The cytochrome P450 24A1 interaction with adrenodoxin relies on multiple recognition sites that vary among species

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*Running title: Cytochrome P450 24A1/adrenodoxin interactions

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Keywords: cytochrome P450, vitamin D, NMR, mitochondria, membrane protein, ferredoxin, adrenodoxin, CYP24A1, P450-ferredoxin complex

ABSTRACT

Mitochondrial cytochromes P450 (P450s) are responsible for important metabolic reactions, including steps involved in steroid and vitamin D metabolism. The mitochondrial P450 24A1 (CYP24A1) is responsible for deactivation of the bioactive form of vitamin D, 1,25(OH)2D3. Its function relies on formation of a P450–redox partner complex with the ferredoxin and electron donor adrenodoxin (Adx). However, very little is known about how the Adx–CYP24A1 complex forms. In this study, we report the results of solution NMR in which we monitor isotopically labeled full-length Adx as it binds CYP24A1 in complex with the P450 inhibitor clotrimazole. The NMR titration data suggested a mode for P450–Adx interactions in which formation of the complex relies on contributions from multiple recognition sites on the Adx core domain, some of which have not previously been reported. To evaluate differences among CYP24A1–Adx complexes from different mammalian species and displaying distinct regioselectivity for 1,25(OH)2D3, all bound spectra were acquired in parallel for human (carbon-23 and -24 hydroxylase), rat (carbon-24 hydroxylase), and opossum (carbon-23 hydroxylase) CYP24A1 isoforms. Binding data from a series of single and double charge–neutralizing substitutions of Adx confirmed that species-specific CYP24A1 isoforms differ in binding to Adx, providing evidence that variations in redox partner interactions correlate with P450 regioselectivity. In summary, these findings reveal that CYP24A1–Adx interactions rely on several recognition sites and that variations in CYP24A1 isoforms modulate formation of the complex, thus providing insight into the variable and complex nature of mitochondrial P450-Adx interactions.

Mitochondrial cytochromes P450 (P450) are responsible for a host of biological reactions, including steps necessary in steroid and vitamin D metabolism. Similar to microsomal P450s, their function requires the transfer of two electrons, delivered sequentially, in order to generate the active oxidizing species necessary for completion of one cycle of P450 catalysis (1). However, in contrast to microsomal P450 enzymes, for which reduction of P450 can be achieved by binding either a P450 oxidoreductase or, in some cases, the small heme protein cytochrome b5, mitochondrial enzymes rely entirely on formation of a transient complex with the soluble ferredoxin protein, Adrenodoxin (Adx). Adx folds into a compact structure consisting of three short α-helices and five anti-parallel β strands, with a [2Fe-2S] cluster coordinated near solvent accessible surfaces between helix-1 and helix-3 (2,3). Changes in the C-terminal region of bovine Adx have been shown to participate in mediating a monomer to dimer transition in response to reduction of the [2Fe-2S] cluster (2), thereby suggesting that dimers of Adx are functionally relevant.

While the prevailing evidence suggests that complex formation between Adx with P450 relies on salt bridge interactions between the side chains of acidic residues on helix-3 (Asp-72 –
Asp-79) with the basic surface residues on the proximal surface of P450 (4-7), there is also evidence to support variability between particular redox complexes. For example, co-crystal structure determination of a CYP11A1-Adx fusion construct supports prior mutagenesis data indicating that the protein complex relies on electrostatic interactions between Asp-72, Glu-73, and – Asp-79 of helix-3, along with Glu-47 of the [2Fe-2S] cluster coordination loop, to interact with basic surface residues of CYP11A1 (4,5). However, a glutamate incorporated at residue Thr-71, designed to mimic phosphorylation of Adx, resulted in enhanced binding with CYP11A1, but had no apparent effect on the interaction with the corticosteroid-metabolizing enzyme CYP11B1 (8). This finding correlated well with previous work in which CYP11B1 was shown to rely, at least in part, on hydrophobic interactions with Adx (9). Nonetheless, more recent analysis of cross-linking data for CYP11B1 and the closely related CYP11B2 reinforces the role of electrostatic interactions with the acidic helix-3, in addition to a previously unreported contact with Adx Asp-113 near the carboxyl terminus, modeled as an interaction with a second molecule of an Adx dimer (10). Taken together, these studies suggest a redox complex with Adx that may be variable between particular P450 systems.

Far less is known regarding the nature of the complex with Adx for the multi-functional vitamin D metabolizing CYP24A1. The maintenance of the bioactive form of vitamin D, 1,25(OH)2D3, is tightly regulated in part by increased expression of CYP24A1, which in turn is responsible for deactivation of the hormone. Mutations affecting CYP24A1 function correlate with incidents of idiopathic infantile hypercalcemia or hypercalcuria or elevated serum 1,25(OH)2D3 in adults (11-14). In humans, CYP24A1 mediates hydroxylation of the vitamin D side chain at either carbon-23 (C23) or carbon-24 (C24), then mediates all subsequent steps in each pathway to produce either the metabolite 1,25(OH)2D3-26,23-lactone or calcitriol acid from the C23 or C24 pathways, respectively (15-17). While calcitriol acid is excreted in urine, 1,25(OH)2D3-26,23-lactone has been demonstrated to modulate vitamin D signaling by binding the vitamin D receptor (18,19). Intriguingly, this first step in the deactivation pathway is also regioselective according to species, with the human isoform hydroxylating at both C23 and C24 sites at an approximate 2:8 ratio while rat CYP24A1 is a pure C24 hydroxylase and opossum CYP24A1 is a pure C23 hydroxylase (20).

Here we report the use of solution NMR to evaluate the redox partner complex between full length bovine Adx and CYP24A1. Analysis of the pattern of differential line broadening in the 15N-Adx HSQC spectra suggests a binding mode for Adx that relies on multiple recognition sites. Candidate binding sites revealed by the NMR titrations were further evaluated by site-directed mutagenesis of Adx, in which contributions from at least three distinct surfaces of Adx were shown to be involved in the redox partner interaction. In order to evaluate differences between the Adx redox complex with CYP24A1 isoforms displaying distinct C23 or C24 regioselective preferences, we also carried out NMR binding studies in parallel with human, rat, and opossum CYP24A1 isoforms. These results provide evidence for a CYP24A1-Adx redox interaction that is complex and variable between species.

RESULTS

**Ligand and redox partner assisted stability of CYP24A1 for NMR analysis** – Due to the low salt, detergent-free requirement of NMR data collection, monitoring formation of the complex between CYP24A1 and Adx requires stabilization of the membrane extracted catalytic domain of CYP24A1. In a previous study, the high affinity inhibitor abiraterone was used to stabilize the catalytic domain of the steroidogenic enzyme CYP17A1 (21,22), thus conferring sufficient sample solubility as required for the acquisition of two dimensional and three dimensional NMR data. A similar approach was utilized here, in which incorporation of the P450 inhibitor clotrimazole resulted in enhanced bacterial expression of recombinant CYP24A1 isoforms when supplemented in growth media as well as conferring enhanced stability and solubility in NMR samples. Generally utilized previously for inhibition of the 1α-vitamin D hydroxylase CYP27B1, clotrimazole has also been demonstrated to inhibit CYP24A1 activity (23). Titration of rat CYP24A1 with clotrimazole induced a type-II spectral shift (Figure 1A) with a
sub µM binding affinity. However, efforts to fit the spectral binding data to a quadratic equation were unsuccessful. Instead, the data were best fit to a sigmoidal binding curve (Figure 2B) with a hill coefficient of 1.8, suggesting that clotrimazole binds CYP24A1 cooperatively.

In order to evaluate the stability of the enzyme-inhibitor-Adx complex under NMR conditions, CYP24A1-clotrimazole was first exchanged into a detergent free buffer followed by exchange into low salt NMR buffer, concentrated to 100 µM, combined with an equal molar equivalent of unlabeled Adx, and stored at 25 °C. Following high-speed centrifugation, the supernatant was periodically assayed over time by monitoring the absorbance intensity of the Soret peak. Minimal CYP24A1 loss was detected over a 96 hour time period. The stability conferred by the CYP24A1-clotrimazole-Adx complex extended toward the 15N labeled NMR samples for human and opossum isoforms, in which no loss of P450 was detected upon completion of data acquisition. However, rat CYP24A1 was observed to decrease in concentration over the course of data collection, in which case P450 concentration was assayed and final rat CYP24A1 (clotrimazole) concentrations are reflected in the enclosed ratios.

**Addition of CYP24A1 induces line broadening of the 15N-Adx NMR spectra** – Formation of the complex was monitored using the two dimensional 15N-HSQC spectrum of oxidized full length bovine Adx. The labeled protein yields a well-dispersed NMR spectrum consistent with that reported previously (Figure 2A) (24). Due to the presence of the paramagnetic [2Fe-2S] cluster of Adx, residues Gly-44 to Cys-55 and Leu-90 to Leu-96 were not detected in the NMR spectra. However, signals corresponding to surfaces residues on helices 1, 2 and 3 flanking the [2Fe-2S] coordination loop, as well as residues comprising the three anti-parallel β strands were present in the spectrum. In total, 64 backbone amides and 14 side chain amides of the folded regions of Adx were transferred from the Biological Magnetic Resonance Data Bank (bmrB entry 4566) (24) and were utilized in this analysis.

In order to identify differences in the CYP24A1-Adx complex for species displaying distinct regioselectivity for hydroxylation of 1,25(OH)2D3, NMR titrations were carried out using unlabeled clotrimazole-bound CYP24A1 corresponding to isoforms found in human, rat, and opossum. For all three isoforms, step-wise addition of CYP24A1-clotrimazole induced pronounced broadening of the NMR line shape for all 15N-Adx resonances (Figure 2A), with approximately 30% of the original peak intensities remaining upon addition of 0.2-0.35 molar equivalents of P450 (Figure 2B). Similar broadening, consistent with a protein interaction in the intermediate chemical exchange NMR time regime, has been observed in other P450 accessory protein complexes (21,22,25,26).

While these titrations reveal a pattern of predominantly uniform broadening for Adx backbone resonances when the P450 is present, the ratios measured at 0.35 molar equivalents of human CYP24A1, 0.25 molar equivalents of rat CYP24A1, and 0.20 molar equivalents of opossum CYP24A1, generally presented a similar pattern of peak broadening (Figure 2B). Modest differences in the extent of broadening were observed for residues in helix-3 (Asp-72 to Asp-79) as determined by ratios that deviated more than one standard deviation from the mean (Figure 2B, highlighted in red), indicating a change in the environment in this region relative to the rest of Adx. This is consistent with the putative role for the acidic side chains of helix-3 as participants in salt bridge formation at the protein interface with other mitochondrial P450 enzymes (4,5,8). Interestingly, additional differential broadening effects were also observed near helix 1 for the interaction with rat and human CYP24A1 as well as for residues near helix-2 for the interactions with all three isoforms.

**Differential line broadening of 15N-Adx side chain amides suggests multiple recognition sites** – In order to further analyze the modest differential broadening of the NMR signal along the 15N-Adx backbone, the resonance intensities corresponding to the Adx glutamine and asparagine side chain amide signals were also quantified with and without CYP24A1 present. Analysis of side chain resonances provides two advantages. First, due to the greater initial intensity of these signals in free 15N-Adx, they retain measurable intensity in the presence of larger amounts of CYP24A1-clotrimazole relative to backbone resonances. Second, the orientation and distribution of
glutamine and asparagine side chains provide adequate coverage of the Adx molecule (Figure 3A), thus allowing for the monitoring of multiple candidate binding sites.

For all titrations, the relative differential broadening was more pronounced than that observed for the $^{15}$N-Adx backbone amides. The most affected resonance corresponded to the side chain of Asn-75 (Figure 3B), in which the signal is entirely broadened upon titration with human and opossum CYP24A1, and is disproportionally broadened beyond one standard deviation upon addition of 0.3 molar equivalents of rat CYP24A1. Asn-75 is the only amide side chain located on helix-3 and is positioned adjacent to the highly conserved contiguous acidic surface comprised of Asp-72, Asp-76, and Asp-79; also considered the putative recognition site in P450-Adx complexes (4,7,8). However, additional line broadening was observed for Adx side chains located opposite helix-3. In particular, Gln-35 on helix-1 was similarly affected in titrations with all three CYP24A1 isoforms, along with differential broadening of Asn-13 in the titration with human CYP24A1. Asn-13 is located on a loop between anti-parallel β strands opposite the site of [2Fe-2S] coordination, thus making it an unlikely candidate as a primary binding interface with CYP24A1. However, Gln-35 of helix-1 shares a surface with the highly conserved residue Asp-31 located directly adjacent to the [2Fe-2S] cluster, and is therefore more likely to participate in formation of the electron transfer complex. In comparison, the side chains of Asn-36 and Asn-37 located outside of helix-1 show no significant differential effect, thereby suggesting a localized difference in chemical exchange on the helix itself. The observation that for all experiments, the Gln-35 side chain is impacted to a lesser degree than Asn-75 also suggests helix-1 plays a secondary role in the interaction. Notably, Asp-31 of Adx has not previously been identified as a recognition site for P450 enzymes, yet the line broadening observed in titrations with all three CYP24A1 isoforms is consistent with a role for this residue in complex formation.

*NMR analysis of single and double mutants of $^{15}$N-Adx* – The apparent contributions from multiple Adx recognition sites, as suggested from analysis of line broadening patterns of side chain amides (Figure 3B), can be explained either by 1) multiple but distinct binding orientations of Adx as it binds the proximal surface of CYP24A1, or 2) a single primary binding orientation that relies on multiple points of intermolecular contact between the proteins, most likely requiring an Adx dimer. In order to distinguish between either possibility, as well as to evaluate the contributions from individual acidic residues for a role in binding CYP24A1, a series of single and double residue mutations of Adx were generated. Aspartate and glutamate residues were substituted with asparagine and glutamine, respectively. All Adx mutants were analyzed by gel filtration and determined to elute at a volume consistent with a 27 kDa protein, similar to the elution profile of wild-type Adx and consistent with the presence of a dimer for the 14 kDa protein. Furthermore, analysis of the $^{15}$N HSQC NMR spectrum of each mutant confirmed that all mutants generally adopted the tertiary structure of full length wild-type Adx, as indicated by a resonance distribution pattern resembling that of the wild-type Adx $^{15}$N HSQC. However, a closer analysis indicated that while D72N, D76N, and E65Q induced chemical shift perturbations only in the immediate vicinity of the substitution, D31N and the double mutant D72N/D76N induced unexpected long-range perturbations. For example, D31N alters the chemical shift values of neighboring residues on helix-1 as well residues in the N-terminal β strand along with broadening of the backbone resonance of M77 on helix-3. (data not shown). Interestingly, the D72N/D76N spectrum includes changes primarily in the neighboring helix-3 residues but also results in the complete broadening of the Asn-36 side chain of helix-1.

Single residue substitutions on helix-3 of Adx are insufficient to disrupt the CYP24A1-Adx complex – For each mutant generated, the $^{15}$N-Adx spectra were acquired in the free state as well as in the presence of 0.25 molar equivalents of unlabeled CYP24A1 and the intensity ratios analyzed for patterns of differential line broadening. Figure 4 summarizes the average line broadening across all wild-type and mutant $^{15}$N-Adx backbone resonances in response to binding the three CYP24A1 isoforms. Among the mutants generated, D72N, D76N, and the double mutant D72N/D76N were designed specifically to target
the P450 interaction, as these sites have previously been established as forming part of the putative recognition domain (4,5,27,28). Upon introduction of CYP24A1, the D72N mutant resulted in moderately less broadening with human and rat CYP24A1, suggesting slight loss of binding to those isoforms, but unexpectedly demonstrated enhanced broadening upon binding the opossum enzyme. The D76N substitution resulted in comparable line broadening to wild-type Adx when bound to human and rat CYP24A1 and showed similarly enhanced broadening with the opossum enzyme as with D72N. The combination of D72N with D76N did result in disruption of the complex across all isoforms. Interestingly, despite apparent overall preservation of the protein complex even with substitutions of conserved acidic residues, a comparison of side chain resonances of \(^{15}\text{N}-\text{Adx}\) D72N with wild-type \(^{15}\text{N}-\text{Adx}\) (Figure 5A) shows that localized line broadening at Asn-75 does appear to be disrupted by the D72N mutation when bound to human and rat enzyme (red arrows), thus indicating a localized loss of the interaction at helix-3. Similar localized differences in line broadening were observed in response to the D76N mutant and upon binding to either human or rat CYP24A1 (data not shown). It should also be noted that while the D72N/D76N double mutant does result in less binding overall (Figure 4), it also did not appear to completely disrupt the redox complexes. These results indicate that a single charge substitution on helix-3 does not disrupt the complex with CYP24A1, as is the case with cytochrome \(b_5\) binding of the microsomal enzyme CYP17A1 (26), and that other regions of Adx are likely involved in recognizing and stabilizing the interaction via additional recognition sites.

Charge neutralization at Asp-31 contributes toward disruption of the CYP24A1-Adx complex for specific isoforms – One possible explanation for the observed differential broadening of backbone and side chain resonances localized at helix 1 would be a conformational change in Adx upon binding CYP24A1, thus potentially reducing the distance between this region and the paramagnetic \([2\text{Fe}-2\text{S}]\) cluster. In light of this possibility, and in order to examine participation of this region in directly binding to CYP24A1, a follow-up mutant (D31N) was designed to replace the lone acidic residue in helix-1. Upon introduction of 0.25 molar equivalents of human CYP24A1, there was a significant loss of line broadening for the D31N backbone resonances (Figure 4), indicating a loss of complex formation in response to charge neutralization at Asp-31. This effect was also observed, albeit to a lesser degree, upon binding opossum CYP24A1. However, there was no loss of line broadening of backbone resonances between the D31N mutant and rat CYP24A1 (Figure 4B). These results are consistent with participation of Asp-31 for binding to CYP24A1, albeit using a species-specific mode in which the human enzyme relies considerably more on the interaction at this site than does the opossum enzyme, while rat CYP24A1 appears able to form the complex without contributions from Asp-31. This finding is supported by analysis of the \(^{15}\text{N} \text{D31N side chain resonances (Figure 5B), in which overall loss of line broadening included greater peak intensities at the Gln-35 side chain when bound to human and opossum CYP24A1 (red arrows), an indicator of loss of binding at helix-1. However, a noticeable loss of broadening also occurred at the Asn-75 side chain of helix-3, in contrast to the corresponding D72N and D76 mutations, for which the change observed at Gln-35 is minimal (Figure 5A). Taken together, this set of NMR titrations provide evidence that the highly conserved residue Asp-31 of helix-1 of Adx participates in the interaction with CYP24A1 as a complimentary recognition site that works in tandem with the acidic residues of helix-3. For the interaction with human CYP24A1, the loss of broadening is more dramatic than even that observed from the helix-3 double mutant. This indicates that while disruption of the highly acidic helix-3 of Adx, in which the remaining acidic residues may still contribute toward interactions with the basic proximal surface of CYP24A1, a single charge neutralization of the less acidic helix-1 may nullify all major electrostatic contributions toward the interaction from that specific region.

Charge neutralization at helix-2 of Adx promotes selective loss or modulation of the CYP24A1-Adx complex – In addition to a modest change in the backbone resonance intensities along helix-1 and helix-3, a minor clustering of peak broadening was also identified along the short helix-2 of Adx.
comprising Gln-61 to Lys-66 (Figure 2B). Helix-2 of Adx is generally not as well conserved across species in comparison to helices 1 and 3 and in bovine contains only a single acidic surface residue (Glu-65). Notably, the side chain resonances of Gln-61, one full turn from Glu-65, were not disproportionately affected by binding to CYP24A1 like the side chain amides of Gln-35 and Asn-75 (Figure 3B). However, in order to further evaluate the contribution from this site, the charge neutralizing mutation E65Q was introduced into ^15N-Adx and the NMR titration data acquired. Unexpectedly, a moderate loss of net backbone line broadening was observed for the interaction with human CYP24A1, along with a significant loss of line broadening for the interaction with rat CYP24A1 (Figure 4). In stark contrast, no net difference in overall line broadening was observed upon binding to the opossum isoform. Similar to the effect of the D31N mutant, loss of binding for E65Q was also accompanied by disruption of the side chain broadening pattern at Asn-75 of helix-3 (data not shown), suggesting that the contribution from Glu-65 is also complimentary to the interaction at helix-3. Such a secondary contribution from helix-2 would be most consistent with an Adx binding mode in which the primary recognition site remains located on helix-3, with stabilizing peripheral interactions from Glu-65 located on the opposite surface of the Adx [2Fe-2S] cofactor, potentially on a second molecule of the Adx dimer.

Interestingly, despite a similar net broadening of backbone resonances for the E65Q interaction with opossum CYP24A1, a detailed analysis of the per-amide broadening pattern indicated that the mutant Adx complex that formed with opossum CYP24A1 was not equivalent to complexes with the other isoforms or to the interaction between opossum CYP24A1 and wild-type Adx. The NMR spectra representing side chain resonances for bound ^15N-Adx E65Q are shown in Figure 6 for the complexes with all three species isoforms. While the complex with human and rat CYP24A1 retained measureable signal of the Asn-36 and Asn-37 side chain resonances (top two panels), the interaction with opossum CYP24A1 resulted in significant loss of signal at Asn-36 with a concurrent gain in signal intensity for the side chain of Asn-75 of helix-3. This same ^15N-Adx E65Q binding data is quantified in Figure 7A in comparison to the intensity ratios from the opossum CYP24A1 complex with wild-type Adx. There is a clear increase in highly localized line broadening at Asn-36 along with a modest increase in broadening at Asn-13 and a lessening of the broadening pattern at Asn-75. A similar non-uniform effect was observed when comparing the backbone resonances as well (Figure 7B). E65Q induced an overall increase of backbone resonance line broadening near the β sheet fold corresponding to the amino and carboxyl terminal domains of Adx, but also resulted in a modest decrease in broadening at helix-3. Mapping of the regions most affected by the interaction between E65Q Adx and opossum CYP24A1 includes the backbone amide signals corresponding to His-10 and Thr-20 and the side chains of Asn-13 and Asn-36. These amides map to a near contiguous cluster that includes the anti-parallel β region of Adx (Figure 7C). Notably, the cluster of affected sites is not located near the cofactor coordination loop. Therefore, the alternate orientation indicated by the mapped sites is unlikely to participate as a primary recognition site for a productive electron transfer complex.

**DISCUSSION**

The redox partner interactions between the mitochondrial P450 enzyme CYP24A1 and its cognate ferredoxin Adx represent the sole avenue for electron delivery as required for C23 or C24 hydroxylation of 1,25(OH)\_2D\_3. In this study, the CYP24A1-Adx complex was adapted for study by solution NMR by first binding the P450 inhibitor clotrimazole. The resulting ligand-enzyme complex, when combined with ^15N-Adx, conferred sufficient stability on the system to withstand the low ionic strength conditions required for analysis by NMR (Figure 1). In addition, growth media supplementation with clotrimazole also allowed for the generation of milligram quantities of purified CYP24A1 corresponding to the human and opossum sequences, thus allowing for a parallel study of the CYP24A1-Adx complex for species that have distinct regioselectivity for vitamin D (Figure 2).

Titration of ^15N-Adx with unlabeled CYP24A1 bound to clotrimazole resulted in a pattern of broadening of the NMR line shape, consistent with previous NMR characterization of
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similar P450 electron transfer complexes (21,25,26). While the overall pattern of broadening for resonances corresponding to the $^{15}$N-Adx backbone showed only modest differential effects, an analysis of the amide containing side chains indicate that at least two surface regions of Adx, corresponding to $\alpha$-helices 1 and 3, are differentially affected upon complex formation with CYP24A1 (Figure 3). This finding is most consistent with an interaction that relies on multiple sites of recognition. In subsequent experiments, the NMR titrations were combined with single and double residue mutants designed to neutralize acidic surface charges of Adx. The resulting spectra confirmed contributions from the putative helix-3 recognition domain comprised in part of Asp-72 and Asp-76, represented by loss of complex formation for a D72N/D76N double mutant. Interestingly, the single residue mutants D31N and E65Q also induced a decrease in overall line broadening when bound to particular isoforms, indicating that the equally conserved helix-1 and the less conserved helix-2 also form contacts with CYP24A1. An alternative interpretation of these data is that the distributed effects observed in this study are a result of disruption of Adx surfaces important in homodimer formation. However, gel filtration analysis of all mutants indicated that the dimer interface is preserved. Therefore, loss of line broadening from the redox complex in response to charge neutralizations in regions other than those flanking the $[2Fe-2S]$ cluster (like E65Q) likely correspond to disruption of the interaction between CYP24A1 and the Adx dimer, in which auxiliary interactions from a second molecule of Adx may contribute toward stabilization of the complex. Currently, the C-terminal domain of Adx is considered the likely dimerization domain, as indicated by the crystal structure of full length bovine Adx (3) as well the loss of dimer formation for C-terminal truncated Adx (residues 1-108) (29). However, the relative orientation of each Adx cofactor domain as part of the homo-dimer is not well understood. Interestingly, the observation from the $^{15}$N HSQC spectrum of Adx D72N/D76N, in which the double mutant unexpectedly affects side chain resonances corresponding to Gln-36, may indicate that these surfaces are located near each other on different molecules of dimerized Adx.

Currently, some of our structural understanding of the P450-Adx interface stems from analysis of the fused CYP11A1-Adx polypeptide (PDB 3NAO) (4), for which binding clearly involves the putative anionic sites at helix 3 of Adx while helix 1 is oriented away from the interface. This orientation of Adx appears to contradict the larger than anticipated binding interface indicated by NMR analysis in this study. However, it should also be noted that the fusion construct with a single Adx molecule attached does not capture interactions with dimeric Adx, where secondary binding sites appear to be involved. The NMR data instead more closely correlate with the structural model presented by Peng et al. (10) between dimeric Adx and CYP11B1 and CYP11B2. While the NMR and mutagenesis data is not in agreement with the specific orientation of Adx in those models, and P450-specific differences may exist when compared to the interaction with CYP24A1, the contributions from a second molecule of an Adx dimer is consistent with the large binding interface indicated here.

As a means of comparing the redox partner complexes for closely related CYP24A1 isoforms (~80 % sequence identity) that nonetheless display distinct regioselective specificity for vitamin D, all of the NMR titrations were carried out in parallel with human, rat, and opossum CYP24A1 bound to clotrimazole. Due to the high sequence conservation of the anticipated Adx recognition domain, as well as the availability of previously reported chemical shift assignments, all three CYP24A1 isoforms were evaluated for their interaction with bovine Adx. Interestingly, while the overall pattern of $^{15}$N-Adx backbone and side chain line broadening suggested similar complexes (Figure 2), the ability of particular charge neutralizing mutations to disrupt the complex strongly indicate that the three P450-redox partner complexes are not equivalent (Figures 4 and 5). The most distinct response appears to be between rat and opossum CYP24A1, in which the D31N substitution of Adx results in loss of complex formation with opossum CYP24A1 but has no effect on the interaction with rat CYP24A1. Conversely, the E65Q substitution results in considerable loss of the line broadening with rat CYP24A1, but is broadened to an equivalent extent with opossum CYP24A1. Closer analysis of the E65Q mutation with opossum CYP24A1 reveals an unexpected pattern of altered

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chemical exchange (Figures 6 and 7) that was not detected with either the human or rat enzyme and that may represent either a previously unidentified P450 binding orientation or a CYP24A1-induced modification in the Adx dimer interface. Meanwhile, the human enzyme interaction with the Adx mutations resulted in a combined effect, losing line broadening in response to both D31N as well as E65Q. Taken together, these findings suggest that while the primary recognition domain (helix-3) is likely similar when binding all CYP24A1 isoforms, the combination of secondary recognition sites appears to be unique for the species isoforms tested.

It is still unclear whether distinct redox partner binding modes would contribute toward regioselective preferences of CYP24A1. In 2007, Prosser et al demonstrated that an active site mutation, A326G, converts human CYP24A1 into a complete C23 hydroxylase, likely by increasing the space available for substrate binding (20). However, there is also mounting evidence that redox partner interactions influence the substrate binding and recognition regions of P450 enzymes (21,22,26,30). For example, the P450 interaction with Adx as measured by hydrogen-deuterium exchange was shown to alter the conformation of substrate binding regions in CYP46A1 (31). Similarly, co-crystallization of the bacterial enzyme CYP101 and its ferredoxin putidaredoxin results in an open form of the P450 structure (32). This allosteric response appears to be mediated in the opposite direction as well, from the active site out toward the redox partner binding site, as ligand bound CYP101 demonstrates an altered affinity for putidaredoxin (33).

Since the NMR titrations reported here reflect the redox partner complex in the presence of clotrimazole-bound CYP24A1, the ability of vitamin D substrate to influence the species-specific redox partner complexes is not captured. Therefore, evaluation of the complex formation while in the presence of vitamin D, combined with reconstituted CYP24A1 assays, represents a clear continuation of this work that will provide further insight toward the role of specific redox partner interactions in modulating CYP24A1 function. Furthermore, despite a generally high level of sequence conservation in Adx, differences do exist with respect to the distribution of acidic surfaces between species, thereby warranting further investigation of sequence variation from the ferredoxin side as well.

In summary, the findings reported herein provide evidence for mitochondrial P450-ferredoxin interactions that are complex and rely on multiple recognition sites, most likely stemming from secondary Adx dimer interactions with CYP24A1. Furthermore, this work demonstrates that species variation in CYP24A1 can modulate the redox protein complex in unanticipated ways, thereby indicating that redox partner interactions in the mitochondria, particularly those between different P450 enzymes (CYP24A1 and CYP27B1), are not functionally equivalent and warrant further examination.

**EXPERIMENTAL PROCEDURES**

*Production of 15N Adx* – The gene encoding for full length bovine Adx (1-128) was synthesized (Genscript) and cloned into a pET-15b vector. A 6x histidine tag was incorporated into the amino terminus. The expression plasmid was transfected into BL21(DE3) cells (Novagen) and selected for using 100 μg/ml carbenicillin. Bacterial cultures were started from a single colony and grown overnight in LB containing 50 μg/ml carbenicillin, then used to inoculate 500 ml cultures of minimal media in a 2.8 L Fernbach flask. Minimal media was comprised of the following components: 45 mM KH2PO4, 100 mM Na2HPO4, 17 mM NaCl, 50 mM glucose, 5 mM MgSO4, 150 μM thiamine, 7.5 mM (NH4)2SO4, 1 X trace minerals, and 50 mg/ml carbenicillin. Cultures were grown at 37 °C with shaking at 220 rpm, then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression was carried out for 24 hours at 22 °C while shaking at 160 rpm. Cells were harvested and resuspended into lysis buffer consisting of 50 mM TrisHCl (pH 7.4), 300 mM NaCl, 5 mM imidazole, and 1X Halt Protease Inhibitor Cocktail (Pierce). Cell lysis was initiated by the addition of 0.5 mg/ml lysozyme and allowed to continue for 30 minutes, followed by sonication in 30 second intervals for a total of 3 minutes. The lysate was recovered by ultracentrifugation at 46000 x g for 20 minutes, then loaded onto an equilibrated nickel-nitrilotriacetic acid column (Qiagen). Bound 15N Adx was cleaned by running 500 ml of wash buffer (50 mM TrisHCl (pH 7.4), 300 mM NaCl, 20 mM imidazole) and eluted with a similar
buffer containing 100 mM imidazole. A subsequent ion exchange purification step was carried out by diluting the eluent 10-fold with 50 mM Tris and binding onto an equilibrated Q ion exchange column (Bio-Rad), washing with 10 column volumes of binding buffer and eluting into 50 mM TrisHCl (pH 7.4), 300 mM NaCl. Purified $^{15}$N-Adx was quantified using an extinction coefficient of 11 mM$^{-1}$cm$^{-1}$ (34,35) and its purity verified by a single band by gel electrophoresis and by a $A_{416}/A_{280}$ ratio above 0.8.

Single and double point mutations of Adx were synthesized (Genscript) from the Adx(1-128)pET15b plasmid and the $^{15}$N samples were generated according the same protocol. The proper folding of each mutant was monitored by a 2D $^{15}$N-HSQC NMR spectrum. The oligomeric state of each mutant in comparison to wild-type Adx was determined using unlabeled samples of each that were passed through a pre-calibrated Superdex 200 resin gel filtration column (GE Healthcare).

Production of CYP24A1 from rat, human, and opossum – Genes encoding the CYP24A1, minus the 35 amino acid leader sequence, were synthesized (Genscript) according to the sequences from human, rat, and opossum, and the insert cloned into a pTrc vector (Novagen). The sequence for rat CYP24A1 included the S57D mutation described previously as a stabilizing substitution (36). The expression and purification protocol for rat CYP24A1 was adapted from those reported previously (36,37). Briefly, the expression plasmids were transfected into JM109 cells and a starter culture grown at 37 °C and 220 rpm from a single colony. Overnight cultures were used to inoculate 6 X 1 liter preparations of TB media. Cultures were then grown at 37 °C and 220 rpm and induced for overexpression at an optical density of 0.7 with 1 mM IPTG, 80 mg of 5-aminolevulinic acid, and a sublethal dose (20 ug/ml) of chloramphenicol. In order to incorporate ligand during protein expression, clotrimazole was dissolved into 5 ml of ethanol and added to culture at induction at a final concentration of 100 uM. Expression was carried out for 48-72 hours at 28 °C and 180 rpm. Cells were harvested and stored as paste at -80 °C until purification. Upon thawing, cell paste was thoroughly resuspended into 100 ml lysis buffer (50 mM potassium phosphate, 20% glycerol, 2 mM BME, pH 7.4). While stirring at 4 °C, 1 X Halt Protease Inhibitor Cocktail (Pierce), 2 ug/ml DNAseI (Alfa Aeser), and 0.5 mg/ml lysozyme (Gold Biotechnology) were added and the cells allowed to stir for 30 minutes. Subsequently, membrane extraction was carried out by addition of 3-{[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent (Gold Biotechnology) to a final concentration of 1% (v/v), and the suspension allowed to stir for an additional 30 minutes, followed by brief sonication using a Brrson SFX Sonifier in 30 second bursts (3 minutes overall) at 60% amplitude. The extracted fraction was then separated by ultracentrifugation for 1 hour at 104,000 x g. Separately, unlabelled full length bovine Adx was bound in batch to Ni NTA resin (Qiagen) and the bound protein packed into an XK-16/20 column (GE Healthcare). The Adx affinity column was pre-equilibrated with 3 column volumes of binding buffer (50 mM potassium phosphate, 0.5% CHAPS, 20% glycerol, 2 mM BME, pH 7.4). CYP24A1 affinity column binding was allowed to occur by slow passage (0.5 ml/min) of the extracted fraction, followed by a washing with 6 column volumes of additional binding buffer, and elution with a high salt buffer (500 mM potassium phosphate, 200 mM NaCl, 0.5% CHAPS, 20% glycerol, 2 mM BME, pH 7.4). The peak elution fractions were further purified by passage through a gel filtration column using a similar elution buffer modified to reduce the final detergent concentration to 0.1%. Eluted fractions containing a $A_{418}/A_{280}$ ratio above 0.9 were pooled for analysis. Purified enzyme was quantified as described previously (36,38). Preparation of human and opossum CYP24A1 followed the above protocol with the following modifications. At induction, growth media was augmented with clotrimazole (Gold Biotechnology) to a final concentration of 100 uM. Clotrimazole was reintroduced during lysis and maintained at 20 μM in all purification buffers. Bound clotrimazole was verified by the presence of the Soret peak at 424 nm.

NMR data acquisition and analysis – All $^{15}$N-Adx HSQC spectra were acquired at 25 °C on a Varian Inova-600 MHz spectrometer equipped with a cryogenic probe. The distribution pattern of chemical shift values closely resembled the spectra reported previously (24), thereby allowing most
assignments in the dispersed regions of the spectrum to be transferred from entry 4566 of the Biological Magnetic Resonance Data Bank. Preparation of the titration samples was carried out as follows. Briefly, in order to ensure removal of unbound detergent, the CYP24A1 buffer was exchanged during gel filtration into a similar buffer containing 0.1% CHAPS, followed by a 10-fold buffer exchange into a detergent-free high salt buffer (500 mM potassium phosphate, 200 mM NaCl, 20% glycerol, 20 µM clotrimazole, pH 7.4) and passed through a 50 kDa molecular weight cutoff filter (Millipore Amicon). Varying amounts of CYP24A1 were then combined with 50 nmoles of $^{15}$N-Adx and the mixture transferred into a 10 (Kd) a molecular weight cutoff filter, followed by an additional 10-fold exchange into NMR buffer (50 mM potassium phosphate, 50 mM NaCl, 20 µM clotrimazole, 10% D$_2$O, pH 6.8). All NMR samples were adjusted to a final concentration of 100 µM of $^{15}$N-Adx, with the exception of the D72N/D76N mutant, which due to lower overall yields was adjusted to 35 µM for all free and bound samples. Raw data was first processed using nmrPipe (39) and further analyzed using NMRView (40). Quantification of NMR line broadening was carried out similar to the statistical approach described previously (41). Briefly, individual peak intensities were measured for each spectrum both with unlabeled CYP24A1 and without (free $^{15}$N Adx). The individual residue backbone and side chain amide peaks were then calculated as a ratio from these data using the following equation, with the free $^{15}$N Adx representing a value of 1.

$$Intensity \ ratio \ (Ir) = \frac{(I \ Adx \ with \ P450)}{(I \ free)}$$

For each ratio, the error bar was calculated from the root mean square of the signals being compared as measured using the NMRViewJ software for a peak-free region of the 2D spectrum and applying the following equation in which each $\sigma$ is derived from the corresponding spectrum.

$$\sigma (Ir \ ratio) = \sqrt{(\frac{\sigma(I Adx \ with \ P450)}{I Adx \ with \ P450})^2 + (\frac{\sigma(I free)}{I free})^2}$$

In order to determine $^{15}$N Adx resonances that experience statistically significant line broadening, the root mean square deviation was calculated from the averaged ratios within each titration experiment, and this deviation from the mean utilized to identify resonances that were disproportionally broadened from the average peak intensity.

Spectral ligand binding assays – Ligand binding assays were carried out using a Shimadzu UV-2700 spectrophotometer. Each assay was performed in triplicate using 1 µM of rat CYP24A1 in 100 mM potassium phosphate buffer, pH 7.4. Clotrimazole was titrated by stepwise addition from an ethanol stock, with 8-minute incubation intervals between additions. The difference spectra were calculated from the type-II Soret shift beginning at 416 nm and terminating at 424 nm upon saturation. The spectral response was plotted against the ligand concentration and the resulting plot analyzed using GraphPad Prism software (version 7.02) and fitted to a sigmoidal binding curve with variable slope.
Acknowledgments – NMR data was acquired at the Statler NMR facility located at the University at Buffalo. Mr. James Lioi (laboratory technician) contributed protein purification of $^{15}$N labeled Adx and unlabeled opossum CYP24A1.

FOOTNOTES
* This work was supported by the National Institutes of Health Grant R00 GM112862 (DFE).

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The abbreviations used are: P450; cytochrome P450; Adx, adrenodoxin; HSQC, heteronuclear single quantum coherence

Author contributions:
Participated in research design: DFE
Conducted experiments: DFE
Performed data analysis: DFE
Wrote or contributed to the writing of the manuscript: DFE

Conflict of Interest:
The author declares that there are no conflicts of interest with the contents of this article.
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Figure 1. Clotrimazole spectral binding assay. (A) Step-wise clotrimazole addition induced a type-II spectral binding perturbation in rat CYP24A1 at ligand concentrations between .05 µM (blue trace) and 3.0 µM (black trace). (B) The difference in absorbance between $A_{432}$ and $A_{411}$ is plotted against ligand concentration. The data are fitted to a sigmoidal binding curve.
Figure 2. Peak broadening of the $^{15}\text{N} \text{Adx}$ HSQC spectra upon addition of CYP24A1. (A) Overlay of $^{15}\text{N} \text{Adx}$ alone (gray) and with increasing amounts of human CYP24A1 (clotrimazole) corresponding to the red and blue spectra, respectively. All spectra are shown at an equivalent contour level. (B) Peak broadening of the $^{15}\text{N}-\text{Adx}$ backbone resonances quantified as ratios of free $^{15}\text{N} \text{Adx}/^{15}\text{N} \text{Adx}$ in the presence of CYP24A1 from human (top), rat (middle) and opossum (bottom). The red markers represent residues broadened beyond one standard deviation from the mean.
Figure 3. Differential line broadening of $^{15}\text{N}$ Adx side chain amides. (A) Distribution of Gln and Asn residues of Adx in relation to the conserved acidic residues (PDB ID 1CJE)(3). (B) Line broadening pattern of $^{15}\text{N}$ Adx side chain resonances upon titration with unlabeled CYP24A1 corresponding to clotrimazole-bound human, rat, and opossum P450 isoforms. Red arrows indicate broadening resulting in ratios that are greater than one root mean square standard deviation from the mean.
Figure 4. Effect of $^{15}$N-Adx charge neutralizing mutations on the CYP24A-Adx complex formation. Amino acid intensity ratios representing broadening of the backbone $^{15}$N-Adx resonances for wild-type and all charge neutralizing mutations were examined. Data are shown for each mutant in a 1:0.25 molar ratio with human (A), rat (B), and opossum (C) CYP24A1 bound to clotrimazole. An increase in the intensity ratio reflects loss of line broadening for each redox complex, consistent with disruption of the CYP24A1-Adx interaction.
Cytochrome P450 24A1/adrenodoxin interactions

Figure 5. Side chain intensity ratios for D31N and D72N mutations of $^{15}$N-Adx. (A) $^{15}$N-Adx containing a D72N substitution resulted in a localized loss of line broadening for the Asn-75 side chain in the complex with human and rat CYP24A1 (red arrows) while displaying an enhanced line broadening pattern overall when bound to opossum CYP24A1 (bottom panel). (B) $^{15}$N-Adx containing the D31N substitution resulted in a loss of line broadening at both the neighboring Gln-35 side chain as well as the helix-3 residue Asn-75 side chain when bound to human and opossum CYP24A1 (red arrows), while resembling the pattern observed for wild-type $^{15}$N-Adx when bound to rat CYP24A1 (middle panel).
Figure 6. $^{15}$N HSQC spectra of the E65Q side chain resonances upon binding to human, rat, and opossum CYP24A1(clotrimazole). 2D resonances corresponding to the Asn-36 and Asn-37 resonances maintain observable intensity when bound to human and rat CYP24A1(clotrimazole) (top two panels). However, upon complex formation with opossum CYP24A1(clotrimazole) (lower panel), the Asn-36 side chain undergoes significantly enhanced broadening (dashed lines indicate the expected location of these resonances) concurrent with an increase in intensity at the Asn-75 side chain resonances. All spectra are displayed at a normalized contour level.
Figure 7. Modulation of the opossum CYP24A1 complex with the E65Q mutation of $^{15}$N-Adx. Redistribution of peak broadening was observed for the complex between opossum CYP24A1 clotrimazole and $^{15}$N-Adx harboring the E65Q substitution on helix 2. (A) Intensity ratios for the $^{15}$N-Adx E65Q side chains reflect a non-uniform pattern of broadening in which the Asn-36 side chain has become significantly more broadened along with that of Asn-13 (red asterisks) combined with a loss of line broadening at the Asn-75 side chain. In the mutant $^{15}$N HSQC, only one of the Gln-61 side chains (located one helical turn from the mutated site) is detectable. (B) The non-uniform line broadening was also observed along the backbone resonances of $^{15}$N-Adx E65Q, in which N and C-terminal $\beta$ strands underwent enhanced broadening compared to wild-type $^{15}$N Adx, particularly at residues His-10 and Thr-20, while the helix-3 resonances were concurrently less broadened. (C) Mapping of the more affected sites, consisting of the His-10 and Thr-20 backbone amides along with the Asn-36 and Asn-13 side chain amides, forms a near contiguous surface on E65Q Adx.
The cytochrome P450 24A1 interaction with adrenodoxin relies on multiple recognition sites that vary among species
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J. Biol. Chem. published online January 25, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001145

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