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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 O_2. 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, af) 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.

1. 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 1*A*) [4–7] The human enzyme converts androstenedione (ASD), testosterone (TST) and 16α-hydroxytestosterone to estrone (E_1), 17β-estradiol (E_2) and 16α-hydroxy-17β-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

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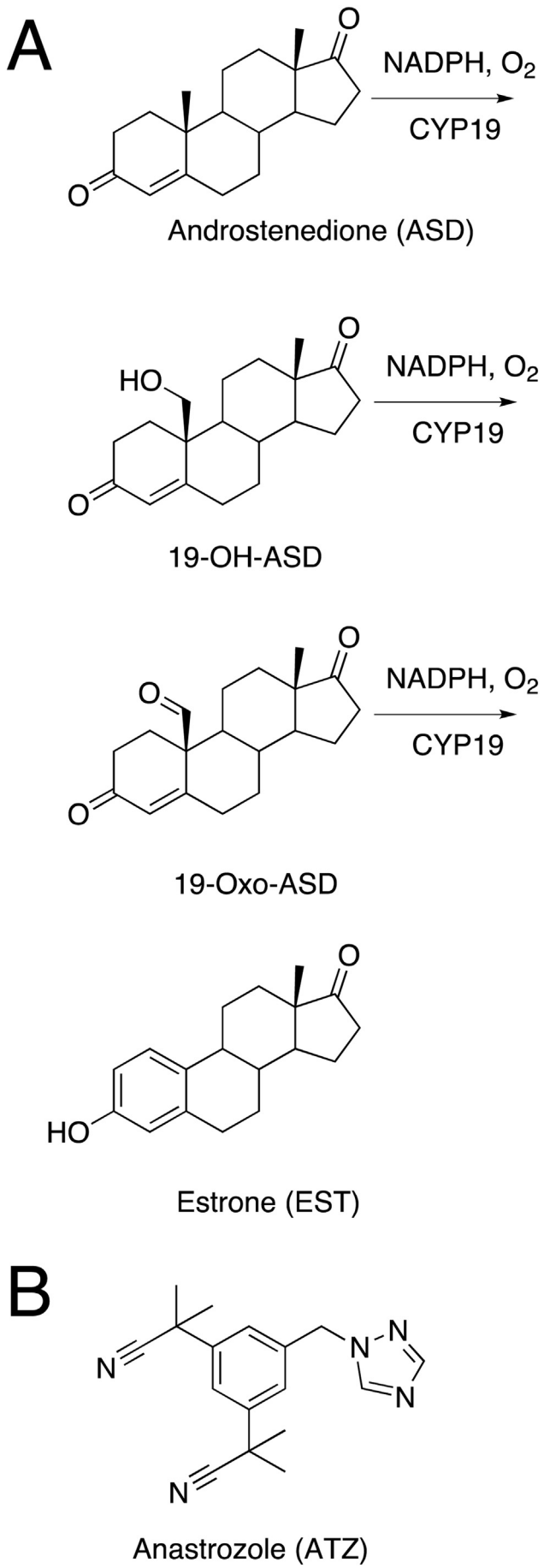

Scheme 1. (A) Summary of aromatase reaction with the conversion of androstenedione to estrone. (B) Chemical structure of anastrozole (ATZ).

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 E_1 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.

2. Materials and methods

2.1. 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 E_1 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.

2.2. 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 1 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 1 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