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Biophysical Characterization of *Aptenodytes forsteri* Cytochrome P450 Aromatase

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Abstract

Cytochrome P450 19 (CYP19, aromatase) catalyzes the conversion of androgens to estrogens in a sequence of three reactions that each depend on NADPH and O₂. Aromatase is a phylogenetically-ancient enzyme and its breadth of expression in other species has highlighted distinct physiological functions. In songbirds, estrogen production is required for programming the neural circuits controlling song and in the determination of sex in fish and reptiles. This work describes the expression, purification, and biophysical characterization of *Aptenodytes forsteri* (Emperor penguin) aromatase. Using human cytochrome P450 reductase as a redox partner, afCYP19 displayed similar substrate turnover and LC/MS/MS confirmed that afCYP19 catalyzes the transformations through the intermediates 19-hydroxy- and 19-oxo-androstenedione. Androstenedione and anastrozole had the highest affinity for the enzyme and were followed closely by 19-hydroxyandrostenedione and testosterone. The affinity of 19-oxo-androstenedione for afCYP19 was ten-fold lower. The time-dependent changes in the Soret bands observed in stopped-flow mixing experiments of the steroidal ligands and the inhibitor anastrozole with afCYP19 were best described by a two-step binding mechanism. In summary, these studies describe the first biophysical characterization of an avian aromatase that displays strikingly similar enzyme kinetics and ligand binding properties to the human enzyme and could serve as a convenient model system for studies of the enigmatic transformation of androgens to estrogens.

Synopsis

The putative *Aptenodytes forsteri* (Emperor penguin) aromatase catalyzes the transformation of androstenedione to estrone through the same steroidal intermediates utilized by the human enzyme

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and pre-steady state analyses of their binding kinetics reveal that these, as well as the inhibitor anastrozole, utilize a multi-step mechanism.

Keywords

Aptenodytes forsteri; Cytochrome P450; Aromatase; Stopped-Flow Kinetics; Resonance Raman Spectroscopy; Enzyme Kinetics

Introduction

Cytochrome P450 19 (CYP19; aromatase) catalyzes the conversion of androgens to estrogens in a three-reaction sequence, where each step depends on NADPH and O_2 .(1–3) The first two steps are accepted to be hydroxylations of the steroid C19 methyl group, whereas the final step relies on a debated mechanism that deformylates the C19 aldehyde and aromatizes the steroid A-ring. (Scheme 1A)(4–7) The human enzyme converts androstenedione (ASD), testosterone (TST) and 16 α -hydroxytestosterone to estrone (E_1), 17 β -estradiol (E_2) and 16 α -hydroxy-17 β -estradiol, respectively. E_1 from adipose tissue and skin fibroblasts represents the dominant circulating estrogen.(8) Aromatase-derived estrogens mediate control of ovulation, cyclical preparation of the reproductive tract for fertilization and implantation of the blastocyst, and exert major actions on mineral, carbohydrate, protein, and lipid metabolism.(9) In the brain, gonadotropin secretion is modulated by locally-produced E_2 and elsewhere, neural aromatase produces estrogens that serve to maintain brain plasticity.(10) Extremely high levels of aromatase are present in endometriosis and breast cancer tissues that greatly enhance local estrogen concentrations. Consequently, aromatase inhibitors have proven to be valuable therapies for these pathologies.(11,12)

Aromatase and estrogen biosynthesis are ubiquitous among the vertebrates, extending to phylogenetically-ancient jawless fishes (hagfish and lampreys).(13) While the physiological importance and the pharmacological value of aromatase for the treatment of a myriad of diseases are established, its physiological roles in other species are comparatively understudied. Nevertheless, it is clear from available studies that the physiological functions extend beyond those observed in mammals. Estrogens are increasingly being recognized to have pronounced effects on cognitive function.(14) In songbirds, estrogens are of critical importance in both learning and discriminating song as well as in spatial memory functions. (15) In fish, reptiles, and amphibians that rely on temperature-dependent sex determination, thermosensitive mechanisms alter aromatase expression and in turn, ovarian differentiation. (16) Gonadal tissues of the invertebrate species *Branchiostoma* have demonstrated the ability to aromatize androgens(17) and bioinformatics analyses have identified a putative *Cyp19* gene.(18,19) This discovery challenges the dogma that aromatase originated with the evolution of vertebrates.(20) When more aromatases are identified in phylogenetically-distant organisms from vertebrates, their distinct physiology will illuminate new aromatase functions and possibly provide insight into the evolutionary origins of estrogen signaling.

Herein, the recombinant production and biophysical characterization of an engineered *Aptenodytes forsteri* aromatase (afCYP19 hereafter) are described. Enzyme kinetic analysis and characterization of products confirmed that afCYP19 and its human counterpart catalyze the transformation of ASD to E₁ through a common set of intermediates and mechanistic features. In addition, yields of ligand-free afCYP19 permitted characterization of steroid and anastrozole (ATZ) (Scheme 1B) complexes by resonance Raman (RR) spectroscopy. Finally, pre-steady state kinetic analyses by stopped-flow UV-vis spectroscopy support multi-step binding mechanisms for both the steroidal ligands as well as ATZ.

Materials and Methods

Chemicals

L-Histidine HCl was purchased from Acros Organics. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) was from A.G. Scientific (San Diego, CA). Complete protease inhibitor cocktail (EDTA-free) was from Roche Diagnostics (Mannheim, Germany). ASD, 19-OH-ASD, 19-oxo-ASD, TST, and E₁ were from Steraloids (Newport, RI). Safety-Solve Scintillation Cocktail was from Research Products International (Mt. Prospect, IL) Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available.

Protein Expression and Purification

The gene for afCYP19 with amino acids 1–39 replaced by MAKKTSSKGR and 4-His tag on the C-terminus was codon-optimized for expression in *E. coli*, synthesized, and inserted into the pCWori+ vector with flanking *NdeI* and *HindIII* restriction sites by GenScript (Piscataway, NJ). Following transformation of DH5α cells with pCW-afCYP19, a single colony was selected and grown for 16 h in Luria-Bertani media containing 100 µg·mL⁻¹ ampicillin. A 10 mL aliquot was used to inoculate one L of terrific broth medium containing 100 µg·mL⁻¹ ampicillin that was grown to an O.D. of 0.5–0.8 at 600 nm. The temperature

was decreased to 28 °C, supplemented with 1 mM 5-aminolevulinic acid, and allowed to incubate for one h. An additional 100 µg·mL⁻¹ of ampicillin was added upon induction with isopropyl β-D-1-thiogalactopyranoside. After 48 h, the bacteria were harvested by centrifugation at 4000×g for 10 min. Cell pellets were resuspended in lysis buffer (100 mM potassium phosphate, pH 7.4, 10% glycerol, 0.2% CHAPS, and 1 mM phenylmethylsulfonyl fluoride). Following addition of 4 mL of lysis buffer g⁻¹ of cell pellet, 1 mg·mL⁻¹ of lysozyme and complete protease inhibitor cocktail were added and the mixture was stirred for 30 min at 4 °C. The suspension was then supplemented with 1% Tween-20 and stirred for an additional 30 min. Cells were disrupted using a Sonicator 3000 (Misonix Inc., Farmingdale, NY) and cell debris were pelleted by ultracentrifugation at 45,000×g.

The supernatant was loaded onto a HisPrep FF 16/10 Ni²⁺-NTA column previously equilibrated with buffer A (100 mM potassium phosphate, pH 7.4, 100 mM NaCl, 20% glycerol). The column was washed with two column volumes of buffer A and afCYP19 was eluted with a linear gradient of buffer A containing 250 mM histidine. Red colored fractions were pooled and concentrated in Amicon Ultracel centrifugal concentrators (30 kDa MWCO; Millipore). Aromatase containing fractions were pooled, concentrated and loaded onto a Superdex 200 column previously equilibrated in with 25 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 % glycerol. The purified protein was exchanged with storage buffer (50 mM potassium phosphate, pH 7.4, 100 mM KCl, 20 % glycerol, 2 mM β-mercaptoethanol), concentrated using a 30 kDa Ultracel centrifugal device (Amicon, Millipore) and stored at -80 °C until required. Human CYP19A1 (hCYP19A1) and human cytochrome P450 reductase (hCPR) were expressed and purified as previously described.(3,21)

Immunoblot analysis

Five pmoles of purified hCYP19A1 and 15 pmoles of afCYP19 were separated by Tris-glycine-buffered SDS-PAGE on 4–20% Mini-PROTEAN TGX gradient precast gels (Bio-Rad) and transferred to polyvinylidene fluoride membrane (Immun-Blot PVDF, Bio-Rad) using a wet transfer method. The membrane was activated with methanol, blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20, then probed with a primary antibody directed against hCYP19A1 (Abcam, rabbit polyclonal, ab35604) at 1:1000 dilution and a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, ThermoFisher) at 1:10000 dilution. Antibodies were detected with the luminol-based SuperSignal™ West Dura Extended Duration Substrate (ThermoFisher) according to the manufacturer's protocol. Image capture was performed on a LI-COR Odyssey instrument using ImageStudio software.

Enzyme Kinetics

afCYP19 catalytic activity was determined by measuring the amount of ³H₂O released as the enzyme converts [1β-³H] ASD to E₁.(22) 900 µL reaction premixes were prepared in 50 mM potassium phosphate buffer pH 7.4 containing 25 nmoles afCYP19 [1β-³H] ASD, 250 nmoles human CPR, 140 µM 1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and [1β-³H] ASD (0.4 µCi/nmole). Triplicate samples were transferred to a shaking water bath and allowed to equilibrate for 10 min. The reactions were initiated by the addition of 100 µL 5 mM NADPH and incubated for an additional 10 min. Reactions were terminated by the the addition of an

equal volume of chloroform and subsequently extracted twice with the same solvent to remove unconverted [1β - ^3H] ASD. A 0.5 mL aliquot was added to 10 mL Safety-Solve cocktail and the $^3\text{H}_2\text{O}$ was quantified by scintillation counting.

Identification of afCYP19 Metabolites

An enzyme-substrate premix containing 25 nM afCYP19, 1 μM hCPR, and 10 μM ASD was prepared in 50 mM potassium phosphate buffer (pH 7.4). The reconstituted enzymes were transferred to a 37 °C shaking water bath and allowed to incubate for 10 min. The reaction was initiated with the addition of 500 μM NADPH and terminated with the addition of 2% zinc sulfate. Precipitated material was pelleted by centrifugation. The supernatant was applied to a ZipTip (Millipore) and eluted with 0.1% acetic acid:50% acetonitrile. The eluate was concentrated by vacuum centrifugation to 10 μL and diluted with 20 μL of 0.1% acetic acid. The samples were loaded on a self-packed fused silica (Polymicro Technologies, Phoenix, AZ) trap column (360 μm outer and 100 μm internal diameter, respectively) with a Kasil (PQ Corporation, Valley Forge, PA) frit packed with 5–15 μm irregular phenyl C_{18} YMC packing. The trap column was connected to an analytical column (360 $\mu\text{m} \times 50 \mu\text{m}$) with a fritted tip of 5 μm or less (New Objective, Woburn MA) packed with 5 μm phenyl C_{18} YMC packing. The steroids were trapped and then eluted into a Thermo Finnigan LCQ deca XP max mass spectrometer (Thermo Finnigan San Jose, CA) with an elution gradient from 0 % buffer A (0.1% acetic acid: water) to 80 % buffer B (0.1% acetic acid:acetonitrile) over 15 minutes. The mass spectrometer was operated in an alternating mode. This sequence included an MS scan followed by two MS/MS scans of the most abundant ions detected, 4 targeted MS/MS scans were then employed to monitor ASD, 19-OH-ASD, 19-oxo-ASD and E_1 abundance.

UV-vis spectroscopy

Measurements of UV-visible absorbance spectra were performed on a dual-beam Olis Cary-14 spectrophotometer (On-Line Instrument Systems, Bogart, GA). Samples of afCYP19 in storage buffer were split equally into two cuvettes and difference spectra (300–700 nm) were collected after a baseline and after the addition of ligands dissolved in ethanol. The difference between the absorbance maxima and minima were fit to the quadratic equation (Eq. 1) for tight binding to a single site:

$$\Delta A = \frac{\Delta A_{\max} [([P]_{\text{total}} + [L]_{\text{total}} + K_d) + (([P]_{\text{total}} + [L]_{\text{total}} + K_d)^2 - 4[P]_{\text{total}}[L]_{\text{total}})^{1/2}]}{2[P]_{\text{total}}}$$

Eq. 1

where $[P]_{\text{total}}$, $[L]_{\text{total}}$, and $A_{\max}$ are the total protein concentration, total ligand concentration, and maximum absorbance change, respectively.(23) The final volume of ethanol did not exceed 2% of the total sample volume. P450 content was determined using Fe^{2+} -CO- Fe^{2+} difference spectra using the method initially described by Omura and Sato. (24) In turn, these data were used to determine an extinction coefficient of $\epsilon^{417\text{nm}} = 84,000$

$\text{M}^{-1} \cdot \text{cm}^{-1}$ for Fe^{3+} afCYP19 that was used for concentration determinations in subsequent studies.

Resonance Raman Spectroscopy

RR spectra of Fe^{3+} afCYP19 enzymes were measured with a f/9.7 single grating monochromator (Acton SP2750, Princeton Instruments) with a $100 \mu\text{m}$ slit using 2400 grooves/mm gratings and imaged using a 1340×400 pixel back-illuminated CCD camera with UV-optimized coatings (PyLoN 400BR eXcelon, Princeton Instruments). Enzyme samples were illuminated with the 406.7 nm line from a Kr^+ (Coherent Innova 302C) laser with a power of ~ 30 mW at the sample. All samples were kept spinning during data collection. Reference calibrations were performed with respect to a Hg vapor lamp. The nonlinear fluorescence background of RR spectra were removed using asymmetric least squares smoothing(25) in MATLAB (MathWorks, Natick, MA).

Pre-steady State Kinetics

Stopped-flow absorbance measurements were made with an Olis RSM-1000 spectrophotometer equipped with a stopped-flow system at 25°C in rapid-scanning mode (10^3 scans $\cdot \text{s}^{-1}$). Rate constants were determined by least-squares fitting of the maximum positive absorbance changes in the Soret bands with respect to time (392 nm for steroids, 418 nm for ATZ) to the solutions of the differential equations for a two-step, reversible binding mechanism:

$$\begin{aligned}\frac{d[E]}{dt} &= -k_1[E][L] + k_{-1}[LE] \\ \frac{d[LE]}{dt} &= k_1[E][L] - (k_{-1} + k_2)[LE] + k_{-2}[EL] \\ \frac{d[EL]}{dt} &= k_2[LE] - k_{-2}[EL]\end{aligned}\quad \text{Eq. 2}$$

where $[E]$, $[L]$, $[LE]$, and $[EL]$ are the concentrations of enzyme, ligand, encounter complex, and bound state, respectively. In the fitting procedure, solutions were subjected to the constraint $k_{-1}k_{-2}/k_1k_2 - K_d = 0$, where the K_d values were derived from the equilibrium titration experiments. The nonlinear constrained optimizations were performed using the *fmincon* nonlinear solver with the interior point algorithm(26) and the differential equations were solved using the *ode113* solver(27), both in MATLAB.

Results and Discussion

afCYP19 Expression and Purification

We deferred to Nature's protein engineering prowess to produce an aromatase ortholog with enhanced stability for *E. coli* expression in the absence of stabilizing ligands. The general strategy was to identify a *Cyp19* among the available genomes that maintained an identical active site to the human enzyme, defined by those residues within $5 \text{ \AA}$ of ASD in the 3EQM crystal structure.(28) We identified the predicted *Cyp19* from *Aptenodytes forsteri* aromatase as a viable candidate. Accordingly, full-length afCYP19 maintains 70%, 72% and

74% identity with the rat, human, and pig orthologs, respectively, although those in the active site are completely conserved. (Boxed residues in Figure 1) The afCYP19 gene was modified so that the first 39 amino acids comprising the N-terminal transmembrane domain were replaced with the MAKKTSSKGR sequence (Figure 1) previously used to facilitate expression of rabbit CYP2C3 and CYP2C11. (29–31) Four additional histidines were added to the C-terminus for purification by Ni²⁺-NTA affinity chromatography. Purification of afCYP19 by affinity and size exclusion chromatography typically yielded 170 to 210 nmoles of purified enzyme L⁻¹ of culture in the absence of stabilizing ligands. The UV-Vis spectrum of purified afCYP19 was characterized by a Soret band at 417 nm and Q-bands at 536 nm and 571 nm. The spectra of the Fe²⁺-CO complex displayed Soret bands at 445nm and 420 nm with a A₄₂₀/A₄₄₅ ratio of 1.2. (Figure 2A) While the former Soret band is telling of an intact cysteinate ligand for the heme iron, the latter P420 form could be attributable to incomplete ligation of the iron or partial degradation of the enzyme under these conditions. SDS-PAGE and immunoblotting of both hCYP19A1 and afCYP19 revealed that the latter is likewise reactive with a polyclonal antibody raised against the C-terminus of hCYP19A1. (Figure 2B) Both enzymes predominately ran as monomers in the SDS-PAGE gels, although bands with apparent molecular weights consistent with dimers of hCYP19A1 and afCYP19 were visible. Given the denaturing conditions of the experiment and the tendency of membrane proteins to run to anomalous apparent molecular weights on SDS-PAGE gels(32), observation of these oligomers are not endorsements of their functional relevance.

Product and Enzyme Kinetic Analyses

Two approaches were used to confirm that afCYP19 was a catalytically-active aromatase. In these experiments, a truncated form of hCPR that lacks its N-terminal transmembrane domain was used to support the reactions.(21) First, an assay that monitors ³H₂O release as the enzyme converts [1β-³H] ASD to E₁ was used to determine enzyme kinetic parameters. (22) The afCYP19 *k_{cat}* (0.02 s⁻¹) and *K_m* (0.20 μM) were comparable to those reported previously. Using a similarly engineered hCYP19A1, Sohl and Guengerich reported a four-fold lower *K_m* (0.044 μM) and two-fold higher *k_{cat}* (0.05 s⁻¹). (3) Likewise, Baravalle *et al.* reported a *k_{cat}* (0.02 s⁻¹) and *K_m* (0.46 μM) for hCYP19A1 that are more similar those measured for afCYP19.(33) Hence, the catalytic activity of afCYP19 is comparable to that previously measured for the human enzyme. The accumulation of 19-OH-ASD and 19-oxo-ASD in the afCYP19-catalyzed conversion of ASD to E₁ were confirmed using LC/MS/MS. ASD was incubated with afCYP19 reconstituted with hCPR and NADPH. Following termination of the reactions, ASD and its metabolites were subjected to solid-phase extraction from the reaction mixture. Guided by MS spectra of ASD, 19-OH-ASD, 19-oxo-ASD, and E₁ standards, a selective reaction monitoring method that scans for specific fragmentation patterns in the MS/MS spectra was used for the detection as each metabolite eluted from the column into the mass spectrometer. MS spectra of the standards, MS and MS/MS scans of each metabolite, and the selected ion chromatograms can be found in the Supplemental Figures S1–S4. ASD, 19-OH-ASD, 19-oxo-ASD, and E₁ eluted at 14.9 min, 12.6 min, 13.2 min, and 13.2 min, respectively, and the identities of each were confirmed by comparing their mass spectra to the authentic standards. These data confirm that afCYP19 catalyzes the transformation of ASD to E₁ by the same 19-OH-ASD and 19-oxo-ASD intermediates as observed for hCYP19A1.

UV-Visible absorbance analysis of ligand binding

The observation that afCYP19 can remove the 1 β -H atom from ASD as well as the detection of common catalytic products as those established for the hCYP19A1 supports that the two enzymes share mechanistic features. Accordingly, we determined the K_d values for ASD, 19-OH-ASD, and 19-oxo-ASD binding to afCYP19 using UV-Visible difference absorption spectroscopy. To a lesser extent, hCYP19A1 also catalyzes the conversion of TST to E₂.⁽³⁴⁾ Consequently, the K_d values for TST as well as the inhibitor ATZ were likewise determined. Illustrated in Figure 3, all of the steroidal ligands induced type-I difference spectra characterized by ligand concentration-dependent increases at 387 nm and decreases at 419 nm characteristic of displacement of coordinated water from the heme iron with a transition to the high-spin state. K_d values derived from fitting these absorbance changes to the quadratic equation for ligand binding to a single⁽²³⁾ site are summarized in Table 1. ASD displayed the highest affinity for the enzyme, followed closely by 19-OH-ASD. The affinity of the subsequent intermediate, 19-oxo-ASD, decreased by an order of magnitude. While this trend of decreasing affinities with progressive oxidation of ASD was likewise observed for hCYP19A1⁽³⁾, there was a notable difference in the relative affinities of ASD and its oxidized products. In hCYP19A1, an order of magnitude difference between the K_d values for ASD (0.13 μ M) and 19-OH-ASD (1.5 μ M) was reported and the K_d for 19-oxo-ASD (3.6 μ M) was only slightly more than two-fold that for 19-OH-ASD. No difference spectrum was observed when afCYP19 was titrated with E₁. The affinity of TST was slightly lower than that of both ASD and 19-OH-ASD. To our knowledge, a K_d value for TST binding to hCYP19A1 obtained by similar experimental means has not been reported, although an apparent K_m for the human placental enzyme is available.⁽³⁵⁾ The reported K_m value for TST (0.041 μ M) is greater than that for ASD (0.014 μ M), so to the extent that the difference in these K_m values represent the relative affinity of these ligands, they are consistent with the K_d values found for afCYP19. ATZ displayed a concentration-dependent type-II difference spectrum, with minima and maxima at 410 nm and 427 nm, respectively, characteristic of triazole nitrogen coordination to the heme iron.⁽³⁶⁾ afCYP19's affinity for ATZ was similar to our previous results obtained with hCYP19A1 (K_d = 0.29 μ M).⁽³⁷⁾ *Resonance Raman Spectroscopy*. RR spectra of afCYP19 were measured to extend our insight into changes in heme electronic structure and dynamics beyond that provided by UV-Vis spectra of the ligand complexes. The high-frequency regions of the RR spectra are illustrated in Figure 4A. This region contains bands that are sensitive to the oxidation (ν_4), and spin state (ν_3) as well as those that are sensitive to the spin state and coordination environment (ν_2 and ν_{10}). The oxidation state marker bands (ν_4) occur at 1374–1376 cm^{-1} as expected for a CYP enzyme in the Fe³⁺ oxidation state. Conversely, the dominant position of the spin-state sensitive bands ν_3 , ν_2 , and ν_{10} , are strongly ligand-dependent. In the ligand-free enzyme, the dominant components are centered at 1503 cm^{-1} , 1587 cm^{-1} , and 1638 cm^{-1} and each attributable to a low-spin, six-coordinate (6CLS) population of the enzyme. Due to its relative isolation in the spectrum, the so-called “pure” spin marker band at ν_3 has a minor component at 1490 cm^{-1} telling of a minor five-coordinate, high-spin (5CHS) population. In the presence of ASD, ν_3 , ν_2 , and ν_{10} , shift to 1490 cm^{-1} , 1570 cm^{-1} , and 1627 cm^{-1} , respectively, consistent with nearly complete conversion to the 5CHS state. The shift pattern observed in the presence of TST is similar; however, the shoulder near 1503 cm^{-1} is indicative of a minor population of the 6CLS enzyme. There was a greater admixture of the

afCYP19 6CLS states when 19-OH-ASD and 19-oxo-ASD are bound compared to the enzyme complexes with ASD and TST. These spectra were performed under saturating concentrations of the ligand, so these minor 6CLS populations are unlikely attributable to the ligand-free enzyme. In the TST case, the 17 β -hydroxyl lowers the affinity for the enzyme and possibly imparts additional orientational heterogeneity to this ligand in the active site. Consequently, some of these orientations could permit maintenance of the 6CLS aqua-ferric heme. Conversely, 19-OH-ASD and 19-oxo-ASD are expected to orient oxygen atoms within iron-coordinating distance thereby replacing coordinated water. In aqueous solution, 19-oxo-ASD exists primarily in its hydrated, geminal-diol form.(7) To the extent that this equilibria is maintained in the active site, the geminal-diol is expected to sample more binding orientations that present a coordinating hydroxyl group to the heme iron. No detectable 5CHS ν_3 component was present when the enzyme was saturated with ATZ consistent with near complete coordination of the 1,2,4-triazole to the heme iron. In summary, the high-frequency RR spectra illustrate that ASD and ATZ induce nearly complete 5CHS and 6CLS transitions of the heme iron, respectively, while TST and the oxidized ASD products induce only partial transitions through partial water or hydroxyl coordination to the iron.

The low-frequency regions of the RR spectra of afCYP19 are illustrated in Fig. 4B. Typical of RR spectra of P450 enzymes, the ν_7 and ν_{15} bands corresponding to heme skeletal modes are centered at 678 cm^{-1} and 755 cm^{-1} . The heme stretching mode at ν_8 is indeed sensitive to the presence of ligand. In the ligand-free enzymes and ATZ-bound enzymes ν_8 is centered at 347 cm^{-1} , but shifts upward to 349 cm^{-1} and 350 cm^{-1} with the oxidized ASD products and ASD/TST, respectively. In addition, the low-frequency regions contain two additional pairs of modes that provide insight into the interactions between the heme peripheral substituents and the protein environment. These include the vinyl bending modes (δ_{vinyls} ; 410–430 cm^{-1}) and those that have been historically attributable to propionate bending ($\delta_{\text{propionates}}$, 370–390 cm^{-1}). (38,39) RR studies of hemes with labeled methyl groups support that the $\delta_{\text{propionates}}$ are more adequately described as out-of-plane distortions of the heme C and D pyrroles. (40,41) Notwithstanding, these modes are still expected to be sensitive to changes in heme propionate hydrogen bonding and/or electrostatic interaction patterns. In ligand-free afCYP19, the $\delta_{\text{propionates}}$ are represented by a doublet with apparent maxima at 372 cm^{-1} and 380 cm^{-1} . Following addition of ASD, 19-OH-ASD, 19-oxo-ASD, and TST the $\delta_{\text{propionates}}$ band coalesces into a band centered at 381 cm^{-1} with an intensity that parallels the extent of high-spin induction by the ligand. In contrast, saturation by ATZ maintains the doublet pattern, however the low-frequency component is shifted to 374 cm^{-1} while the high-frequency component is shifted to 390 cm^{-1} . Conversely, the positions of the peripheral vinyl bending modes (δ_{vinyls}) between 413–427 cm^{-1} are largely resistant to ligand binding. While the presence of ligand does not induce a spectroscopically detectable change in the local vinyl environments, the ligand-free, substrate, and ATZ complexes are characterized by distinct environmental perturbations of the heme pyrroles, possibly due to changes in the propionate-active site interactions.

Ligand Binding Kinetics

Using stopped-flow UV-Vis absorption spectroscopy, rates of ligand binding were performed to gain insight into the binding mechanism. The results of symmetrical mixing of equal concentrations of afCYP19 and each ligand are illustrated in Figure 5. Binding of ASD, TST, 19-OH-ASD, and 19-oxo-ASD result in a rapid shift in the Soret band from 416 nm to 392 nm; characteristic of 6CLS to 5CHS shifts. ASD induces a nearly complete shift of the Soret band to 392 nm, whereas the shift induced by 19-OH-ASD, 19-oxo-ASD, and TST are incomplete. Conversely, ATZ rapidly induces a shift from 415 to 418 nm consistent with coordination of theazole nitrogen to the afCYP19 heme. These spectral shifts are consistent with the 6CLS/5CHS populations inferred from RR spectroscopy.

To gain insight into the ligand binding mechanisms, the time dependence of the absorbance changes in the Soret band were analyzed to test several mechanistic hypotheses for ligand binding. The increases in absorbance at 392 nm and 418 nm for the steroids and ATZ, respectively, were subject to nonlinear least squares fit to the solutions of differential equations (Eq. 2) for the complete set of one- and two-step models with and without reversibility in all steps. In the fitting of one- and two step models with complete reversibility, the search was constrained so that the derived rate constants conformed to the K_d values determined in the ligand titration experiments. Of the models considered, only the two-step reversible model yielded satisfactory fits and physically-realistic rate constants.

In the two-step mechanism, the ligand (L), interacts with the enzyme (E) to form an encounter complex (LE) that subsequently rearranges to a fully-bound state (EL) with both steps reversible:

Rate constants for this model are summarized in Table 2. Versions of the model that enforced that allowed LE to contribute to the absorbance change were also evaluated; however, this did not change the quality of the fits or the resulting rate constants. Hence the binding intermediate appears to spectroscopically silent as previously reported for hCYP19A1.(3) The second-order rate constants defining the initial on-rates (k_1) for all of the ligands were on the order of $\sim 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ as expected for the otherwise diffusion-limited formation of a protein-ligand encounter complex.(42) Within the standard error of the measurements, ASD and TST have similar k_1 values, while those of 19-OH-ASD, 19-oxo-ASD, were somewhat higher. Within the confines of this two-step model, the observed differences in the K_d values become clear upon examination of the remaining rate constants. Since $k_{-1} > k_2$ for the steroidal ligands, dissociation of LE is consistently favored over rearrangement to EL. The k_{-1}/k_2 is least for ASD and thereby contributes to the low K_d value for this ligand; however, this is dampened by a greater k_{-2} . This brings the K_d for ASD in line with that for TST and 19-OH-ASD. The $k_{-1} > k_2$ for 19-oxo-ASD is four- to five-fold that determined for the remaining steroids, contributing to the order of magnitude decrease in the relative affinity of this ligand.

The binding kinetics of ASD, 19-OH-ASD, and 19-oxo-ASD to a similarly engineered form of hCYP19A1 have been reported and the rate constants bear similarities to those for afCYP19.(3) The binding kinetics of hCYP19A1 likewise were best fit to a two-step reversible model with k_1 values on the order of 10^6 – 10^7 $\text{M}^{-1}\cdot\text{s}^{-1}$. The values of k_{-1} and k_2 were somewhat smaller for ASD binding to hCYP19A1, although k_{-1}/k_2 was consistent with those for afCYP19. The k_{-1}/k_2 ratios for 19-OH-ASD and 19-oxo-ASD were two orders of magnitude greater than that for ASD, that in contrast to afCYP19, results in comparably lower affinity for both intermediates.

The k_{-1}/k_2 for ATZ is near unity, imparting this ligand with higher affinity than the steroid substrates or catalytic intermediates. However, its rate constant for reversion from EL to LE is also largest of these ligands. Compared to the steroidal ligands, this apparent destabilization can be partially rationalized with the crystal structure of hCYP19A1 bound to ASD.(28) The structure reveals an active site that is optimized to recognize androgens. If the configuration observed in the crystal structure is representative of the solution ensemble of EL and the ligand binding configuration in LE involves a more promiscuous site, it is not surprising that the K_d values for nonsteroidal ligands such as ATZ would be tempered by greater rates of reversion to the encounter complex.

Conclusions

Herein we describe the first expression and biophysical characterization of an avian cytochrome P450 aromatase from *Aptenodytes forsteri*. Hundreds of nanomoles of a pure, N-terminally truncated form of the enzyme can be purified L^{-1} of *E. coli* culture in the absence of a stabilizing ligand. The means to produce ligand-free afCYP19 makes it a convenient model for spectroscopic studies of ligand-aromatase interactions that typically require considerable amounts of material. afCYP19 catalysis is supported by hCPR with comparable kinetics to those previously reported for hCYP19A1. In addition, the apparently selective removal of the 1β -hydrogen atom of ASD, common oxidized intermediates in the transformation of ASD to E_1 , and similar heme-ligand interactions indicate that afCYP19 and hCYP19A1 share mechanistic features. Like the human enzyme, ASD appears to be the preferred substrate for afCYP19 and the affinity of the intermediates progressively decrease with increased oxidation.(3,35) In addition, afCYP19 binds the aromatase inhibitor ATZ with near identical affinity to that measured for the human enzyme. Like hCYP1A1, ASD, TST, 19-OH-ASD, and 19-oxo-ASD appear to bind to the enzyme using at least a two-step mechanism with a spectroscopically-silent intermediate. In addition, the kinetic data also support a two-step mechanism for ATZ. In summary, the similarities between the human and *Aptenodytes forsteri* enzymes demonstrate that the latter could serve as a convenient model system for studies of the enigmatic transformation of androgens to estrogens.

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Abbreviations

5CHS	Five-coordinate, high-spin
6CLS	Six-coordinate, low-spin
ATZ	Anastrozole
afCYP19	<i>Aptenodytes forsteri</i> Cytochrome P450 19
ASD	Androstenedione
CCD	Charge Coupled Device
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
E₁	Estrone
E₂	17 β -Estradiol
hCPR	Human Cytochrome P450 Reductase
19-OH-ASD	19-Hydroxyandrostenedione
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)
19-oxo-ASD	19-Oxoandrostenedione
RR	Resonance Raman
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TST	Testosterone

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Highlights

- Recombinant expression, purification, and characterization of the *Aptenodytes forsteri* (Emperor penguin) aromatase are described.
- The enzyme has remarkably similar kinetics and ligand-binding properties to the human enzyme.
- Stopped-flow kinetics experiments support that steroid substrates and the inhibitor anastrozole bind to aromatase in multiple steps.

Figure 1.

Partial sequence alignment of *Aptenodytes forsteri*, *Rattus norvegicus*, *Homo sapiens*, and *Sus scrofa* (isoform 1) aromatase. Sequences were retrieved from the National Center for Biotechnology Information Protein Database accession numbers XP_009274754_1 (*Aptenodytes forsteri*), NP_058781_2 (*Rattus norvegicus*), NP_001334185_1 (*Homo sapiens*), and NP_999594_1 (*Sus scrofa*). α -Helical and β -sheet regions are colored in purple and green, respectively. The active site residues (surrounded by black boxes) are defined as those that are within 5 Å of ASD in the crystal structure of human placental aromatase (PDB ID:3EQM). The sequence alignment was performed with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). afCYP19(t) specifies the modifications made to the N-terminus for *E. coli* expression.

Figure 2.

(A) UV-Vis spectra of Fe^{3+} (solid line) and Fe^{2+} -CO (dotted line) afCYP19. The latter spectrum was obtained by reducing the enzyme with a few crystals of sodium dithionite following by blanketing the solution with CO. (B) Immunoblot of human CYP19A1 (center lane) and afCYP19 (right lane). Molecular weight marker is positioned in the left lane.

Figure 3. Representative afCYP19 (2 μ M) absorbance difference spectra (top) and binding isotherms (bottom) for (A) ASD, (B) TST, (C) 19-OH-ASD, (D) 19-oxo-ASD, and (E) ATZ.

Figure 4.

RR spectra of afCY19 and ligand complexes obtained with 406.7 nm laser excitation. High- and low- frequency regions of the RR spectra are illustrated in panels (A) and (B), respectively. Spectra in the high- and low- frequency regions were normalized to ν_4 and ν_7 , respectively. Samples contained 10 μM afCYP19 and 100 μM ligand. LF refers to the ligand-free afCYP19.

Figure 5.

Representative UV-Visible absorbance spectra collected at rate of 1 ms^{-1} following mixing of equal concentrations of afCYP19 and ligands (left) as well as the corresponding changes (right) in the absorbance at 392 nm (418 nm for ATZ) for (A) ASD, (B) TST, (C) 19-OH-ASD, (D) 19-oxo-ASD, and (E) ATZ. The observed changes in absorbance are depicted by grey circles and the superimposed fits are depicted with black lines.

Scheme 1.

Table 1

Fitted K_d values for afCYP19 ligands. Reported values are the mean of three experiments $\pm$ standard deviation.

	K_d (μ M)
Androstenedione (ASD)	0.36 ± 0.02
Testosterone (TST)	0.56 ± 0.02
19-OH-Androstenedione (19-OH-ASD)	0.43 ± 0.04
19-Oxo-Androstenedione (19-Oxo-ASD)	3.9 ± 0.3
Anastrozole (ATZ)	0.20 ± 0.03

Table 2

Rate constants for ligand binding to afCYP19 by a two-step, reversible mechanism. The rate constants are the mean of at least five experiments $\pm$ standard deviation. In the experiments with ASD, TST, and 19-OH-ASD the concentrations of afCYP19 and ligand following mixing were 4 μ M, for 19-oxo-ASD and ATZ the concentrations were 8 μ M.

	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
Androstenedione (ASD)	$3.5 \pm 0.9 \times 10^7$	5.2 ± 1.4	1.6 ± 0.2	3.9 ± 0.3
Testosterone (TST)	$3.3 \pm 0.8 \times 10^7$	4.9 ± 1.2	0.2 ± 0.0	0.8 ± 0.0
19-OH-Androstenedione (19-OH-ASD)	$5.5 \pm 1.6 \times 10^7$	6.5 ± 2.0	0.2 ± 0.0	0.9 ± 0.0
19-oxo-Androstenedione (19-oxo-ASD)	$7.8 \pm 0.9 \times 10^7$	24.0 ± 3.0	0.2 ± 0.0	2.6 ± 0.2
Anastrozole (ATZ)	$5.7 \pm 0.0 \times 10^7$	0.8 ± 0.1	1.0 ± 0.2	15.7 ± 0.4