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Molecular characterization of aromatase

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Abstract

Aromatase is an estrogen synthetase. Estrogens are female sex hormones involved in the development and growth of breast tumors. It has been of significant interest to elucidate the structure-function relationship of aromatase since its inhibitors have shown great promise in fighting breast cancer. Aromatase belongs to the cytochrome P450 family, and forms an electron-transfer complex with its partner, NADPH-cytochrome P450 reductase. Because of the membrane-bound character and heme-binding instability, no crystal structure of aromatase has been reported so far. Much remains to be investigated, including the 3-dimensional structure of aromatase, interaction between aromatase and reductase, catalytic mechanism of estrogen synthesis by aromatase, and the binding mechanism of aromatase inhibitors. This review will present current knowledge about structural and functional characteristics of aromatase to address unsolved mysteries about this enzyme.

Keywords

aromatase; aromatase inhibitors; breast cancer; computer modeling; estrogen; NADPH-cytochrome P450 reductase; structure-function studies

Introduction

Aromatase is the rate-limiting enzyme in estrogen biosynthesis. Estrogen plays an important role in breast cancer development. Upon binding to estrogen, estrogen receptor activates transcription of its target genes, which are responsible for cancer cell proliferation in estrogen-dependent breast tumors. Third-generation aromatase inhibitors, including two triazole derivatives, anastrozole (Arimidex) [1] and letrozole (Femara) [2], and one steroid analogue, exemestane (Aromasin) [3], are currently used clinically for the endocrine treatment of hormone-dependent breast cancer in postmenopausal patients [1, 4, 5]. Investigators have a long-term interest in the structure-function relationship of aromatase, due to its important roles in breast cancer and reproductive dysfunction. To date, no crystal structure of aromatase has been reported. Although the percentage sequence identity between aromatase and other P450 isozymes range from 13-20%, the overall 3-dimensional (3-D) fold is maintained in all P450s [6], thus crystal structures of mammalian P450s offer reliable templates for modeling aromatase 3-D structure. Based on a theoretical 3-D model of aromatase by homology modeling on the crystal structure of human P450 2C9, docking analyses of a series of substrate, product, and inhibitors into aromatase have helped us to

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better understand the molecular mechanism of this enzyme [7-9]. In this 3-D model of aromatase, four β sheets and fifteen α helices define two domains. The small β domain, which is predominantly β sheets, is closely associated with the membrane and involved with substrate recognition and access channel. The major α domain is associated with the catalytic center [7, 9, 10].

This review will summarize current progress in understanding the structure-function relationship of aromatase, including structural and evolutionary analysis of aromatase family based on multiple sequence alignment; protein-protein interaction of aromatase with NADPH-cytochrome P450 reductase (CPR) based on structural docking; and molecular basis for the interaction of androgen, estrogen, and non-steroidal inhibitor with aromatase.

Aromatase functional domain evaluation based on sequence alignment

Aromatase belongs to the cytochrome P450 family and is encoded by the *CYP19* gene. It is expressed within the gonads and brain of vertebrates, including fish, amphibians, reptiles, birds, and mammals, and also in the placenta of primates and artiodactyls, with broad expression in humans [11-13]. To date, the aromatase family has been found to have a single form in amphibians, reptiles, birds, and most mammals, with pigs having ovarian, embryonic, and placental forms, and fish having brain and ovarian forms. All these forms are active with the same substrates: testosterone and androstenedione [11, 12, 14-17]. Phylogenetic analysis of 41 aromatase proteins among mammalian, amphibian, reptile, bird, and fish were investigated, and it was found that aromatase is well conserved among all the vertebrates [17]. The aromatase family shows a divergence of functional characteristics, reflecting differences in K_m , V_{max} , binding affinity for androgen, and response to aromatase inhibitors [15, 18-23]. However, the function differences were marginal from DIVERGE analysis, and divergent residues were random across the protein without clustering, which demonstrates the entire protein sequence of aromatase family is conserved [17].

In this review, 169 non-redundant members of the aromatase family from database Ensembl, UniProt/TrEMBL, and UniProt/Swiss-Prot were applied for multiple sequence alignments (ensembl: www.ensembl.org). The conservation histogram (Fig.1) shows that the most diverse sequences are in the N-terminal transmembrane domain, and the C-terminal loop region, including β sheets 3 and 4, while the major internal sequence is highly conserved, especially the I and L helices, between which the heme is sandwiched. The β domain, including helices A', A, and B, and β sheets 1 and 2, is conserved. The F-G loop, which is diverse among P450s, is highly conserved among aromatase family members. Human aromatase active site residues I133, F134, E302, D309, T310, and S478 predicted from previous studies [8, 9] are highly conserved. Their percentage identities are 99.4%, 100%, 100%, 98.2%, 98.8%, and 95.9%, respectively. Aromatase amino acid sequence is relatively divergent in *Tetraodon nigroviridis* (green spotted puffer) and *Takifugu rubripes* (pufferfish or fugu), which creates several gaps in multiple sequence alignments as shown in Fig.1.

Although the exact physiological significance of sequence divergence in the N, C-terminus of aromatase family is not well understood, it suggests that the enzyme can tolerate sequence modification at the N- and, C-termini for its expression and purification. The N-terminal transmembrane domain serves a structural rather than functional role, thus deletion of the N-terminal transmembrane sequence of human aromatase for its expression in *E. coli* doesn't affect its catalytic activity [9, 24, 25]. Attachment of a His-tag to the C-terminus of aromatase to facilitate purification also maintains its activity [9, 25]. Unexpectedly, the β 4 sheet, which has been proposed to be located in the active site of aromatase, exhibits sequence divergence. This may actually contribute to functional characteristics divergence of the aromatase family. Interestingly, active site residue serine in the β 4 sheet (human

aromatase S478) remains highly conserved among all the species. Another active site residue histidine (human aromatase H480) exhibits species divergence: histidine in amphibians, reptiles, birds, and mammals, while glutamine at this position in fish.

Protein-protein interaction between aromatase and reductase

The cytochrome P450 multicomponent monooxygenase system consists of CPR and one of many cytochrome P450 isozymes. CPR contains flavins FAD and FMN, and catalyzes electron transfer from NADPH to all known cytochrome P450s, including aromatase (Fig. 2). A number of studies indicate that CPR and cytochrome P450s are attached to the endoplasmic reticulum by portions that are deeply embedded in the membrane. The interactions between CPR and cytochrome P450 may comprise interaction of the hydrophobic membrane binding portions and electrostatic attraction [26]. Chemical cross-linking and modification studies have shown that CPR contains multiple carboxylate groups, presumably aspartate and glutamate, which form ion pairs with basic amino acids on cytochrome P450s [27-29]. Multiple sequence alignments of cytochrome P450s have shown that there are five homologous positions of positively charged amino acids which have been identified by protein modification or site-mutagenesis as being involved in the interaction with CPR [30-33]. In human aromatase, these candidate residues are K108, K262/ R263/ R264/R265, K389/K390, and K420 through sequence alignments with cytochrome P450 1A1, 1A2, 2B1, and 2B4.

The crystal structure of CPR was solved in 1997. CPR is composed of four domains, which are the FMN-binding domain, connecting domain, FAD-binding domain, and the NADP-binding domain, from N to C terminus [34]. The membrane binding site of CPR is situated around V64 and near some hydrophobic patches of the surface, most likely, containing loops 516-525, 250-281, and 553-557 [34]. These membrane binding sites enable a CPR molecule to sit on the membrane surface, thus CPR adopts an orientation in which the FMN, FAD, and NADP binding sites are facing towards the cytoplasmic side. The crystal structure of aromatase is still unknown. According to the known crystal structures of mammalian P450s, the F-G loop of aromatase may be involved in membrane binding [10].

In this review, we dock the 3-D structure model of aromatase into the crystal structure of CPR. It is reasonable to assume that the FMN-binding domain of CPR provides a major surface for the electrostatic interaction with aromatase since an electron exits from the FMN-binding domain and enters the heme of aromatase (Fig. 3A). Another assumption, that the proximal surface of aromatase is close to CPR, was made based on the fact that substrate binds to the distal side of aromatase (Fig. 3B). The proximal side of P450 is defined as the surface of protein closest to the cysteine thiolate ligand of the heme iron, while the distal side forms part of the substrate-binding pocket. Protein-protein docking decoys were produced from ZDOCK [35] software version 3.0.1. A total of 13500 decoys were generated by selecting dense rotational sampling. The docking scores were then re-calculated based on the consensus of five equally-weighted measurements including: 1. distance between the FMN of CPR and the heme of aromatase; 2. whether the proximal surface of aromatase interacts with CPR; 3. whether aromatase forms ion pairs with CPR through predictive residues K108, K262, R263, R264, R265, K389, K390, and K420; 4. average distance of C-alpha atoms in the membrane binding portion of aromatase with the plane determined by the membrane binding surface of CPR; and 5. angle of two planes determined by the membrane binding surfaces of CPR and aromatase. The model with the highest docking score is shown in Fig. 3C.

This docking model of aromatase and CPR allows a maximum contact area between the FMN-binding domain of CPR and the proximal surface of aromatase, and an ion-pair

between D121 of CPR and K108 of aromatase. In addition, the N-terminal transmembrane domains and the membrane binding surfaces of both molecules are facing the same orientation. In this docking model, the distance between the C(2)O group of the FMN and the heme iron is 35 Å. The FMN-to-heme distance is 18.4 Å in P450BM-3, a self-sufficient monooxygenase with the heme domain and the FMN/FAD reductase domain linked together on a single polypeptide [36]. P450BM-3 is a prototype for the complex of cytochrome P450s and CPR. It is likely that P450 and CPR form a complex similar to P450BM-3. However, FMN is oriented toward FAD in CPR, thus a structural rearrangement of the FMN domain must occur during the electron transfer to make FMN accessible for P450, in other words, closer to the heme. A flexible hinge between the FMN and FAD domains allows back-and-forth motions of FMN [34, 36]. The docking model shown in Fig. 3 presents an initial contact between aromatase and CPR, while the structure of protein-protein complex reformed during the electron transfer reaction still remains unclear.

Molecular basis for the interaction of androgen and estrogen with aromatase

Upon receiving electrons from CPR, aromatase synthesizes estrogen from androgen by three hydroxylation steps. The first and second hydroxylations occur at the 19-methyl group of androgen, while the third hydroxylation results in the cleavage of the C10-C19 bond and the aromatization of the A ring. The major structural difference between estrogen and androgen is that estrogen has a planar aromatic A ring, while androgen has a non-aromatic six-carbon A ring. In a recent publication [9], the way in which androgen binds to aromatase and the particular mechanism by which the aromatization reaction occurs were discussed. In addition, how the structural conversion by the aromatization reaction leads to the release of estrogen from aromatase was also investigated [8].

The theoretical 3-D model of human aromatase based on the crystal structure of human P450 2C9 [7] provides useful structural information and a reliable structural basis for ligand docking. In this model, the heme iron is ligated by a conserved cysteine (C437), and the propionates of heme interact with the side chain of residues R115, W141, R145, R375, and R435. I125-P138 of the B'-C loop penetrates into the interior of the molecule so that residues I132-F134 are located in or adjacent to the active site. The β 4 sheet protrudes into the active site, placing residues L477-H480 above the active site pocket. By carefully examining this structural model and the results from our recent structure-function studies of aromatase, a clamping mechanism of steroidal substrate/inhibitor binding to the active site has been proposed [9]. Together with D309 (in the I helix), S478 and H480 (in the β 4 sheet) are thought to participate in a charge relay system that leads to the aromatization of the A ring of androgen. In addition, S478 forms a hydrogen bond with the C-3 keto oxygen of androgen. The D-ring of androgen is anchored by Van der Waals forces from I133 and F134. The 3'-flanking loop (P368-M374) of the K helix is thought to participate in forming the hydrophobic ligand-binding pocket and hence residue V373 possibly interacts with the B ring of androgen. This loop (P368-M374), together with the I helix (between E302 and T310), B'-C loop (containing I133 and F134) and the β 4 sheet (containing S478 and H480), hold the substrate above the heme.

The structure difference between androgen and estrogen is the A ring. The fact that this structural conversion causes dramatically different binding affinities of androgen and estrogen leads us to assume that the β 4 sheet plays an important role in steroid binding since the β 4 sheet is found to be near the A ring in our studies. In fact, residue S478 is also involved in estrogen binding because site-directed mutagenesis experiments showed that the S478T mutant increased the binding affinity of estrogen [9]. However, the planar aromatic A ring of estrogen probably has weaker interaction with the β 4 sheet when compared to the

non-aromatic six-carbon A ring of androgen, and leads to the release of estrogen from the active site of aromatase. In addition, our docking model showed that estrogen is localized high above the heme due to the removal of the C-19 methyl group of androgen, which may leave extra space allowing a water molecule to re-ligate to the iron, and in turn trigger the release of estrogen from the active site.

The hypothesis that a steroid with a planar A ring is not a favorite form to bind to aromatase is also supported by the study on the inhibition of aromatase by coumestrol [9]. Coumestrol is a phytoestrogen that exhibits structural and functional similarity to estrogen, but is a weak competitive inhibitor for aromatase [37]. The $\beta 4$ sheet of aromatase, particularly residues S478 and H480, plays an important role in the binding of coumestrol to aromatase based on mutagenesis studies showing that the S478T and H480Q mutants have dramatic effects on the binding affinity of coumestrol. In the docking model of a aromatase-coumestrol complex [8], due to the planar structure and the small size of a coumestrol molecule, coumestrol enters deep into the cleft between the I helix and the $\beta 4$ sheet, which results in close contacts between the A ring of coumestrol and residues S478 and H480. Similar to E2, the planar A ring of coumestrol might have weaker interaction with the $\beta 4$ sheet, when compared to the non-aromatic six-carbon A ring of androgen. In addition, planar flavones in previous studies were shown to have low binding affinity to aromatase [38]. Phytoestrogen derivatives with substitution at the planar rings can increase its binding affinity to aromatase, for example, 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin with a K_i value of 84 nM [39].

Docking of non-steroidal inhibitor into aromatase

It is easier to predict the interaction of steroidal inhibitors such as exemestane with aromatase than to predict the interaction of nonsteroidal inhibitors with the enzyme. The binding of exemestane to aromatase has been extensively discussed in a previous publication [9]. There have been a number of modeling studies on triazole derivative inhibitors, including anastrozole, letrozole, and vorozole. Although there is a consensus that the triazole nitrogen (N-4) coordinates the heme iron, various opinions about the orientation of these triazole derivatives in the active site of aromatase exist. In a previous study [40], we proposed an orientation for anastrozole in the active site of aromatase, using a 3-D model of aromatase based on the crystal structure of P450bm3. This docking model provides an overlap between the triazole substituent of anastrozole and the imidazole ring of 2-phenylimidazole in the crystal structure of the complex of P450cam and 2-phenylimidazole, but both the cyanoisopropyl groups of anastrozole occupy a region of space close to the $\beta 4$ sheet, leaving a space near the B'-C loop unoccupied.

Recently the interaction between aromatase and anastrozole was re-investigated by docking anastrozole into the new 3-D model of aromatase (Fig. 4). In the current model of the aromatase-anastrozole complex, one cyanoisopropyl group protrudes into the cleft between the I helix and the $\beta 4$ sheet, and the other one occupies a region of space close to the B'-C loop. Besides the primary coordination between the triazole group (N-4 atom) of anastrozole and the heme iron, anastrozole interacts with the active site of aromatase through two hydrogen bonds. They are between the T310 residue and the triazole group (N-1 atom) of anastrozole and between the D309 residue and the cyano group (C-N) of anastrozole. This binding model of anastrozole provides new insights into the structural basis of the interaction between triazole derivative inhibitors and aromatase.

Among sixteen aromatase mutants at residues I133, F235, I395, I474, E302, P308, D309, T310, S478, and H480 that have been tested in our previous studies [40, 41], the D309A mutant dramatically decreases the binding affinity of anastrozole, the I133Y, E302D, S478T, and H480K mutants also decrease its binding, while the T310S, I474Y, I474M, and

I474N mutants increase its binding. Although it is difficult for one docking model to account for all the mutations, these inhibition profile studies helped us to determine the docking orientation for anastrozole, and to evaluate the docking model. The current anastrozole docking model shows one cyanoisopropyl group towards the β 4 sheet and the other one towards the B'-C loop, which is consistent with inhibitory studies which demonstrated mutants located in both the B'-C loop and the β 4 sheet affect the binding affinity of anastrozole. Two newly proposed hydrogen bonds between the D309 and T310 residues and anastrozole explain effects of the D309A and T310S mutants on the binding affinity of anastrozole: the significantly higher IC₅₀ value of anastrozole in the D309A mutant, when compared to WT aromatase, may result from the loss of the hydrogen bond provided by the D309 residue; higher binding affinity of anastrozole in the T310S mutant might be due to less steric clash in the triazole ring of anastrozole because of the smaller side chain of serine.

In summary, structure-function studies of aromatase allow us to better understand the reaction mechanism of this enzyme and the binding nature of different classes of inhibitors. This information is critical for the development of new aromatase inhibitors as well as for the identification of novel anti-aromatase phytochemicals for the prevention/ treatment of hormone-dependent breast cancers.

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Fig.1.

Conservation of amino acid sequence of 169 aromatase family members.

The extent of sequence conservation is indicated by the histogram quality. The secondary structure of human aromatase is indicated by *box* (α -helix) and *arrow* (β -sheet) under the conservation histogram. The sequences were aligned using Ensembl BLAST Server (www.ensembl.org).

Fig. 2.
Electron flow in the complex of CPR and aromatase.

Fig.3.

Electrostatic potential surface of CPR, aromatase, and the CPR-aromatase docking complex. The positive potential is shown in *blue*, and the negative potential is shown in *red*. The electrostatic potential was calculated with the APBS Plugin in PyMOL [42].

A. The electrostatic potential surface mapped onto the surface of CPR. The binding surface of CPR onto which aromatase binds is the FMN binding domain (bottom-right), which is highly negatively charged. B. Diagram showing the proximal surface of aromatase, which is largely positively charged. C. A docking model of CPR (*left*) with aromatase (*right*). The distance between the C(2)O group of the FMN and the heme iron is 35 Å. FMN, FAD, NADP, and heme are shown in *stick* representation, and colored in *yellow*, *cyan*, *purple*, and *green*, respectively.

Fig.4.

A docking model of the aromatase-anastrozole complex.

The N-4 atom in the triazole group of anastrozole coordinates with the heme iron. The N-1 atom in the triazole group and one cyano group of anastrozole form hydrogen bonds with residues T310 and D309, respectively. Anastrozole is shown in *purple* above the heme group (colored in *red*). The heme iron is shown in *sphere* representation. The 3-D model of aromatase is displayed as secondary structure cartoon and colored according to sequence succession from blue to red. Helices I, F, and G are displayed as helical structures. The B'-C loop and the β_4 sheet are displayed as loop structures. Amino acid side chains are shown in *stick* representation. Oxygen atoms are colored in *red*, nitrogen in *blue*, sulfur in *yellow*, hydrogen in *white*. The red dashed line is drawn between the atoms involved in a hydrogen bond.