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Binding features of steroidal and nonsteroidal inhibitors

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ABSTRACT

Aromatase is the rate-limiting enzyme in estrogen biosynthesis. As a cytochrome P450, it utilizes electrons from NADPH–cytochrome P450 reductase (CPR) to produce estrogen from androgen. Estrogen is a key factor in the promotion of hormone-dependent breast cancer growth. Aromatase inhibitors (AIs) are drugs that block estrogen synthesis, and are widely used to treat estrogen-dependent breast cancer. Structure–function experiments have been performed to study how CPR and AIs interact with aromatase to further the understanding of how these drugs elicit their effects. Our studies have revealed a strong interaction between aromatase and CPR, and that the residue K108 is situated in a region important to the interaction of aromatase with CPR. The published X-ray structure of aromatase indicates that the F221, W224 and M374 residues are located in the active site. Our site-directed mutagenesis experiments confirm their importance in the binding of the androgen substrate as well as AIs, but these residues interact differently with steroidal inhibitors (exemestane) and non-steroidal inhibitors (letrozole and anastrozole). Furthermore, our results predict that the residue W224 also participates in the mechanism-based inhibition of exemestane, as time-dependent inhibition is eliminated with mutation on this residue. Together with previous research from our laboratory, this study confirms that W224, E302, D309 and S478 are important active site residues involved in the suicide mechanism of exemestane against aromatase.

1. Introduction

Aromatase, a cytochrome P450 enzyme, catalyzes three consecutive hydroxylation reactions to convert C19 androgen to aromatic C18 estrogen. After estrogen is synthesized by aromatase, it binds to the estrogen receptor (ER) to form an estrogen–ER complex. This estrogen-bound ER activates target genes that promote the growth of estrogen-dependent breast carcinomas. During estrogen biosynthesis, aromatase forms an electron transfer complex with the NADPH cytochrome P450 reductase (CPR). In the past two decades, researchers have analyzed the interactions between the members of cytochrome P450 family and the CPR enzyme. Structural data showed that the CPR enzyme consists of the following electron transfer domains: NADP-binding domain, FAD-binding domain and FMN-binding domain [1]. During the synthesis of estrogen, electrons are transferred from NADPH through the FAD and FMN domains of CPR to the heme of aromatase (Fig. 1). The recently solved crystal structure of the aromatase–androstenedione complex [2] provides structural information on how androgen binds at the active site of aromatase. This protein (residues 45–495) contains twelve α-helices (labeled from A to L) and ten β-strands (numbered from 1 to 10). The active site of aromatase was found to be relatively small (<400 $Å^3$), and the androgen molecule fits tightly into this androgen-specific cleft. The opening of the active site channel rests on the endoplasmic reticulum membrane and allows the substrate to enter directly from the membrane site [2]. Based on the three-dimensional structural information from the CPR and aromatase crystals, we initiated a study to address a fundamental question: In what form does the aromatase–CPR complex exist under cellular conditions? The molecular docking model of the aromatase–CPR complex, published from our laboratory [3], revealed that the FMN domain of CPR undergoes a structural rearrangement, allowing the proximal surface of aromatase to fit into the cleft between the FMN and FAD domains of reductase. We identified key residues, including K108 on the surface of aromatase, which are involved in the interaction with reductase [3].

About 60 percent of premenopausal and 75 percent of postmenopausal breast cancer patients have estrogen-dependent carcinomas [4]. To treat these patients, the Food and Drug Administration (FDA) approved three aromatase inhibitors (AIs): exemestane, letrozole and anastrozole. These AIs showed greater clinical benefits with higher specificity than anti-estrogen drug tamoxifen. These drugs were also found to effectively decrease recurrence rates of the hormone-dependent breast cancer [5–10]. Exemestane [brand name: Aromasin] is a steroidal aromatase inhibitor that primarily affects enzyme catalysis by binding as a pseudo substrate (Fig. 2), making it a mechanism-based inhibitor. The reactive intermediate of exemestane irreversibly binds to aromatase and causes permanent inhibition of the enzyme. Due to structural similarity with the substrate, it is easier to predict the

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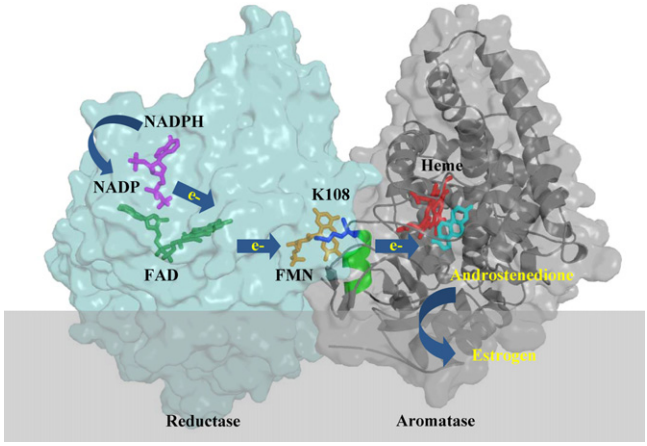

Fig. 1. Schematic demonstration of the CPR–aromatase complex model. NADP (purple), FAD (green), FMN (orange), heme (red) and androstenedione (cyan) are shown in stick representation. During the synthesis of estrogen, electrons are transferred from NADPH, through FAD and FMN of CPR (light blue) to the heme of aromatase (grey). The K108 (blue) residue is located at the B′ helix (green) of aromatase and plays an important role in CPR and aromatase interaction and the electron transfer process. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

interaction of exemestane with aromatase [2,11]. Alternatively, letrozole [brand name: Femara] and anastrozole [brand name: Arimidex] are not androgen analogues and are referred to as nonsteroidal inhibitors. These two AIs contain a triazole functional group, which perturbs the catalytic properties of the heme prosthetic group of aromatase, and act as competitive inhibitors with respect to the androgen substrate [12]. The exact nature of nonsteroidal inhibitor interactions with aromatase is still unclear due to their structural diversity. In our laboratory, we have performed site-directed mutagenesis experiments to evaluate how steroidal and nonsteroidal AIs bind to the active site of aromatase. These results will be discussed in this paper.

2. Molecular characterization of the aromatase–NADPH cytochrome P450 reductase (CPR) complex

One of the research goals of our laboratory is to analyze the interaction of aromatase with its electron transfer partner, CPR. Our published data from the last two years are reviewed here. To study aromatase–CPR interaction, we used ZDOCK software to apply a flexible, step-wise docking approach. At first, aromatase was docked together with the FMN domain of reductase. The FAD and NADP domains were then added into the complex. The first support of the model came from the distance between FMN and heme which was found to be 18.8 Å [3]. This distance corresponds to the one found in the crystal structure of the bacterial cytochrome P450BM-3 complex, where the heme and the reductase domains linked together on a single polypeptide chain [13]. Earlier sequence alignment data noted five positively charged amino acids of cytochrome P450 that are present at the CPR interaction surface [14–16]. For aromatase, the corresponding residues are K99, K108, K389/K390, K420 and R425. In our model, we noticed interactions between the K108 and K420 residues of aromatase with the N175/T177 and E115 residues of CPR, respectively.

Based on our structural model, we investigated the role of the K108 residue in detail through site-directed mutagenesis. The conserved K108 residue is located on the B′ helix of aromatase, and the B′ helix intrudes into the cleft between the FMN and FAD domains of reductase (Fig. 1). We generated a K108Q mutant in CHO cells, and used the 'in cell' aromatase assay to measure and compare enzyme activity between the wild-type and the mutant proteins. Results from Western blot analysis showed that similar protein levels were detected in the wild-type and mutant expressed cells, but a significant decrease in aromatase activity was observed in the K108Q cells [3]. We then performed kinetic analyses with recombinant CPR and aromatase enzymes from *Escherichia coli* to determine if the decreased activity was due to a change in CPR–aromatase interaction. Results showed that the *Km* value of CPR for the interaction with the K108Q mutant (20 nM) was twentyfold weaker than the wild-type aromatase (1 nM). Our kinetic analysis further showed that the K108Q mutant had a similar androstenedione binding affinity as the wild-type protein. Therefore, the decreased activity of the K108Q mutant is most likely due to a decrease in the efficiency of the electron transfer process from CPR to aromatase. The IC50 values (half maximal inhibitory concentration) of exemestane were equivalent for the K108Q mutant and the wild-type protein (unpublished data). In addition, the time-dependent inhibition profile of exemestane, for both the mutant and the wild-type protein, was also similar. Thus, our data indicated that the K108 residue has no role in

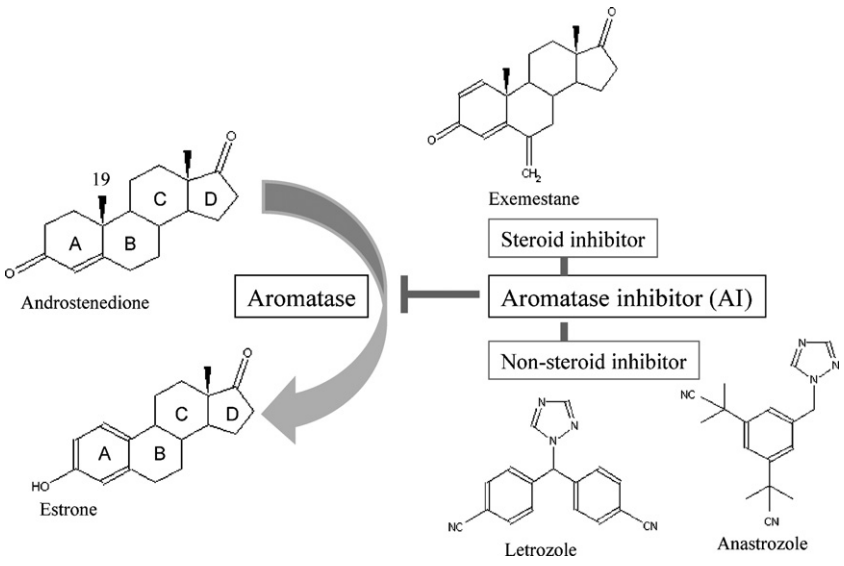

Fig. 2. Structures of androstenedione, estrone, exemestane, letrozole and anastrozole.