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Functional interactions of adrenodoxin with several human mitochondrial cytochrome P450 enzymes

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ABSTRACT

Seven of the 57 human cytochrome P450 (P450) enzymes are mitochondrial and carry out important reactions with steroids and vitamins A and D. These seven P450s utilize an electron transport chain that includes NADPH, NADPH-adrenodoxin reductase (AdR), and adrenodoxin (Adx) instead of the diflavin NADPH-P450 reductase (POR) used by the other P450s in the endoplasmic reticulum. Although numerous studies have been published involving mitochondrial P450 systems, the experimental conditions vary considerably. We compared human Adx and bovine Adx, a commonly used component, and found very similar catalytic activities in reactions catalyzed by human P450s 11B2, 27A1, and 27C1. Binding constants of 6–200 nM were estimated for Adx binding to these P450s using microscale thermophoresis. All P450 catalytic reactions were saturated at 10 μM Adx, and higher concentrations were not inhibitory up to at least 50 μM. Collectively these studies demonstrate the tight binding of Adx (both human and bovine) to AdR and to several mitochondrial P450s and provide guidance for optimization of Adx-dependent P450 reactions.

1. Introduction

The cytochrome P450 (P450 or CYP) family of heme monooxygenases has been of continued interest since the discovery of the enzyme more than 50 years ago [1,2]. With the sequencing of the human genome, the number of *CYP* genes was determined to be 57, and these enzymes play various roles in the body [3]. Most frequently they catalyze the insertion of a single atom from molecular oxygen into a *C*–H bond, using electrons supplied by NADPH, although they can perform reactions as wide-ranging as epoxidation, desaturation, *C*–C bond cleavage, and ring expansion [4,5]. In the liver, they are responsible for the activation and detoxication of xenobiotics, while in the adrenals, gonads, and placenta they catalyze key reactions in steroid hormone biosynthetic pathways [6]. P450 enzymes that metabolize exogenous chemicals, such as P450s 2C9 and 3A4, are generally localized to the endoplasmic reticulum and are dependent on the flavoprotein NADPH-P450 reductase (POR) for the transfer of electrons to support mixed-function oxidation. P450s in the mitochondria rely instead on a two-component electron transfer system comprised of a [2Fe–2S] ferredoxin, adrenodoxin (Adx), and an FAD-dependent ferredoxin reductase, NADPH-adrenodoxin reductase (AdR). Deficiencies in any of the mitochondrial P450s can cause disease states in humans involving not only steroidogenesis but also vitamin D homeostasis [6–8]. Of the six P450 enzymes involved in steroid hormone biosynthesis [3,6], three (P450s 11A1, 11B1, and 11B2) are mitochondrial P450s that use the Adx pathway. The remaining mitochondrial P450s also catalyze reactions in the bile acid pathway (P450 27A1 performs sterol 27-hydroxylation), vitamin D_3 hydroxylation in the kidney (P450s 24A1 and 27B1), and retinoid desaturation in the skin (P450 27C1).

Because transfer of electrons to the P450 heme is mediated by the interaction of AdR, Adx, and the P450 itself, there has been significant interest over the years regarding the affinity and thermodynamics of the interactions between these components. The question of what complexes these form and how the formation affects the rate and specificity

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of electron transfer has been of interest since the first amino sequence determination of bovine Adx [9] and subsequent cloning and expression in *Escherichia coli* [10]. The first crystal structure of Adx was suggestive of dimerization [11]. More recently, Ivanov and associates used surface plasmon resonance (SPR) to estimate K_d values for POR and Adx interactions with 12 different P450s [12]. The effect of substrate binding on the P450-Adx complex has been examined recently both by SPR [13] and solution NMR [14,15]. Solution NMR indicated that substrate binding altered the interaction of microsomal P450 17A1 with another auxiliary protein, cytochrome b_5 [16], which is consistent with more recent SPR studies [13]. In contrast, no effect of substrate was observed on P450/POR and P450/Adx binding with microsomal P450 17A1, P450 21A2, and P450 2C19 by SPR [13], while solution NMR showed that the mitochondrial P450 24A1/Adx interaction was sensitive to the presence of specific substrates [15]. These studies, taken together, indicate that Adx may play a role beyond simple delivery of electrons to P450s and, as a corollary, may additionally affect substrate binding and P450 conformation.

Studies of mitochondrial P450-catalyzed reactions have used a variety of experimental conditions in the interactions with Adx. Many of the early studies with Adx were done with the bovine protein, particularly prior to heterologous expression [17–21]. Taking P450 11B2 as an example, both (recombinant) human [22–24] and bovine [12,25,26] sources of Adx have been commonly used in catalytic studies. The concentrations of Adx and AdR used are generally described with regard to the concentration of P450 in a simple molar ratio. For typical steady-state enzyme kinetic studies, the Adx:P450 ratio has varied from 8:1 [24] to 60:1 [23] with Adx concentrations varying from 1 μM [25] to 30 μM [26].

In this work, we sought to understand the effects of these experimental variables in studies of mitochondrial P450s with Adx. Specifically, we examined the effects of different sources of Adx and concentrations on enzyme reaction parameters. Thermodynamic binding constants (K_d) for Adx and redox enzymes were estimated utilizing microscale thermophoresis (MST), again comparing the two Adx sources (bovine and human). The two Adx forms interact with the P450s differently, but the effect was found to be rather negligible for most enzyme kinetic studies. These results suggest optimized conditions for the study of interactions between Adx with mitochondrial P450s.

2. Materials and methods

2.1. Chemicals

All chemical reagents were purchased from MilliporeSigma (Burlington, MA) or Thermo Fisher Scientific (Waltham, MA) and used without further purification unless otherwise noted.

2.2. Recombinant proteins

Bovine Adx and AdR [27], human P450 11B2 [25], human P450 27A1 [28], and human P450 27C1 [29] were all expressed in *E. coli* and purified as previously described. The plasmid containing human Adx (pLW01) was a gift from Prof. R. Auchus (U. Michigan) [22], and the protein was expressed and purified according to a similar method as bovine Adx [27], with some modifications. Briefly, the plasmid was transformed into *Echerichia coli* BL21 cells and plated on LB_{amp} agar. A single colony was inoculated into 50 mL of Luria-Bertani (LB) media containing 100 μg/mL ampicillin and incubated overnight at 37 °C with shaking at 220 rpm. This pre-culture was used to inoculate bulk culture media at a 1:100 v/v dilution (500 mL Terrific Broth (TB) containing ampicillin (100 μg/mL). The cultures were incubated at 37 °C and 200 rpm until the OD_{600} reached approximately 0.6 (~5 h), at which point isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM) and trace elements (0.025% v/v) [30] were added, and the temperature and speed were reduced to 26 °C and 150 rpm, respectively. The cell pellet was harvested by centrifugation (3000×*g* for 10 min) after a further 24 h of growth. The pelleted cells were resuspended in 100 mM potassium phosphate (pH 7.4) buffer containing 20% glycerol (v/v) and 0.1 mM dithiothreitol (DTT) and sonicated for 8 × 30 s at 70% amplitude. The sonicated cells were then centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was loaded onto a DEAE-Sepharose column (2.5 × 10 cm), equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT. The column was washed with the same buffer, and the protein was eluted using a linear gradient of KCl (0–200 mM). The fractions containing Adx (as identified by A_{414} measurements and SDS-PAGE) were pooled, concentrated by centrifugal filtration (MilliporeSigma), and loaded onto a Sephadex G-75 column (2.5 cm × 100 cm) equilibrated with 10 mM potassium phosphate (pH 7.4) containing EDTA (0.1 mM). The fractions containing Adx (based on A_{414} measurements) were combined and purity > 95% was confirmed by SDS-PAGE. The total yield was 2000 nmol of Adx from 3 L of culture, which was then aliquoted and stored at −80 °C.

A P450 27A1 cDNA was purchased as an *E. coli* codon-optimized g-block, containing a *C*-terminal His_6 tag, from Integrated DNA Technologies (Coralville, IA). Details of the g-block and the primers used for amplification can be found in the Supplementary Material. Using *Nde*I and *Hin*dIII restriction enzyme sites, the cDNA was cut and ligated into the vector pCWori$^+$ [28]. Correct insertion was confirmed by nucleotide sequence analysis (GenHunter, Nashville, TN). The plasmid was transformed into *E. coli* DH5α cells along with a plasmid for expressing the molecular chaperone GroEL/ES, according to a method described previously for P450 27A1 [28]. A colony was picked, grown in 50 mL of LB media containing 100 μg/mL ampicillin and 50 μg/mL kanamycin, and incubated overnight at 37 °C at 220 rpm. This starter culture was used to inoculate 3 L of TB media (500 mL/2 L flask) supplemented with antibiotics (*vide supra*), 1 mM thiamine, trace elements (250 μL/L of culture) [30], and glycerol (4 mL/L culture), at a 1:100 v/v dilution. The resulting cultures were incubated at 37 °C at 220 rpm until the OD_{600} reached 0.7–0.75 (~2.5 h). Expression was induced by addition of 1 mM IPTG, 1 mM δ-aminolevulinic acid, and 6 mM arabinose. Cultures were incubated for another 48 h at 28 °C and 150 rpm, following which the cells were harvested by centrifugation (3000 × *g* for 20 min). The pellet was resuspended in TES buffer (100 mM Tris-acetate buffer (pH 7.4) containing 0.5 M sucrose and 0.5 mM EDTA) and a total of 150 mg lysozyme was added. Cells were shaken at 4 °C for 45 min and the spheroplasts were harvested by centrifugation (3000 × *g* for 20 min). The resulting spheroplasts were resuspended in 100 mL of sonication buffer (100 mM potassium phosphate (pH 7.4) containing 16% glycerol (v/v), 9 mM magnesium acetate, 100 μM DTT, 1.0 mM phenylmethylsulfonyl fluoride, and two protease inhibitor tablets (Roche)/L of cell culture) and sonicated on ice (8 × 30 s cycles at 70% amplitude). The resulting solution was centrifuged at 6500×*g* for 20 min, and then the recovered supernatant was further centrifuged at 10^5 × *g* for 60 min. The pellet was collected and solubilized overnight at 4 °C in 200 mL of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM DTT, 0.5 M KCl, and 1.0% sodium cholate (w/v). The homogenate was centrifuged at 100,000×*g* for 60 min, after which the supernatant was loaded onto a Ni^{2+}-NTA column, previously equilibrated with the solubilization buffer. The column was washed with the same buffer containing 20 mM imidazole, and P450 27A1 was eluted with 200 mM imidazole. The eluted fractions containing P450 27A1 (as confirmed by SDS-PAGE) were dialyzed against 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v, 24 h with three buffer changes for a total of 4 L). The dialyzed protein was stored at −80 °C until further use.

3. Experimental procedures

3.1. Catalytic assays

3.1.1. Bovine AdR

The Adx concentration-dependence of the catalytic activity of bovine AdR was tested with both human and bovine Adx. Reaction solutions were prepared in the wells of a polystyrene 96-well microplate (Greiner Bio-One North America, Monroe, NC). A 200 μL solution was prepared in each well including 0.015 μM AdR, 100 μM horse heart cytochrome *c*, 100 mM potassium phosphate buffer (pH 7.4), and various concentrations of either human or bovine Adx varying from 0.1 to 150 μM. NADPH (1.1 mM) was added to initiate the reaction, providing reduction equivalents to AdR, which can then reduce Adx. Reduced Adx is able to reduce cytochrome *c*. The increase in cytochrome *c* absorbance at 550 nm in this system indirectly reports on the reduction of Adx by AdR. Under these conditions, the reduction of Adx by AdR is the rate-limiting step in this series of reactions [18]. After initiating reactions by addition of NADPH, the absorbance at 550 nm was recorded every 3 s for 4 min using a Synergy H1 microplate reader (BioTek, Winooski, VT). A linear fit to the absorbance over the initial 90 s of the experiment was used to estimate the initial rate of reduction; during this observation period less than 15% of total cytochrome *c* was reduced. The absorbance changes were converted to reduced cytochrome *c* concentration using an extinction coefficient of 21,100 M^{-1} cm^{-1} [31]. Reactions were performed in duplicate.

3.1.2. Human P450 11B2 assays

The activity of human P450 11B2 with both human and bovine Adx was tested using 11-deoxycorticosterone as the substrate. These assays were performed based on previous work in this laboratory using P450 11B2 [25]. The conditions used for this assay allow for the assay to primarily report on the production of corticosterone because aldosterone and 18-OH corticosterone production is low. Levels of 18-OH corticosterone and aldosterone are below the detection limit of the experiment (2 pmol); whereas, corticosterone production levels are 2.5- to 12-fold higher than the detection limit [25]. Briefly, 500 μL samples (final volume) including 1 nM P450 11B2, 0.5 μM AdR, 50 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine (added as lipid vesicles after sonication of a 1 mg/mL stock), 4 μM 11-deoxycorticosterone, and various concentrations (0–100 μM) of either human or bovine Adx were incubated for 5 min at 37 °C (shaking water bath, final concentrations). NADPH was then added to the mixtures at a final concentration of 1.5 mM to initiate the reactions. Reactions were allowed to proceed at 37 °C for 7.5 min before quenching with the addition of 2 mL of ethyl acetate. Steroids in the sample were extracted into the organic solvent and an aliquot was removed, dried under a N_2 stream, and then dissolved in a CH_3CN:H_2O mixture (1:1, v/v). The resulting samples were analyzed using a Waters Acquity UPLC system (Milford, MA), separated with an Acquity BEH C18 UPLC octadecylsilane column (2.1 mm × 100 mm, 1.7 μm, solvents and gradient conditions previously described [25]), with detection using a photodiode array detector, and quantified using external standards. The production of corticosterone from 11-deoxycorticosterone was analyzed in this study. Assays were done in duplicate.

3.1.3. Human P450 27A1

The activity of human P450 27A1 was assayed in a similar way to that of P450 11B2 described above. The concentration of P450 27A1 was 0.2 μM, and the reaction tested was the oxidation of vitamin D_3 (cholecalciferol) to 25-hydroxyvitamin D_3. The initial concentration of vitamin D_3 used was 20 μM, and the reaction proceeded for 10 min after the addition of NADPH. The reaction samples were processed in a similar way as for the P450 11B2 reactions. The chromatography instrumentation was the same, with changes made for this reaction. The mobile phases used were A (95% H_2O, 5% CH_3CN, v/v) and B (95% CH_3CN, 5% H_2O, v/v) at a flow rate of 0.3 mL/min. The mobile phase linear gradient was: 0–1 min, 50% A (v/v); 5.5 min, 0% A (v/v); 9.5 min, 0% A (v/v); 9.75–11 min, 50% A (v/v). The retention times of vitamin D_3 and the 25-hydroxy product were 8.9 and 5.3 min, respectively. The peak area was quantified at a wavelength of 265 nm, based on a standard curve using an authentic standard of 25-hydroxyvitamin D_3. Experiments were performed in duplicate.

3.1.4. Human P450 27C1

The activity of human P450 27C1 was tested in a similar way to P450 11B2 described above and as described previously [32,33]. The concentration of P450 27C1 was 20 nM, and the concentration of L-α-dilauroyl-*sn*-glycero-3-phosphocholine was 16 μM. The reaction tested was the oxidation of all-*trans* retinol to 3,4-dehydroretinol, and the initial concentration of all-*trans* retinol used was 500 nM. Reactions were initiated with the addition of NADPH (1.5 mM) but quenched by the addition of 1 mL *tert*-butyl methyl ether containing 20 μM butylated hydroxytoluene after 1 min (to avoid autoxidation artifacts). After drying under a nitrogen stream, samples were dissolved in 50% ethanol (v/v).

Reaction samples were processed in a similar manner as for the P450 11B2 reactions. The chromatography instrumentation was the same with changes made to the method to optimize for this reaction. The mobile phases used were A (95% H_2O, 4.9% CH_3CN, 0.1% HCO_2H (v/v)) and B (95% CH_3CN, 4.9% H_2O, 0.1% HCO_2H (v/v)) at a flow rate of 0.5 mL/min. The mobile phase linear gradient was: 0–0.1 min, 40% A (v/v); 5–6 min, 25% A (v/v); 6.5–8 min, 0% A (v/v); 8.5–10 min, 40% A (v/v). The retention times of all-*trans* retinol and the 3,4-dehydroretinol product were 6.6 and 5.2 min, respectively. 3,4-Dehydroretinol formation was identified by co-elution with a commercial standard (Santa Cruz, Dallas, TX) and quantification was based on the A_{350} peak areas. Concentrations of retinoids were determined spectrophotometrically and samples were kept in amber glass vials at all times. Experiments were performed in duplicate.

3.2. Microscale thermophoresis (MST)

Binding affinity of Adx forms for bovine AdR and human P450s was studied using a Monolith NT.115 MST system (NanoTemper Technologies GmbH, Munich, Germany) in the Vanderbilt Structural Biology Core Facility. Either human or bovine Adx was labeled using a NanoTemper Monolith Protein Labeling Kit RED NHS 2nd Generation, which reacts with amine groups in a protein sample to label the protein with a fluorescent dye (RED) (proprietary). The extent of labeling was determined by a modification of the manufacturer instructions. Briefly, the Adx concentration was initially determined using the extinction coefficient ε_{414} = 9800 M^{-1} cm^{-1} [34]. Then, dilution of the Adx from the dye addition procedure was determined using the ratio of 280 nm absorbance before and after adding the dye. The 414 nm Adx absorbance band was too faint to be measured with the small reaction volume. The dye concentration in the final reaction volume, after unreacted dye was removed using a desalting spin-column, was determined using the manufacturer-provided extinction coefficient of 195,000 M^{-1} cm^{-1}. A ratio of [dye]/[Adx] in the final purified sample was used to determine extent of labeling. The kit labeling instructions were modified because initial attempts at labeling resulted in a degree of labeling of ~10%. By increasing the dye-protein incubation time from 30 min to 2 h and increasing the dye:protein ratio from 3.3 to 10, the extent of labeling was increased to ~50%, as determined by UV–visible absorbance measurements at 650 and 280 nm.

MST samples contained 20 nM either RED-labeled bovine Adx or RED-labeled human Adx, 100 mM potassium phosphate buffer (pH 7.4), 0.05% Tween 20 (v/v), and various concentrations of either bovine AdR or the human P450s (100 pM–39 μM). Samples were loaded into the standard Monolith NT.115 capillaries and tested by MST analysis at 25 °C. The results were analyzed by plotting the baseline-corrected normalized fluorescence (ΔF_{norm} [%]) versus enzyme concentration.

Data from two or three independently pipetted experiments were analyzed using a single site binding model, and the resultant K_d values were averaged with error propagated through the averaging (only one data set was done examining the interaction of bovine Adx with human P450 11B2).

4. Results

4.1. Enzyme catalytic activity dependence on Adx concentration

Steady-state kinetic measurements were made with three human mitochondrial P450 enzymes— P450s 11B2, 27A1, and 27C1—to compare the effectiveness of human and bovine Adx to act as redox mediators with human P450s. In each case the concentration of the P450 enzyme, the initial substrate concentration, and the concentration of bovine AdR were fixed. The concentration of the Adx was varied to examine the concentration-dependence of the observed P450 catalytic rate. Additional experiments to test the ability of bovine AdR to reduce human and bovine Adx were also performed. These reactions were studied indirectly by observing the reduction of cytochrome *c* by Adx under conditions where the AdR reduction of Adx is rate-limiting (Fig. 1).

In each case the observed reaction rate increased with Adx concentration until a maximal rate was achieved, as expected. This relationship was hyperbolic in nature and accordingly the data were fit using a typical Michaelis-Menten equation for enzyme kinetics. This is a different application of the Michaelis-Menten equation than usual because one would typically treat the small molecule being oxidized as the substrate in P450 reactions. However, the reduced Adx acts as the rate-limiting co-substrate, so it can also be treated as the dependent-substrate variable in a Michaelis-Menten analysis. This treatment allows for a comparison of the maximal rate achieved by the enzyme (k_{cat}), the sensitivity of the reaction to Adx concentration ($K_{m,app}$), and the overall efficiency of the reaction with respect to Adx concentration ($k_{cat}/K_{m,app}$). The fit parameters from this analysis (Table 1) indicate that in all parameters both bovine and human Adx were equally effective with human P450 11B2 and P450 27C1. A 5-fold increase in $k_{cat}/K_{m,app}$ was observed with bovine Adx when used with human P450 27A1, driven by a 1.5-fold increase in k_{cat} and a 3-fold decrease in $K_{m,app}$. A similar 2.3-fold increase in $k_{cat}/K_{m,app}$ was observed with human Adx when used with bovine AdR, driven by a decrease in $K_{m,app}$.

4.2. Estimates of enzyme-Adx dissociation constants by MST

MST was used to measure the dissociation constants (K_d) describing the interaction of both human and bovine Adx with human P450 11B2, human P450 27A1, human P450 27C1, and bovine AdR. In these experiments, Adx used was labeled with a fluorophore by conjugation of an NHS-ester to a free amine, kept at a constant ratio. The concentration of the enzyme was varied within the experiment.

Each MST data set (Fig. 2) was independently fit to a single-site binding model assuming a 1:1 stoichiometry to solve for the dissociation constant. Our analysis was based upon a complex of a single molecule of Adx and P450 (or AdR), although evidence for the interaction of two Adx molecules with P450 11B1 has been presented by Seybert et al. [35]. The K_d values were then averaged with error propagated to produce the values presented in Table 2. For most of the experiments, the multiple trials resulted in reproducible binding isotherm plots. The inter-day amplitude of the isotherm varied significantly among the trials for human P450 27C1 with bovine Adx and bovine AdR with bovine Adx, so in general three trials were averaged for data analysis. Only one successful trial was obtained for the interaction of

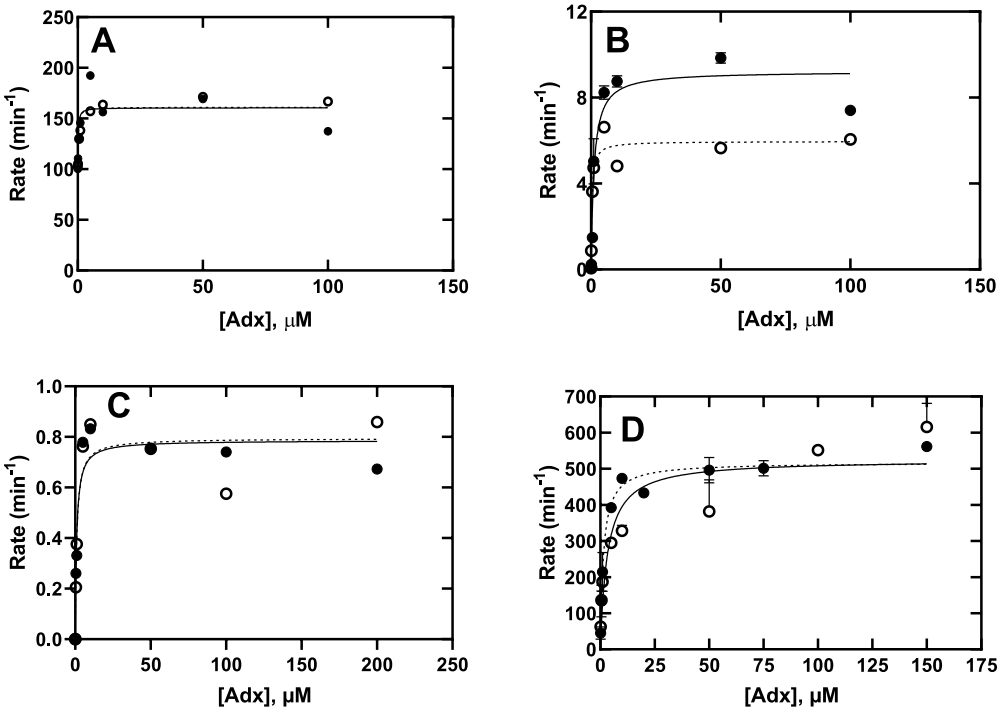

Fig. 1. P450-mediated oxidations supported by varying concentrations of either bovine (○—○) or human (●—●) Adx, to hyperbolic plots. (A) P450 11B2 oxidation of 11-deoxycorticosterone to corticosterone; (B) P450 27A1 oxidation of vitamin D_3 to 25-hydroxyvitamin D_3; (C) P450 27C1 oxidation of retinol to 3,4-dehydroretinol; (D) reduction of cytochrome *c* by AdR. Rates are presented as nmol product formed min^{-1} (nmol P450)$^{-1}$ in Parts A-C and nmol cytochrome *c* reduced min^{-1} (nmol AdR)$^{-1}$ in Part D. Data are shown as points with a hyperbolic fit to the data shown with the lines. Note: Under steady-state conditions of limiting bovine adrenodoxin a rate of 520 nmol cytochrome *c* reduced min^{-1} (nmol AdR)$^{-1}$ was estimated for the reduction of cytochrome *c*, which can be compared with the pre-steady-state rate of 4.6 s^{-1} (275 min^{-1}) previously reported for reduction of cytochrome *c* by a bovine AdrR-Adx complex by Lambeth and Kamin [18].

Table 1
Catalytic parameters from hyperbolic plots of reaction rates versus human and bovine Adx concentrations for P45011B2, 27A1, and 27C1. Rates are presented as nmol product formed min^{-1} (nmol P450)$^{-1}$.

	Bovine Adx			Human Adx		
	k_{cat} ± SD (min^{-1})	$K_{m,app}$ ± SD (μM)	$k_{cat}/K_{m,app}$ ± SD (μM^{-1} min^{-1})	k_{cat} ± SD (min^{-1})	$K_{m,app}$ ± SD (μM)	$k_{cat}/K_{m,app}$ ± SD (μM^{-1} min^{-1})
P450 11B2	160 ± 20	0.053 ± 0.006	3000 ± 500	160 ± 20	0.066 ± 0.008	2400 ± 400
P450 27A1	9.2 ± 0.5	0.33 ± 0.06	28 ± 5	6.0 ± 0.3	1.1 ± 0.3	5 ± 1
P450 27C1	0.79 ± 0.06	1.0 ± 0.4	0.8 ± 0.3	0.79 ± 0.05	1.0 ± 0.3	0.8 ± 0.3
AdR[a]	520 ± 40	3 ± 1	160 ± 50	520 ± 10	1.4 ± 0.2	360 ± 10

[a] Cytochrome *c* reduction assay.

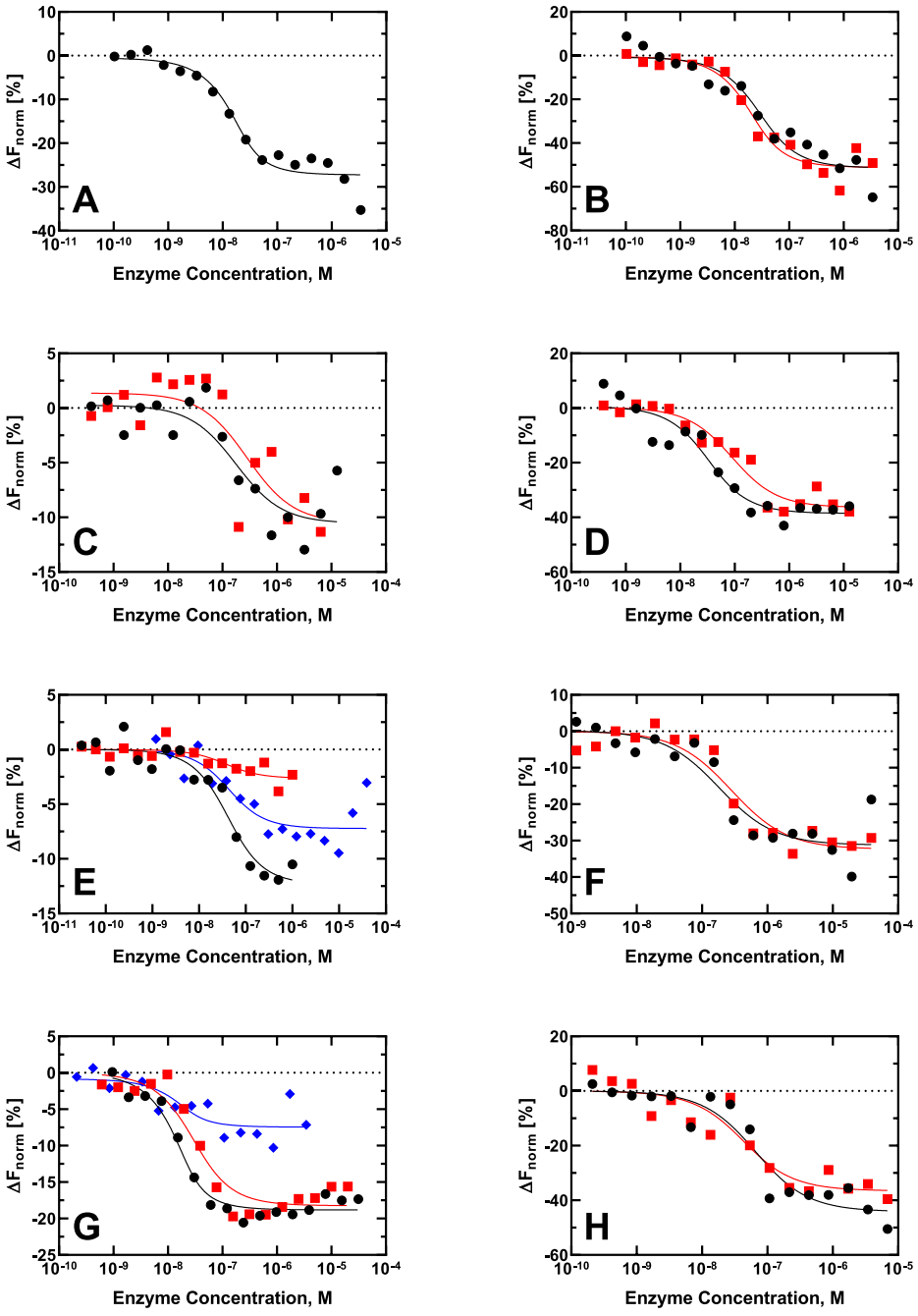

Fig. 2. MST data for binding of P450 11B2 (A & B), P450 27A1 (C & D), P450 27C1 (E & F), and bovine AdR (G, H) with bovine (A, C, E, F) and human (B, D, F, H) Adx. Each trace (shown with different colors) is a different independently determined experiment. The fit is a modified quadratic based on a single-binding site model, provided by the NanoTemper MST Analysis Software. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Dissociation constants for mitochondrial P450s with bovine and human Adx, derived from fitting MST data presented in Fig. 2 n: number of replicates.

	Bovine		Human	
	$K_d \pm$ SD (M)	n	$K_d \pm$ SD (M)	n
P450 11B2	6 (±3) × 10^{-9}	1	1.4 (±0.5) × 10^{-8}	2
P450 27A1	2 (±1) × 10^{-7}	2	5 (±2) × 10^{-8}	2
P450 27C1	3 (±1) × 10^{-8}	3	2.2 (±0.7) × 10^{-7}	2
AdR	1.0 (±0.4) × 10^{-8}	3	4 (±2) × 10^{-8}	2

human P450 11B2 with bovine Adx. K_d values ranged from 6 nM to 200 nM. The bovine and human Adx trials did not result in the same K_d values with the same enzyme. The degree and direction of this variation differs among the enzymes, with human P450 11B2 favoring bovine Adx by 2-fold, human P450 27A1 favoring the human Adx by 4-fold, human P450 27C1 favoring bovine Adx by 7-fold, and bovine AdR favoring bovine Adx by 4-fold. With exception of P450 27C1, these differences in K_d are relatively small and are likely insignificant when we consider that the K_d values are all close to or below the experimental Adx concentration of 20 nM. Despite this potential limitation of the fits, these results indicate tight binding between Adx and its various enzyme redox partners.

5. Discussion

The purpose of this study was to understand how variations in studies of P450- and Adx-catalyzed oxidations of steroids and vitamins affect the results. These variations include the species form of Adx used and the concentration of Adx used. The first issue is whether using bovine or human Adx as the electron carrier in P450 reactions changes the observed reaction kinetics. The primary sequences of the two proteins are very similar (76% identity), with most of the differences in the *N*-terminal region that is cleaved during mitochondrial transport [22,27] (https://www//uniprot.org/uniprot/P00257). The recombinant proteins used have an even higher identity of 89.4% (Supplementary Material Fig. S2). Identification of the residues related to the small differences of the two Adx proteins in the catalytic activities (Fig. 1) can only be speculative. In an ideal experimental setup, the concentration of Adx would be saturating and not be rate-limiting, so the most useful steady-state kinetic parameter to compare is k_{cat}. With the four enzymes that we tested, the k_{cat} values observed when using human or bovine Adx were less than 2-fold different, a fairly small difference indicating that either form of Adx is an effective electron transporter for these human enzymes (three different human P450s plus bovine AdR). Only the P450 27A1 data indicates a difference in k_{cat} between the two forms of Adx. The bovine Adx yielded a 1.5-fold higher k_{cat} value with P450 27A1.

The second variation among published studies utilizing P450 enzymes is the concentration of Adx used. Decreased P450 activity was not seen at even very high Adx concentrations (100–200 μM). The sensitivity of each studied reaction to Adx concentration is reflected in the measured $K_{m,app}$ values (Table 1); however, these values are not directly comparable as the concentration of the P450 used in each reaction varied (or the AdR in the cytochrome *c* reduction assays). The enzyme system used at the lowest concentration (P450 11B2, 1 nM) required the least amount of Adx ($K_{m,app} \sim 0.06$ μM). Despite the variation in the P450 concentration in the other P450-catalyzed reactions, the reactions with P450 27A1 and P450 27C1 produced similar $K_{m,app}$ values of ~1 μM, possibly indicating that there may be other factors determining these $K_{m,app}$ values. The reduction of Adx by AdR must also occur for the P450-catalyzed reactions to occur, so the Adx concentration-dependence of this reaction was independently examined. The resultant $K_{m,app}$ values for the AdR reactions were similar to those from the P450 27A1 and P450 27C1 reactions, i.e. with human Adx a $K_{m,app}$ of 1.4 μM and bovine Adx a $K_{m,app}$ of 3.3 μM. Overall, the answer to the question "How much Adx is optimal?" appears to vary by P450 and reaction conditions but in general 10 μM (bovine or human Adx) was found to be optimal for activity assays using sub-μM P450 concentrations.

MST was used to quantify the equilibrium binding dissociation constant (K_d) for each Adx form with the enzymes studied (Fig. 2, Table 2). To our knowledge (and a PubMed search, July 2020), microscale thermophoresis has not been utilized with P450 enzymes. In all cases, the measured K_d was low, ≤200 nM, indicating relatively tight interactions between the proteins. These values are similar in scale to those obtained by Yablokov et al. using an SPR approach [12], who reported K_d values from 10 to 80 nM with three different mitochondrial P450s. The only direct comparison of the measurements that can be made is between human P450 11B2 and its interaction with bovine Adx. Yablokov and coworkers reported a K_d of 80 ± 4 nM [12], based on SPR, compared to our value of 6 ± 3 nM (Table 2). The difference in observed K_d is probably due to the technique used, and both studies are consistent with very strong interactions between P450s and Adx. Schiffler et al. [35] reported a K_d value of 0.9 μM for an AdR-Adx complex and 13 nM for an Adx-P450 11A1 complex, using SPR methods. SPR utilizes one protein partner immobilized on the surface of a chip in order to determine the K_d, whereas MST is performed in solution, allowing for free diffusion of the protein partners (i.e., closer to native conditions). Having a protein attached to a surface limits its conformational freedom and can lead to a change in binding interactions, and a major problem is due to "mass transfer," the diffusion of the ligand from the solution though the matrix to reach the receptor [36]. K_d estimates of 160 nM for Adx-P450 11A1 [19] and 5 nM for AdR-Adx [34] have been made using spectroscopic methods.

A direct correlation between the K_d values and the calculated $K_{m,app}$ values was not observed (Pearson correlation coefficient 0.14), which is not surprising in that we only examined the interactions of oxidized Adx with the enzymes. It is possible that reduced Adx would have different binding affinities for P450s and AdR as well; this is a possible driving factor for the entire P450-Adx-AdR shuttle mechanism [21]. Further, the interactions of a P450 with Adx could be affected by the presence of individual substrates, in that Adx has been show to affect substrate affinities [25] and thermodynamic box analysis [37] would indicate that, as a corollary, substrate binding can affect Adx affinity, as reported for P450 24A1 [15]. The exact meaning of $K_{m,app}$ here is unknown, and a high affinity for either AdR or a P450 could sequester it away from the other redox partner. Therefore, it is not possible at this point to predict the ideal concentrations of Adx and AdR for steady-state analysis of all mitochondrial P450-catalyzed reactions. In practice, when a low concentration of P450 is used (≤500 nM) with an AdR concentration of 500 nM, Adx concentrations of 1–20 μM have been used, depending on the P450 being utilized and its concentration [22–26,32]. For studies using higher concentrations of P450 (≥1 μM), e.g. for pre-steady-state experiments [25], higher concentrations of AdR and Adx are required and should be optimized first.

6. Conclusions

This goal of this work was to determine the effects of several experimental variables on reactions catalyzed by mitochondrial P450 enzymes, specifically understanding the effects of redox partner source and redox partner concentration. To achieve optimal enzyme catalysis, the Adx form utilized can be checked for optimal activity, but both bovine and human Adx led to rates within a factor of two. Regardless of the species origin of Adx, the concentration of Adx used in these studies must be sufficiently high to support optimal turnover rates, i.e. 5-10 μM for typical steady-state kinetic experiments utilizing mitochondrial P450s (with the P450 concentration ≤ 500 nM).

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Abbreviations

Adx	adrenodoxin
AdR	NADPH-adrenodoxin reductase
b_5	cytochrome b_5
DH	dehydrogenase
G-6-P	glucose 6-phosphate
MST	microscale thermophoresis
P450 or CYP	cytochrome P450
POR	NADPH-cytochrome P450 reductase
SPR	surface plasmon resonance

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2020.108596.

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