steap3 may function as a homodimer in cellular membranes (10). However, a co-precipitation experiment utilizing FLAG-tagged Steap3 as bait and StrepII-tagged Steap3 as prey with Strep-tactin affinity resin did not produce evidence of an interaction (data not shown). Thus, dimerization may be transient or incompatible with co-precipitation conditions. To characterize a potential homodimer in vivo, a BiFC assay, which enables visualization of protein interactions in their natural cellular environment, was performed (17, 18). In the BiFC assay, proteins of interest are tagged with two complimentary fragments of a split fluorescent protein. If the proteins interact, the non-fluorescent fragments are brought together, which in turn facilitates folding and “maturation” of an active protein and results in a fluorescent readout. For the Steap3 BiFC assay, HEK-293 cells were co-transfected with Steap3 fused to N-terminal (Steap3.VN173) and C-terminal (Steap3.VC155) fragments of Venus. Transfected cells visualized at Venus emission wavelengths exhibited a distribution of cellular fluorescence similar to that seen in cells transfected with Steap3 fused to full-length Venus (Fig. 3). This strongly suggests that a Steap3 oligomer does indeed form in vivo.

We also wished to analyze the ferric reductase activity of the BiFC dimer; thus the Steap3.VN173 and Steap3.VC155 fusions were cloned into the pIRES dual expression vector (pIRES.Steap3VN.Steap3VC). This ensured the presence of both constructs in each transfected cell. Using this dual expression vector, we found that the specific activity of the BiFC pair was 9.9 pmol of Fe$^{3+}$ reduced/min/fluorescence unit, which is actually greater than the specific activity of cells transfected with Steap3.Venus. The increased specific activity was due primarily to decreased fluorescence, which is expected due to the presence of, at most, a single mature Venus per Steap3 dimer. Interestingly, it is believed that once the split fluorescent protein has matured, it remains stable, and the N- and C-terminal fragments do not dissociate, leading to a constitutive oligomer (17, 18). Thus, not only is Steap3 able to form homodimers in the relevant cellular compartments but it is also enzymatically active in the dimeric state.

**Steap3 Electron Transfer Follows an Intrasubunit Pathway**—Steap3 is composed of two linked domains: the transmembrane domain and a cytoplasmic oxidoreductase domain. Upon dimerization, protomers may thus be capable of performing intersubunit electron transfer wherein the oxidoreductase domain of one protomer transfers electrons to the transmembrane domain of the other (Fig. 4A). To examine the possibility of such “domain swapping,” cells were again transfected with the BiFC dual expression vector containing two Steap3 genes (pIRES.Steap3VN.Steap3VC). In this case, the two Steap3 constructs were either both wild type or contained two different
Supplemental Fig. 1 and a list showing Steap3.VC155 alone did not exhibit a fluorescence signal (not shown).

The identification and characterization of a flavin binding site for any full-length Steap family member has not been reported, and the development of the enzymatic assay described above is the first indication that FAD is the preferred flavin for Steap3 (Fig. 1). To this end, we measured Fe$^{3+}$ reductase activity as a function of FAD concentration. When fit to the Michaelis-Menten model (Fig. 5), we found a $K_m$ of 0.9 $\mu$M for FAD, indicating the presence of a physiologically relevant FAD binding site. Relative to the isolated N-terminal oxidoreductase domain, the $K_m$ value for full-length Steap3 is lower by more than 2 orders of magnitude, indicating that the transmembrane domain plays a critical role in the recognition of FAD.

Multiple sequence alignments are frequently used to identify residues critical to protein function. For Steap3, specifically, the utility of this approach can be assessed by mapping strictly conserved residues to the surface of the Steap3 oxidoreductase domain for which crystal structures have been determined (10). Toward this goal, the NCBI non-redundant protein sequence database was utilized to obtain a total of 461 Steap2, Steap3, and Steap4 sequences (Steap1 lacks the oxidoreductase domain), which were then aligned with Clustal Omega (19). We found that 10 residues in the oxidoreductase domain are strictly conserved, seven of which are surface-exposed. Importantly, five of the seven are found in the NADPH binding pocket of the Steap3 crystal structure (Fig. 6A). Thus, Steap family sequence alignments are able to discriminate residues involved in substrate recognition.

We then undertook a similar analysis but with the inclusion of Steap1 sequences to identify potential FAD binding site residues in the transmembrane domain. 607 Steap family sequences were aligned, and strictly conserved amino acids were assigned approximate positions within the membrane bilayer as predicted by the membrane topology program TopPred (Fig. 6B and supplemental Fig. 1 and a list showing Steap family alignment species) (20). Of the 15 strictly conserved residues in the transmembrane domain, nine are predicted to lie on cytoplasmic loops between transmembrane helices II and III (the $\alpha$II/III loop) or helices IV and V (the $\alpha$IV/V loop). As these residues lie in between the N-terminal oxidoreductase domain and histidine residues implicated in heme binding, we considered these as potential residues involved in recognition of FAD.

The nine strictly conserved residues in the $\alpha$II/III and $\alpha$IV/V loops of the transmembrane domain were individually mutated to alanine, and the kinetic parameters for FAD-dependent ferric reductase activity were determined (Table 3). With an increase in the $K_m$ from the wild-type value of 0.9 to 17.7 $\mu$M,
the E395A variant showed the most significant decrease in affinity for FAD; however, the $V_{\text{max}}$ was essentially unchanged. Although not as large, we also found a significant increase ($p < 0.05$) in the FAD $K_m$ value for the E390A variant ($K_m = 4.5 \mu M$), which is also in the αIV/V loop. Because the hallmark of residues involved predominately in substrate recognition is an increase in $K_m$ with little or no change in $V_{\text{max}}$, these data strongly implicate Glu-390, Gln-395, and the αIV/V loop in formation of the FAD binding site.

The exact role of residues in the αII/III loop was more difficult to assess. Simple alanine substitution for Gln-281, Lys-287, and Leu-295 all resulted in complete loss of activity. It is not clear whether this was because the variants were unable to bind FAD or because these are instead key catalytic or structural residues. However, it does clearly indicate a critical role for the αII/III loop. However, measurable activity was found for the W298A, R302A, and K303A variants, and in each case, we did find increased $K_m$ values for FAD. However, compared with wild type, the differences were not statistically significant ($p > 0.05$). We thus considered the possibility that Arg-302 and Lys-303 might utilize their positive charge to interact with negative charge on FAD and that negatively charged substitutions might interfere with such interactions. Indeed, the K303E variant gave a significantly increased $K_m$ (4.6 μM) with a minimally disturbed $V_{\text{max}}$. The R302E mutation however, showed a complete loss of activity, confounding interpretation of the results. Finally, we also investigated the R302A/K303A double mutant and found a slightly increased $K_m$ relative to each individual alanine mutant indicative of an additive effect for these residues. More importantly, the double mutant also showed a statistically significant increase in $K_m$ (4.0 μM) over wild-type Steap3. Collectively, our mutational analysis thus also implicates Arg-302, Lys-303, and the αII/III loop in general in FAD binding. In conjunction with previous work on the Steap4 oxidoreductase domain demonstrating the lack of a high affinity flavin binding site in the oxidoreductase domain, we conclude that major elements of the high affinity FAD binding site lie in the transmembrane domain of Steap3 and that conserved residues within the αII/III and αIV/V loops are of particular importance.

**Fe**^{3+} Binding Site of Steap3—Ferric and cupric reductase activities have been reported for Steap2, Steap3, and Steap4 (2, 3). More recently, the first determination of $K_m$ values for any Steap family member for Fe^{3+} and Cu^{2+} was reported for rat Steap4 using the cell surface metalloreductase assay (11). Collectively, this work suggested that Steap proteins contain high affinity binding sites for both Fe^{3+} and Cu^{2+} and that Steap family members are likely to be physiologically relevant cupric and ferric reductases involved in homeostasis of these metals. However, to date, metal $K_m$ values have not been reported for Steap3. For this reason, using Fe^{3+}-NTA as the substrate, we

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**FIGURE 4. Investigating Steap3 electron transfer pathway.** A, a Steap3 dimer could potentially transfer electrons in an intra- or intersubunit manner. In intrasubunit electron transfer (A, left side), electrons from the NADPH-binding oxidoreductase domain would be transferred to the subunit's own transmembrane domain. In intersubunit electron transfer (A, right side), a domain-swapped dimer would result in the electrons from one oxidoreductase domain transferred to the transmembrane domain of the other subunit. B, to differentiate between these two alternatives, mutations were introduced to inactivate the cytoplasmic NADPH oxidase domain of one subunit (S58I.VN173) and the transmembrane domain of the other subunit (H409A.VC155). Cells were transfected with a dual expression vector containing the S58I.VN173 and H409A.VC155 constructs (pIRES.S58I.VN.H409A.VC). If the Steap3 dimer undergoes intrasubunit electron transfer, the mutational lesions in the respective subunits would prohibit electron transfer in both subunits (red arrow). In intersubunit electron transfer, the BIFC heterodimers would complement one another, restoring one of the two electron transfer pathways (green arrow). Thus, the presence of an intersubunit electron transfer pathway would be indicated by the partial restoration of ferric reductase activity. C, in contrast to cells expressing the wild-type BIFC pair (pIRES.Steap3VN.Steap3VC), ferric reductase activity was not detectable (N.D.; limit of detection, 0.4 pmol of Fe^{3+} reduced/min/fluorescence unit) in cells expressing the mutant BIFC pair (pIRES.S58I.VN.H409A.VC). Expression levels were not significantly altered in the mutant BIFC pair (0.13 fluorescence unit/μg of protein) from the wild-type pair (0.14 fluorescence unit/μg of protein); thus the lack of activity was not due to loss of stable protein expression. The lack of complementation indicates an intrasubunit electron transfer pathway as illustrated in B (left side).
determined that the $K_m$ for $Fe^{3+}$ was 5.0 $\mu M$ (Fig. 5 and Table 4), which is nearly identical to that found for Steap4 using the cell surface assay. Unfortunately, $Cu^{2+}$ was spontaneously reduced to $Cu^{+}$ under these assay conditions due to the presence of NADPH, precluding measurement of a $K_m$ value for $Cu^{2+}$.

Specific residues involved in metal recognition have not been reported for any member of the Steap family. However, our success in identifying residues involved in FAD recognition suggested that conserved residues in the luminal or extracellular half of the transmembrane domain, i.e. residues above the heme-coordinating histidine residues (Fig. 6B), might participate in iron recognition. Two strictly conserved residues, Tyr-229 and Trp-355, are predicted by TopPred to lie on the extracellular/luminal side of the heme-coordinating histidine residues (Fig. 5B). Because of its non-polar side chain, Trp-355 was not chosen for mutation. In addition, a third residue, Tyr-319, was also selected for mutation because of its high degree of conservation (>98%) and proximity to the heme cofactor. Specifically, Tyr-319 is predicted to lie approximately one helical turn nearer to the luminal face than the heme-binding His-316 and could thus be well positioned to participate in iron binding and reduction. Indeed, when these tyrosine residues were individually mutated to alanine or phenylalanine, impaired iron reduction was observed. For the conservative Y319F substitution, the $V_{max}$ for $Fe^{3+}$ increased by more than 3-fold from 5 to 17 $\mu M$, whereas $V_{max}$ was essentially unchanged. Similarly, the Y229F variant showed an inflated $K_m$ of 15 $\mu M$, suggesting that each of these residues play significant roles in recognition of $Fe^{3+}$. The double mutation Y229F/Y319F was also examined, and the $K_m$ increased to 24 $\mu M$ with only an insignificantly small decrease in $V_{max}$.

Tyr-271, also strictly conserved, is predicted to lie near the midpoint of the bilayer in helix $\alpha III$ (Fig. 5B). This location seemed inconsistent with a residue involved in iron recognition. Nevertheless, we considered the possibility that its location could be mispredicted or that its side chain might reach up toward the iron binding site. However, when mutated to phenylalanine, no effect was seen on ferric reductase kinetics, whereas mutation to alanine resulted in complete loss of activity (Table 4). With no indication of a role in iron binding, the phenyl ring of Tyr-271 serves an as yet undefined structural or functional role in transmembrane electron transfer.

Although additional residues are likely to participate in iron binding and reduction, the characterization of these conserved tyrosine residues provides significant insight into the location and nature of the iron binding site. First, the location of Tyr-319 approximately one helical turn past heme-coordinating His-316 suggests that the iron binding site lies near the “top” edge of the heme cofactor in the transmembrane domain. Second, it suggests that the topological arrangement of the transmembrane helices will bring Tyr-229 and Tyr-319 in close proximity to each other, allowing them to work in concert to form a recessed iron binding site adjacent to the heme.

**Discussion**

**Steap1 Shares the Steap Family FAD and Iron Binding Motifs**—Steap1 is highly expressed in multiple cancers and has received interest as an immunotherapeutic target (21, 22). However, Steap1 lacks the N-terminal oxidoreductase domain found in Steap2, Steap3, and Steap4 and instead substitutes a 70-residue N-terminal tail. In addition, attempts to demonstrate Steap1 ferric or cupric reductase activities have been unsuccessful (3). Thus, it has been unclear whether Steap1 is a functional transmembrane oxidoreductase. However, because the FAD and iron binding motifs that we have identified here in Steap3 are also present in Steap1, it seems that Steap1 might indeed harbor latent transmembrane oxidoreductase activity. If so, this would obviously require a cytosolic source of FAD($H_2$). In addition, it remains possible that Steap1 might oxidize or reduce an alternative extracellular or luminal substrate.

**Steap3 Homodimer**—In conjunction with the crystallographic structures of the isolated oxidoreductase domains of Steap3 and Steap4, the BiFC study presented here suggests that Steap3 is indeed active as a homodimer. One potential explanation for dimer formation was that Steap3 might utilize an intersubunit electron transfer pathway through a domain-swapped dimer. However, the inability of the S58I and H409A mutants to complement each other suggests otherwise. Thus, the functional or regulatory significance of the homodimer remains unclear. However, it is possible that Steap3 may function within a larger complex with the 2-fold symmetric transferrin receptor (23). A Steap3 dimer and its cargo of four electrons is stoichiometrically matched to the transfer-
**FAD and Fe$^{3+}$ Binding Sites in the STEAP Transmembrane Domain**

**A**

![Diagram of Amino acid conservation of Steap family oxidoreductase and transmembrane domains.](image)

**B**

Extracellular/Lumen

Cytosol

**C**

![Diagram of NADPH and Heme coordination.](image)

**FIGURE 6.** Amino acid conservation of Steap family oxidoreductase and transmembrane domains. Amino acid sequences from Steap family members in the NCBI non-redundant database were aligned with Clustal Omega (19). A, strictly conserved residues (magenta) from all Steap2, Steap3, and Steap4 sequences are mapped on the surface of the crystal structure for the Steap3 oxidoreductase domain (Protein Data Bank code 2VQ3 (10)). The NADPH binding site includes five of the 10 strictly conserved residues in the Steap3 oxidoreductase domain (Protein Data Bank code 2VQ3 (10)). The Steap4 sequences are mapped on the surface of the crystal structure for the Steap4 oxidoreductase domain (Protein Data Bank code 2VQ3 (10)). The topology prediction program TopPred (20) was used to predict approximate locations for strictly conserved residues in the Steap3 transmembrane domain. Heme coordination by His-316 and His-409 is represented by a connecting line. Tyr-319 is not strictly conserved (>$98\%$) but was targeted for mutagenesis studies because of conservation in earlier sequence alignments (11) and its proximity to His-316.

**Steap3 Transmembrane Domain Binds a Single, b-type Heme—**Ohgami et al. (2) proposed a model for iron reduction by Steap3 in which a heme cofactor acts as a key intermediary in the transfers of electrons from NADPH and flavin bound in the N-terminal oxidoreductase domain to iron bound at the extracellular or luminal face of the transmembrane domain. Here we confirm their prediction that Steap3 binds a single heme in a bishistidine fashion (His-316 and His-409) and show that it is a b-type heme. These histidine residues lie in transmembrane helices 3 and 5 with predicted locations slightly above the center of the bilayer toward the extracellular or luminal space (Fig. 7). These residues are a core feature of the FRD superfamily, which includes such members as yeast ferric reductase (Fre1) and human NADPH oxidase (9). However, in contrast to a single transmembrane heme in the Steap proteins, other members of the FRD superfamily contain a second heme cofactor localized near the cytoplasmic face of the membrane (9). In this context, the transmembrane domain of Steap family proteins appears unique. In fact, to our knowledge, there exists no functional evidence for monoheme cytochromes that perform transmembrane electron transport beyond the Steap family metalloreductases and their bacterial YedZ homologs (24) for which sulfite oxidase activity has been demonstrated (25).

**Steap3 Iron-binding Residues Adjacent to the Heme Cofactor—**In our mutational analysis, Tyr-229 and Tyr-319 were found to participate in iron binding. Tyr-229 is predicted to reside in the first transmembrane helix at approximately the same “height” as Tyr-319, which is located one $\alpha$-helical turn above His-316 (Fig. 7). The Tyr-319 side chain is thus expected to lie near the top of the porphyrin ring. This in turn suggests that the iron binding site is also near the top edge of the heme cofactor as opposed to a more distant site composed of extracellular loops. This raises the possibility that one or perhaps both of the propionate arms of the heme cofactor might serve to coordinate iron in a manner similar to manganese coordination observed in manganese peroxidase (26). This would require the heme to be oriented with the propionate arms pointed toward the extracellular space. Such an orientation is indeed observed in structures of integral membrane cytochromes such as the ascorbate-dependent ferric reductase duodenal cytochrome $b$ (27) and the cytochrome $b$ subunit of the $bc$ complex (28).

**The Transmembrane Domain Contains a High Affinity FAD Binding Site—**We have shown for the first time that Steap family proteins do indeed utilize a flavin in the reduction of Fe$^{3+}$. In addition, we found that Steap3 exhibits a clear preference for FAD as the addition of exogenous FMN or riboflavin did not increase activity above the flavin-free control. This suggests that the activity in the flavin-free control is due to low levels of endogenous FAD present in the membrane preparations.

We measured a $K_m$ for FAD in full-length Steap3 of $\sim1 \mu M$, which is greater than 2 orders of magnitude lower than the non-physiological flavin $K_m$ values ($>100 \mu M$) measured for the isolated N-terminal oxidoreductase domains of Steap3 and Steap4 (11). Indeed, the structural and kinetic work on these isolated domains previously suggested that the N-terminal oxidoreductase has only a weak interaction with FAD and other endosomal Fe$^{3+}$ and cytosolic NADPH, a transferrin receptor-Steap3 complex might also ensure recruitment of Steap3 into iron-laden endosomes (10).
TABLE 3
FAD kinetic characterization of Steap3 Fe3+ reductase activity
The transmembrane topology prediction was generated by the TopPred program (20). Fluorescence (Fluor unit) per microgram of protein is represented to indicate approximate expression levels of the respective constructs. Values are represented as mean ± S.D. with means from n = 3 independent experiments. ND, not determinable
(limit of detection = 0.4 pmol of Fe3+ reduced/min/fluorescence (fluor) unit).

| Variant | Topology prediction of mutant | pmol Fe3+/min/fluor unit | μmol FAD | Vmax/Km | Fluor unit/μg protein |
|---------|-------------------------------|--------------------------|-----------|----------|------------------------|
| Wild type |                                | 3.7 ± 1.9                | 0.9 ± 0.4 | 4.4 ± 2.5 | 0.47 ± 0.13           |
| Q281A   | all-all loop                   | ND                       | ND        | ND       | ND                     |
| K287A   | all-all loop                   | ND                       | ND        | ND       | ND                     |
| L295A   | all-all loop                   | ND                       | ND        | ND       | ND                     |
| W298A   | all-all loop                   | 2.8 ± 0.3                | 2.2 ± 0.4 | 1.3 ± 0.3 | 0.52 ± 0.05           |
| R302A   | all-all loop                   | 1.4 ± 0.8                | 2.8 ± 1.4 | 0.6 ± 0.4 | 0.35 ± 0.19           |
| R302E   | all-all loop                   | ND                       | ND        | ND       | 0.35 ± 0.18           |
| K303A   | all-all loop                   | 2.5 ± 1.2                | 2.8 ± 0.6 | 0.9 ± 0.4 | 0.54 ± 0.10           |
| K303E   | all-all loop                   | 2.7 ± 1.7                | 4.6 ± 2.9 | 0.6 ± 0.4 | 0.50 ± 0.18           |
| R302A/K303A | all-all loop | 1.4 ± 0.9                | 4.0 ± 2.2 | 0.5 ± 0.5 | 0.50 ± 0.13           |
| W386A   | all-V/V loop                   | 2.3 ± 1.5                | 3.0 ± 0.6 | 0.7 ± 0.4 | 0.56 ± 0.07           |
| E390A   | all-V/V loop                   | 3.0 ± 1.2                | 4.5 ± 1.0 | 0.6 ± 0.4 | 0.55 ± 0.07           |
| Q395A   | all-V/V loop                   | 4.4 ± 1.8                | 18 ± 4.4  | 0.3 ± 0.2 | 0.52 ± 0.10           |

* Statistically significant differences (p < 0.05) versus wild type from one-way analysis of variance and Dunnett’s multiple comparison test.

TABLE 4
Fe3+ kinetic characterization of Steap3 Fe3+ reductase activity
Fluorescence per microgram of protein is represented to indicate approximate expression levels of the respective constructs. Values are represented as mean ± S.D. with means from n = 3 independent experiments. ND, not determinable
(limit of detection = 0.4 pmol of Fe3+ reduced/min/fluorescence (fluor) unit).

| Variant | pmol Fe3+/min/fluor unit | μmol Fe3+/Km | Vmax/Km | Fluor units/μg protein |
|---------|--------------------------|--------------|---------|------------------------|
| Wild type | 3.0 ± 1.6                | 5.0 ± 1.4    | 0.60    | 0.54 ± 0.19           |
| Y229A   | 2.7 ± 2.0                | 12 ± 3.9     | 0.21    | 0.39 ± 0.30           |
| Y229F   | 1.9 ± 1.2                | 15 ± 2.9*    | 0.13*   | 0.42 ± 0.08           |
| Y271A   | ND                       | ND           | 0.10    | 0.50 ± 0.13           |
| Y271F   | 2.6 ± 0.4                | 5.7 ± 0.2    | 0.45    | 0.57 ± 0.10           |
| Y319A   | 3.5 ± 1.3                | 9.9 ± 1.6    | 0.35    | 0.40 ± 0.17           |
| Y319F   | 2.7 ± 0.7                | 18 ± 4.5*    | 0.15*   | 0.52 ± 0.10           |
| Y229F/Y319F | 1.7 ± 0.7       | 24 ± 2.8*    | 0.07*   | 0.49 ± 0.05           |

* Statistically significant differences (p < 0.05) versus wild type from one-way analysis of variance and Dunnett’s multiple comparison test.

flavins and that this low affinity recognition is primarily via the isosaloxazine ring. We now conclude that the elements necessary for high affinity FAD binding reside primarily within the Steap transmembrane domain.

The αII/III and αIV/Ⅴ Loops Form the FAD Binding Site—Among the 15 strictly conserved residues in the transmembrane domain (Fig. 6B), nine are predicted to lie within loops connecting helix II to helix III and helix IV to helix V. Our mutational analysis clearly shows that these loops are critical for Steap3 activity where they contribute to FAD binding. Thus, although these loops are distant within the linear sequence, they are expected to lie close to each other in the folded protein structure, working together to bind FAD (Fig. 7). Interestingly, the αIII/Ⅴ loop also contains the endosomal targeting motif for AP-2 (Y288XXΦ where Φ is bulky hydrophobic Phe-291) (29). Whether AP-2 might alter the activity of Steap3 during clathrin-mediated endocytosis remains an open question.

The Transmembrane Domain FAD Binding Site Replaces the Cytoplasmic Facing Heme—As shown above, Steap proteins contain an extracellular or luminal facing heme that is common to all members of the FRD superfamily (9) but appear to lack the second cytoplasmic facing heme. Interestingly, a bioinformatics analysis of the FRD superfamily shows that YedZ and Steap family proteins are differentiated from other members of the FRD superfamily by replacement of the histidine residues coordinating a cytoplasmic facing heme with conserved arginine and glutamine residues. Importantly, in Steap3, the arginine and glutamine residues in this superfamily sequence alignment correspond to Arg-302 and Gln-395. Here we have shown that these residues contribute to the FAD binding pocket in the transmembrane domain (Table 3). Thus, the Steap and YedZ family proteins have apparently lost their cytoplasmic facing heme binding site and replaced it with a cytoplasmic facing FAD binding site.

Predicted Structure for the Transmembrane Domain—The recently solved crystal structure of duodenal cytochrome b reveals an FRD-like structure in which the cytoplasmic and extracellular facing hemes are surrounded by six transmembrane helices (27). Interestingly, the helices follow one another in succession as they work their way around the heme cofactors. If a similar helical arrangement is considered for the transmembrane domain of Steap3 (Fig. 7), several observations consistent with the biochemical data can be made. First, the arrangement results in placement of the heme-coordinating histidine residues on opposite sides of the heme cofactor. Second, it brings the αIII/Ⅴ loop in close proximity to the αIV/Ⅴ loop so they might collectively work to bind FAD. Third, it could bring the tyrosine residues implicated in iron binding (Tyr-229 and Tyr-319) into close proximity. For these reasons, we propose the structure shown in Fig. 7 for the Steap3 transmembrane domain as it is consistent with the structures of integral membrane cytochromes in general and with the unique structural and biochemical properties of the Steap family that we have elucidated here, namely utilization of a single b-type heme and the location of the iron and FAD binding sites. Furthermore, it is expected that the structure of this transmembrane electron transfer domain is shared by all...
FAD and Fe\(^{3+}\) Binding Sites in the STEAP Transmembrane Domain.

**FIGURE 7. Structural model of the Steap3 transmembrane domain.** Strically conserved residues key to Fe\(^{3+}\), heme, or FAD binding are shown. Residues are oriented based on predictions by the TopPred topology server (20). The electron source, NADPH, is oriented approximately according to that found in the Steap3 oxidoreductase domain crystal structure with "P" representing the heme-containing nicotinamide moiety. The heme is transferred to FAD, which is bound by the all/III and all/V loops, including interactions with strictly conserved residues Arg-302, Lys-303, Glu-390, and Gln-395. Single electrons are transferred to a single b-type heme bound in a bishistidine fashion via His-316 and His-409. Tyr-229 and Tyr-319 are implicated in binding an Fe\(^{3+}\) atom adjacent to the heme cofactor. Foreground segments are represented by dashed boundaries, and Tyr-229 is positioned on the backside of helix I in close proximity to Tyr-319 and Fe\(^{3+}\). Transmembrane α-helices are numbered by roman numerals. The transmembrane helices have been split open for purposes of illustration to reveal the interior heme- and metal-binding residues. However, by analogy to other transmembrane cytochromes, we expect helices I and VI to contact each other such that the helices completely encircle the heme cofactor.

Steap family members, including Steap1, and the related YedZ family of bacterial proteins.

**Author Contributions**—M. D. K. was responsible for the bulk of the data acquisition. M. D. made the Gateway vectors and helped revise the manuscript. M. D. K. and C. M. L. conceived and coordinated the study and wrote and revised the paper.

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