ABSTRACT

Aromatase, a cytochrome P450, catalyzes three consecutive hydroxylation reactions converting C19 androgens to aromatic C18 estrogenic steroids. In this study, eight human aromatase mutants (1133Y, 1133W, F235L, I395F, I474Y, I474W, I474M, and I474N) were prepared to evaluate the active site and a proposed hydrophobic pocket of the enzyme that exists in an aromatase model based on the X-ray structure of cytochrome P450cam. In addition, the binding characteristics of three steroidal inhibitors [4-hydroxyandrostenedione, 7α-(4' -amino)phenylthio-1,4-androstadiene-3,17-dione, and bridge (2,19-methyleneoxy)androstene-3,17-dione (MDL 101,003)] and four nonsteroidal inhibitors [aminogluthethimide, CGS 20267, ICI D1033, and vorozole (R83842)] were investigated through inhibitory profile studies on the eight new and three previously generated mutants (P308F, D309A, and T310S). The latter analyses have provided a molecular basis regarding how seven aromatase inhibitors with different structures bind to the active site of aromatase.

INTRODUCTION

Aromatase, a cytochrome P450, catalyzes three consecutive hydroxylation reactions that convert C19 androgens to aromatic C18 estrogenic steroids. This enzyme has received considerable attention because of the central importance of estrogens in many reproductive and metabolic processes. The synthesis of estrogens is required for the normal expression of secondary sexual characteristics and establishment and maintenance of pregnancy. Fetal expression in the brain is believed to determine male or female metabolic patterns expressed during adult development (1). In pathological situations, an abnormal expression of aromatase has been detected in a significant number of breast tumors, in cancers as well as surrounding adipose stromal cells (2—6). In view of this, the inhibition of the enzyme has been considered as a potential therapy for breast cancer. Throughout the years, a number of very potent and highly selective aromatase inhibitors have been synthesized and tested as drugs for the treatment of breast cancer.

Aromatase inhibitor development has been based primarily on structure-activity relationship studies. Aromatase inhibitors can be categorized into two types: steroidal and nonsteroidal inhibitors. In general, steroidal aromatase inhibitors are analogues of androgen substrates and nonsteroidal inhibitors perturb the catalytic properties of the heme prosthetic group of aromatase. Although a number of the inhibitors have been shown to be very potent and specific inhibitors of aromatase, the exact nature of their interactions with aromatase are not known. This is especially true for nonsteroidal inhibitors since these compounds have very diverse structures. Although the structures of these compounds are different, it is thought that they bind to the active site of aromatase, as indicated by competitive inhibition of the enzyme (further details in “Discussion”).

To provide some insight into this problem, we have carried out structure-function studies of aromatase by site-directed mutagenesis experiments (7—13). We have examined the amino acid sequences of aromatase from five species and aligned them with that of cytochrome P450cam, whose three-dimensional structure has been extensively studied (14). On the basis of this, several regions of aromatase were postulated to be parts of the active site. A number of mutants with changes in these regions were prepared, using a mammalian cell-expression system that was developed in our laboratory, to evaluate the proposed model of the active site of the enzyme. In addition, structure-activity studies on aromatase inhibitors have led to the hypothesis that the active site is quite large, with a pocket distal to the heme group of the enzyme (15, 16). This allows the binding of a large range of inhibitors that have very different structures. A recent structure prediction of aromatase by homology modeling from cytochrome P450cam (17) predicted an extra hydrophobic pocket, unused by natural substrates and extending roughly in the plane of a bound steroid from the position that would be occupied by its C4 and C7 atoms. In a recent study (13), four mutants (G121A, I125N, F235N, and I474P) were generated to evaluate the predicted pocket.

During the last several years, the X-ray structures of three additional cytochrome P450 enzymes have been determined. The cytochrome P450 enzymes can be divided into two classes on the basis of their redox partners. Class I enzymes require a flavin adenine dinucleotide-containing reductase and an iron-sulfur protein for activity, whereas class II enzymes are partners by a flavin adenine dinucleotide and flavin mononucleotide-containing reductase. The four cytochrome P450 enzymes, whose crystal structure has been determined, are P450cam (14), P450bm3 (18), P450erp (19), and P450eryF (20). Of these cytochromes, P450bm3, is unique in being a class II cytochrome P450; the holoenzyme consists of an N-terminal heme- and substrate-binding domain and a C-terminal reductase domain (21, 22). As such it bears a much closer functional similarity to eukaryotic class II enzymes such as aromatase. We have therefore used homology modeling techniques to construct a new model for aromatase based on the cytochrome P450bm3 structure and compared it with our original cytochrome P450cam-based model (17). Amarnah et al. (23) have performed a site-directed mutagenesis study with a cytochrome P450bm3-based model which was recently published by Graham-Lorence et al. (24).

In this study, eight mutants (1133Y, I133W, F235L, I395F, I474Y, I474W, I474M, and I474N) were prepared to evaluate our aromatase models. In addition, we have investigated the binding characteristics of seven aromatase inhibitors using inhibitory profile studies on the eight new and three previously generated mutants [P308F (7), D309A (8), and T310S (10)].

MATERIALS AND METHODS

Chemicals. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. [35S]ATP and radiolabeled androgen were obtained from New England Nuclear. DNA sequencing kits were obtained from United States Biochemical. Seven aromatase inhibitors were obtained from United States Biochemical. 3-Phosphoadenosine 5-phosphosulfate was obtained from United States Biochemical.
Fig. 1. Structures of androstenedione and seven aromatase inhibitors.

were used in this study. AG and 4-OHA were purchased from Sigma. 7α-APTADD, bridge (2,19-methyleneoxy)androstene-3,17-dione (MDL 101,003), CGS 20267, ICI D1033, and vorozole (R83842) were kindly provided by Dr. R. W. Brueggemeier at the Ohio State University, Dr. J. O'Neal Johnston at the Marion Merrell Dow Research Institute, Dr. A. S. Bhatnagar at Ciba-Geigy AG (Basel, Switzerland), Dr. Michael Dukes at Zeneca Pharmaceuticals (Macclesfield, United Kingdom), and Dr. R. De Coster at the Janssen Research Foundation (Beerse, Belgium), respectively. The synthesis of 7α-APTADD has been reported by Snider and Brueggemeier (25). The synthesis of MDL-101,003 has been described by Peet et al. (26). CGS 20267 was first reported to be an aromatase inhibitor by Bhatnagar et al. (27). The synthesis of ICI D1033 was reported by Dukes et al. (28). Vorozole was first indicated as an aromatase inhibitor by Wouters et al. (29). Among the seven inhibitors, 4-OHA, 7α-APTADD, and MDL 101,003 are steroidal inhibitors, and AG, CGS 20267, ICI D1033, and vorozole are nonsteroidal inhibitors. The structures of these compounds are shown in Fig. 1.

Stable Expression and Site-directed Mutagenesis Experiments. A PCR-based mutagenesis method described by Nelson and Long (30) was used to generate aromatase mutant cDNAs. Each desired PCR product was resolved over a 1% or 1.5% agarose gel and then electroeluted into a Centricon 30 ultrafiltration unit (Amicon) using a house-made electroeluter. The gel-purified PCR products were cloned into the PCR1000 vector from the TA cloning kit (Invitrogen Co., San Diego, CA). Mutant clones were then selected by dideoxy sequencing. The resulting mutant constructs were ligated into the mammalian expression vector, pH/L3-Apr-1-Neo (31), through the engineered SalI and HindIII restriction sites. The transfection procedure was the same as described previously (32), except that the selection process underwent 4 weeks with stepwise increased G418 (Promega) up to a final dosage at 680 μg/ml. Eight mutants (1133Y, 1133W, F235L, 139SF, 1474Y, 1474W, 1474M, and 1474N) were expressed in CHO cells and used in this study.

Computer Modeling. The initial alignment of cytochrome P450bm3 and aromatase was taken from the multiple alignments of Nelson (33). This was then adjusted on the basis of the location of secondary structural elements, known in the case of cytochrome P450bm3, and predicted using PHD (34, 35) in the case of aromatase.

Main-chain coordinates for the core regions were taken directly from the cytochrome P450bm3 structure. Main-chain coordinates for the loops were obtained from a loop data base search. Side-chain conformations were predicted using the three-dimensional local homology/Monte-Carlo procedure (36). Refinement of the structure involved energy minimizations using AMBER 4.0 (37) coupled with analyses using Procheck (38) and manual adjustments using MidasPlus (39).

Preparation of Polyclonal Antibera against Aromatase. Two female New Zealand White rabbits were immunized by multiple s.c. injections of 150 μg of purified D38 aromatase, an Escherichia coli-overexpressed recombinant placental aromatase apoprotein whose 38 amino acid residues from the NH2 terminus were removed (12). The recombinant protein was suspended in 1 ml of a 50% emulsion of Freund’s complete adjuvant in PBS. Booster immunizations were given s.c. at 5-week intervals with 75 μg of the same antigen homogenized in Freund’s incomplete adjuvant. The rabbits developed antibodies that reacted to purified placental aromatase in Western immunoblots.

Western Blot Analyses. Microsomes from transfected and untransfected CHO cells were prepared according to the method of Yoshida and Osawa (40). Membranous proteins, 20 μg of each sample, were electrophoresed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter. The filter was then incubated with immune serum at a dilution of 1:1000, washed, and reacted with a secondary antibody. Immune serum reacted in the screening assay at a dilution of 1:1000, serum was collected.

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Fig. 2. Alignment of cytochrome P450bm3 and aromatase sequences. Secondary structural elements (known in the case of cytochrome P450bm3, predicted for aromatase) are shown boxed (heavy lines, helices; lighter lines, sheets).

SDS-PAGE using the discontinuous buffer system described by Laemmli (41). The fractionated proteins were then electrophoretically transferred to nitrocellulose membrane (BA85; Schleicher & Schuell) in 25 mM Trizma base/190 mM glycine. The semidyed electrophoretic transfer was performed in a MilliBlot SDE electrophoretic apparatus (Millipore Ltd.) at 200 mA for 2 h. The membrane was pretreated with 3% BSA in PBS during a 1-h incubation. The nitrocellulose was then washed three times with PBS for 5 min each. Prior to probing, approximately 200 μg of microsomes prepared from untransfected CHO cells were boiled for 5 min and used to absorb 35 ml of diluted antiserum for 24 h, at 4°C, utilizing a gyratory shaker. The antiserum was diluted 1:1000 in PBS containing 3% BSA. After washing, the blocked nitrocellulose filter was incubated with the preabsorbed antiserum for 1 h with gentle agitation. Subsequently, the filter was washed three times with PBS, 5 min each, and followed by a treatment of biotinylated goat anti-rabbit IgG-avidin-D:biotinylated horseradish peroxidase H complex system (Vectastain ABC kit; Vector Laboratories) by following the manufacturer’s instructions. 4-Chloro-1-napthol was used as substrate for the coloring reaction. Unless otherwise stated, all procedures were conducted at room temperature.

RESULTS

Comparison of the Cytochrome P450bm3-based Model and the Cytochrome P450cam-based Model for Aromatase. On the basis of the alignments, it was expected that the cytochrome P450bm3-based model for aromatase would be superior to the original P450cam-based model. The alignment of aromatase with cytochrome P450cam (Fig. 2) had a 17% sequence identity and spans residues Trp-39 to Pro-494 of aromatase. There are 14 core regions separated by 13 indels: six are insertions, and seven are

isolation assay (32). The effects of seven aromatase inhibitors on aromatase and the mutants were evaluated with the in-cell assay method. For the aromatase inhibitor study, inhibitors were added during the assays with 100 nm [1-3H]androstenedione. Both enzyme kinetic and inhibition profile analyses were performed in triplicate. Kinetic constants for the mutants or IC50 values for inhibitors were determined by plots generated with the average values of the results for each data point.

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AROMATASE INHIBITOR BINDING

pair energy extra hydrophobic pocket was not readily identifiable. Suspicions that the model had serious deficiencies were supported by protein structure analysis using the program Prosall (42, 43). This revealed a poor energy profile for the structure in the region between residues 150 and 250 (Fig. 3). In this region, a major difference between the P450bm3-based and P450cam-based models is in the predicted lengths of helices F and G. In P450cam (and in the P450cam-based model of aromatase), helices F and G lie antiparallel and extend across the active site face of the molecule from one edge to the center, so that the C-terminal residues of helix F and the N-terminal residues of helix G make a major contribution to the structure of the active site (Fig. 4A).

In P450bm3 and the derived aromatase model, both helices are longer and therefore extend almost all of the way across the active site face of the molecule (Fig. 4B). This has a major effect on the structure of the active site. It should be noted that in their description of the cytochrome P450bm3 structure (18), the authors note that this region of the structure has the highest temperature factors and suggest that it may show considerable conformational flexibility, further reducing the confidence that can be placed in a homology-built structure in this region. Another major difference between cytochrome P450cam and cytochrome P450bm3 is in the orientation of the B' helix, which in the P450cam-based aromatase structure plays an important role in defining the structure of the extra hydrophobic pocket (Fig. 4).

Characterization of Mutants. All eight mutants were detected in transfected CHO cells using Western blot analysis. Although the antibody used in this analysis cross-react with proteins other than aromatase, aromatase mutant proteins were detected in transfected CHO cells (Fig. 5). Like the controls, aromatase was not detected in microsomes prepared from untransfected CHO cells, and an aromatase band was clearly seen when a small amount of purified aromatase was added to this microsomal preparation (Fig. 5). The presence of aromatase or its mutants in transfected CHO cells was further confirmed by enzyme activity analysis (see below).

The catalytic properties of aromatase mutants have been determined using both an in-cell assay and an assay on microsomal preparations. The apparent $K_m$ and $V_{max}$ values (as determined by both methods) of eight mutants along with three previously prepared mutants (P308F, D309A, and T310S) are shown in Table 1. Both the in-cell and microsomal assays provided comparable $K_m$ and $V_{max}$ values for the wild-type enzyme (F235L, I474Y, I474W, I474M, P308F, and

deletions. The largest insertion is five residues in the loop before helix A, the largest deletions are two of four residues: one between helices E and F and the other between helices H and I. However, with the P450bm3-based model, identifying a suitable orientation for a substrate molecule at the active site proved problematic. The active site cavity had a very different shape from that observed in the cytochrome P450cam-based model and would not permit the same orientation of the substrate relative to the heme. In addition, the putative surface energy

![Fig. 3. Prosa II energy profiles (smoothed with a 50-residue window) for the cytochrome P450bm3-based model of aromatase showing the poor surface energy term for the 150–250 region.](image)

![Fig. 4. Comparison [generated using MOLSCRIPT (43)] of helices B', F, and G in the cytochrome P450cam-based model (a) and cytochrome P450bm3-based model (b) of aromatase.](image)

![Fig. 5. Western blot analysis of microsomal preparations from CHO cells expressing aromatase and eight mutants. The procedures of preparing microsomes and Western blotting are described in "Materials and Methods." Lane 1, 0.9 µg of pure human placental aromatase as positive control; Lanes 2–10, wild-type, I133W, I133Y, F235L, I395F, I474M, I474N, I474W, and I474Y, approximately 30 µg of microsomal preparation each, respectively; Lane 11, 30 µg of microsomal preparation from untransfected CHO cells as negative control; Lane 12, 0.8 µg of pure human placental aromatase spiked into 10 µg of microsomal preparation of untransfected CHO cells.](image)
relatively low level, and the present purification methods for aromatase are complex and have low yields. Since we are not working with purified enzyme preparations, we will discuss the differences in Vmax values for the wild-type aromatase and the mutants with caution. In addition, the Vmax values when the assay was performed with microsomal preparations were often found to be lower and more variable than those determined using the in-cell assay. For example, we have prepared microsomes from the wild-type aromatase-transfected CHO cells five times, and the aromatase activity in these preparations ranged from 3 to 67 pmol/h/mg (27 ± 27 pmol/h/mg: Table 1). We assume that the decrease and the variability are a result of the instability of our microsomal preparations.

**Inhibitor Profile Studies.** We have made an extensive investigation of the interaction of different aromatase inhibitors with the above described mutants to test the accuracy of our computer model as well as to determine the binding characteristics of different steroidal and nonsteroidal inhibitors. As indicated in "Materials and Methods," the analyses were performed using the in-cell method. The aim has been to use results generated with steroidal inhibitors to refine our computer model and then explain results generated with nonsteroidal inhibitors using the refined model. The results are shown in Table 2 and will be discussed in detail in "Discussion." Through the inhibition profile studies, we have determined the relative potency of these seven inhibitors (according to their IC50 values for the inhibition of the wild-type aromatase), i.e., CGS 20267 (IC50 = 1.3 nM) > vorozole (IC50 = 2.5 nM) > MDL 101,003 (IC50 = 12 nM) > ICI D1033 (IC50 = 25 nM) > 4-OHA (IC50 = 60 nM) > 7α-APTADD (IC50 = 165 nM) > AG (IC50 = 6 μM).

Initially, we tried to perform inhibition profile studies using microsomal preparations of various mutants. However, due to inconsistent performance, we decided to carry out the study with the in-cell assay method because this gave more reproducible results. In addition, because of the problems associated with the in-cell assay (as discussed for the enzyme kinetic analysis) and possible differences in the inhibitory mechanism of different inhibitors (competitive versus noncompetitive inhibition), we decided not to determine the Ks value for each inhibitor to each different mutant with data obtained with the in-cell method. Instead, we are comparing them through their IC50 values, the concentrations needed to inhibit the mutants to 50%. In this way, regardless of the original binding affinity for the substrate for each mutant, we are comparing the relative binding affinity of each inhibitor and the substrate for each different mutant. We feel that this should be a valid analysis, free of the problems associated with the in-cell assay method. As indicated in Table 1, through assays on microsomal preparations, mutants I133Y, I133W, I395F, I474N, and D309A were found to have much larger Ks values than that of the wild-type aromatase (as shown in Table 2). We feel that this way, regardless of the original binding affinity for the substrate for each inhibitor to each different mutant with data obtained with the refined model, the results are shown in Table 2 and will be discussed in detail in "Discussion."

### Table 1: Kinetic constants of the CHO cell expressed wild-type aromatase and 11 mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>K&lt;sub&gt;ms&lt;/sub&gt; (nM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pmol/h/mg)</th>
<th>K&lt;sub&gt;ms&lt;/sub&gt; (nM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pmol/h/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>31 ± 6</td>
<td>137 ± 27</td>
<td>16 ± 5 (±&lt;sup&gt;st&lt;/sup&gt;5)</td>
<td>27 ± 27</td>
</tr>
<tr>
<td>I133Y</td>
<td>9 ± 2</td>
<td>3 ± 1</td>
<td>360 ± 210</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>I133W</td>
<td>15 ± 1</td>
<td>1 ± 0</td>
<td>310 ± 110</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>P235L</td>
<td>30 ± 19</td>
<td>36 ± 14</td>
<td>29 ± 10</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>I395F</td>
<td>5 ± 1</td>
<td>13 ± 1</td>
<td>530 ± 180</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>I474Y</td>
<td>12 ± 5</td>
<td>24 ± 5</td>
<td>14 ± 1</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>I474W</td>
<td>25 ± 4</td>
<td>9 ± 1</td>
<td>15 ± 8</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>I474M</td>
<td>10 ± 3</td>
<td>15 ± 3</td>
<td>25 ± 8</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>I474N</td>
<td>7 ± 1</td>
<td>35 ± 3</td>
<td>187 ± 14</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>I398F</td>
<td>16 ± 5</td>
<td>13 ± 4</td>
<td>47 ± 3</td>
<td>14 ± 0</td>
</tr>
<tr>
<td>D309A</td>
<td>23 ± 6</td>
<td>2 ± 0</td>
<td>345 ± 7</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>T310S</td>
<td>32 ± 6</td>
<td>24 ± 2</td>
<td>15 ± 2</td>
<td>4 ± 0</td>
</tr>
</tbody>
</table>

* The kinetic analyses were performed five times with five microsomal preparations of the wild-type aromatase-transfected CHO cells.

### Table 2: IC50 values for the inhibition of the wild-type aromatase and 11 mutants by 7 aromatase inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDL 101,003</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>4-OHA</td>
<td>2.5 ± 0.0</td>
</tr>
<tr>
<td>7α-APTADD</td>
<td>12.0 ± 0.1</td>
</tr>
<tr>
<td>AG</td>
<td>165 ± 6.0</td>
</tr>
<tr>
<td>ICI D1033</td>
<td>60 ± 0.0</td>
</tr>
<tr>
<td>CGS 20267</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Vorozole</td>
<td>60 ± 0.0</td>
</tr>
</tbody>
</table>

* Values are expressed in nM.

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wild-type aromatase, implying that the substrate binds to these mutants poorly. To verify our results of the inhibitor profile analysis generated with the in-cell method, we also determined the IC₅₀ values of MDL 101,003 (as an example of a steroidal inhibitor) and CGS 20267 (as an example of a nonsteroidal inhibitor) for inhibition of 1133Y, 1395F, and 1474N using assays on microsomal preparations of these mutants. The results generated (as shown in Table 2) are comparable to those derived from the in-cell method.

**DISCUSSION**

The catalytic properties of the mutants have been analyzed in relation to the computer models we generated. They are discussed for each individual mutated position as follows.

**1133Y and 1133W.** The eight mutants described in this study were designed based on an aromatase model generated 2 years ago. Based on the recently revised model, Ile-133 is predicted to lie close to the heme but not to form part of the active site cavity. Replacement by bulkier residues might be expected to require a displacement of the heme, which would no longer be optimally positioned for the redox reaction. This would account for the observation that both mutants show a greatly reduced Vₘₐₓ (as indicated by both in-cell and microsomal assays). The mutations are also observed to inhibit substrate binding (as indicated by a great increase in the Kₘ₋ₐp values for androstenedione for mutants 1133Y and 1133W, as determined using the microsomal assay method), but in the current model a direct interaction between this residue and the substrate is not predicted. Therefore, an indirect effect must be postulated, with residues in the vicinity of position 133 that do form part of the active site being displaced to allow for the more bulky side chain of the mutant. Alternatively, it could be hypothesized that the alignment requires an adjustment to place this residue in position 134 where it would then face the cavity and would interact with the β face of the D-ring of the substrate. This hypothesis would lead to the prediction that, with the area normally occupied by the D-ring of a steroid partially blocked, the mutants would show decreased sensitivity to inhibitors that do make use of this part of the active site cavity. There is no strong support for this from the IC₅₀ values (Table 2). Among the seven inhibitors used in this study, mutant 1133Y is slightly more resistant to ICI D1033 and CGS 20267, and mutant 1133W is slightly more resistant to 7α-APTADD than the wild-type enzyme. Results obtained from inhibition profile studies will be discussed further for each individual inhibitor.

In the analysis by Amarneh et al. (23), Phe-134 has been suggested to be an important residue that may interact with the C2 position of the steroid substrate. A mutant F134E was generated and analyzed. It was found to have a similar Kₘ and a higher Vₘₐₓ value than the wild-type enzyme (23). The fact that the kinetic analysis was performed using the in-cell method prevents a more definitive analysis of the results.

**F235L.** In the P450cam-based model of aromatase, Phe-235 lies at the carboxyl-terminal end of helix F and makes a major contribution to the extra hydrophobic pocket in the active site. However, in our previous study (13), the observation that the mutant F235N was inactive suggested that the alignment between aromatase and cytochrome P450cam was incorrect in this region, and that this residue played an important role in stabilizing the structure of the enzyme. The observation that the mutant F235L is active supports this conclusion, and the close similarity to wild-type enzyme in its spectrum of sensitivity to different inhibitors further supports the conclusion that this is a structurally conservative mutation. However, by the same token, the mutant gives little information as to the location of this residue in relation to the active site. It may be noted that this residue lies in the F- and G-helix region which proved so problematic in the cytochrome P450bm3-based structure prediction.

**I395F.** This residue was predicted to lie at the far end of the extra hydrophobic pocket, and therefore it was expected that the mutation would not affect substrate binding or catalytic activity to a great extent. However, in the microsomal assay, the mutant shows a very high Kₘ₋ₐp when compared to the wild-type enzyme, suggesting that Ile-395 may be more important for substrate binding than we had thought. A conversion of Ile-395 to Phe changes relative binding affinities for 4-OHA, 7α-APTADD, AG, and vorozole in comparing the binding of substrate. However, we do not have sufficient information to assign the residue in relation to the active site.

**1474Y, 1474W, and 1474M.** Residue Ile-474 is predicted to lie in the extra hydrophobic pocket. Mutations at this position would be expected to have a limited effect on substrate binding and the aromatization reaction, and to be resistant to inhibitors that make use of the extra pocket. In our previous study (13), the mutant 1474F was found to show some of these characteristics, but was unexpectedly sensitive to 7α-APTADD, despite its bulky 7α substituent. It was hypothesized that this might be due to favorable aromatic-aromatic interactions between the phenyl rings of the substituent and the mutated residue. The mutants analyzed here enable us to test this hypothesis. As before, the mutations show kinetic constants not far removed from the wild-type enzyme, although mutant 1474N shows a much increased Kₘ₋ₐp in the microsomal assay. The results with 1474N indicate that, as we suggest in a previous article (13), this residue is actually closer to the substrate than predicted by the computer model. Examining the sensitivity of the mutants to 7α-APTADD, an analogue of 7α-APTA with the same extended aromatic substituent in the 7α-position, it is pleasing to note that the profile of activity is in agreement with our previous hypothesis. The mutations that maintain the aromatic character of the residue at position 474 show increased sensitivity to 7α-APTADD (1474Y and 1474W), but this is abolished if the residue is bulky and hydrophobic but not aromatic (1474M) nearly as much as if it converted to a hydrophilic residue (1474N). The fact that no mutations at position 474 actually reduce the potency of inhibitors that are expected to make use of the extra hydrophobic pocket leads us to believe that this may be better regarded as a hydrophobic surface than a true pocket. This is in agreement with the observations that there appears to be no limit on the size of substituents that can be accommodated at position 474. Additional mutations in this region will be carried out to evaluate further the existence of an extra hydrophobic pocket or a hydrophobic surface.

**P308F.** A characteristic of cytochrome P450 structures is the distortion in the central region of the I-helix, with the formation of a cleft in the region of the heme iron. In the cytochrome P450cam-based model of aromatase, Pro-308 forms part of this cleft. The fact that this mutant shows kinetic constants very similar to the wild-type enzyme indicates that Pro-308 may not be directly involved in substrate binding. One unexpected observation is the remarkable sensitivity of this mutant to 7α-APTADD and 4-OHA. In the cytochrome P450cam-based model, Pro-308 is on the side of the I-helix remote from the substrate; therefore, mutations at this position are not expected to directly alter the structure of the portion of the active site interacting with the substrate. It would appear that the mutation produces some indirect effect on the active site structure which maintains the efficacy of the catalytic mechanism, but increases the binding of these two inhibitors (for further discussion see below).

**D309A.** Previous studies by us (8) and Amarneh et al. (23) have suggested that this mutant has major alterations in the active site geometry, and the inhibition data in this study support this. The mutant is remarkably resistant to all of the inhibitors except AG. The resistance to steroidal inhibitors may be associated with general steric
problems, but in the case of vorozole, ICI D1033, and CGS 20267 one could hypothesize that it is the loss of a specific interaction between the inhibitor and Asp-309 which is responsible for the resistance. Koymans et al. (44) have used a simple “I-helix model” to explain the superior inhibitory activity of S-vorozole over its enantiomer. In their model, the 1-methylbenzotiazole moiety in S-vorozole binds near Asp-309. Both ICI D1033 and CGS 20267 can be modeled in a similar orientation, in which one of their nitrile groups appears to be suitably placed to interact with the same residue. However, in the case of ICI D1033, accommodation of the bulky α-dimethyl group requires that the active site in this region be considerably larger than that currently modeled. In addition, since there is no particular affinity between the nitrile and carboxylate groups, we prefer an alternative explanation for the particular resistance of this mutant to these inhibitors. The effect of the D to A mutation at this position could be to cause a partial collapse in the active site cavity in a region of particular importance to such nonsteroidal inhibitors. This is discussed further below.

T310S. A threonine in a homologous position to Thr-310 in aromatase is observed in all of the known cytochrome P450 structures except cytochrome P450coryF. It appears to be involved in stabilizing the distortion in the I-helix by providing a replacement hydrogen bond to the carbonyl of residue Ala-306; in addition, it may act as a proton donor during the redox reactions, and from our model it may also interact with the C3-carbonyl group of the substrates. In view of the multiple roles this residue may play, it is not surprising that even the conservative mutation to serine results in a considerable reduction in $V_{\text{max}}$. As well as being involved in the mechanism of aromatization, the resistance of the mutant to 4-OHA and (to a lesser extent) the inhibition of MDL 101,003 to a very similar degree. This is supported by the observation that the binding affinity of the inhibitor for the active site, but also to alterations in the ability of the mutant enzyme to catalyze the reaction that produces the irreversible inhibition. For enzyme inhibition by mechanism-based inhibitors, including aromatase inhibition by 4-OHA, the irreversible inactivation step normally precedes at a significantly slower rate than the initial reversible binding step (48). Thus, the profile of inhibition should resemble ordinary competitive steroidal inhibitors. An interpretation of our results is that the presence of 4-hydroxyl group changes the binding orientation of the inhibitor in comparison to that of the substrate. Such a subtle change in its binding orientation may bring the sterol closer to Asp-309 and Thr-310. Conversion of Asp-309 to Ala and Thr-310 to Ser may bring a change in the size of the active site pocket, explaining the decreased potency for mutants D309A and T310S. The conformation near the active site region may be modified slightly, increasing the binding affinity and potency of 4-OHA for mutant P308F. However, it is also possible that the rate of irreversible inactivation step decreases upon mutations on aromatase. This could be the case for the mutant T310S. Thr-310 may be involved in the mechanism of suicide inactivation (as discussed above), and the mutation reduces the rate of the inactivation step gradually, resulting in a very large increase of the IC50 value (Table 2). This latter possibility certainly exists since the binding orientation of 4-OHA in the active site is not much different than that of the substrate (Fig. 6B), and stereohindrance cannot explain adequately the large increase in the IC50 value for T310S. Because the 4-hydroxyl group is in the direction of the extra hydrophobic pocket, the slight sensitivity to mutations of Ile-474 is inexplicable.

7α-APTADD. This is also a mechanism-based inhibitor with a $K_i$ value of 9.9 nm (49); therefore, the caveats discussed above for 4-OHA apply. The inhibitor is expected to adopt a conformation in the active site similar to that adopted by the substrate (Fig. 6C). In this orientation, aromatic-aromatic interaction between the phenyl rings of the 7a substituent and an aromatic residue at position 474 [i.e., I474Y, I474W, and I474F (13)] can explain the affinity of this inhibitor for these mutants. In addition, the binding of 7α-APTADD may differ from that of the substrate in having the D-ring closer to Ile-133 or Phe-134. This would account for a slight decrease in its binding affinity to I113W and I113Y in relation to the substrate (as indicated by increases in IC50 values).
Fig. 6. Models (ribbon plots) of the predicted orientations of seven aromatase inhibitors in the active site of aromatase. In these models, the substrate androstenedione is shown in green, and the heme is shown in red. A, MDL 101,003 (magenta); B, 4-OHA (magenta); and C, 7a-APTADD (magenta). In this plot, the side chain of Ile-133 is shown in its alternative position (see text for details); D, AG (magenta); E, vorozole (magenta); F, vorozole (cyan), CGS20267 (yellow), and ICI D1033 (magenta). The orientation of 2-phenylimidazole in the crystal structure of its complex with cytochrome P450cam is also shown (green); G, ICI D1033 (magenta); and H, a plot comparing the predicted orientations of vorozole (magenta), CGS20267 (yellow), and ICI D1033 (orange) in the active site.
AG. This inhibitor is thought to bind to the active site of the enzyme with its p-aminophenyl group directed toward the heme iron ($K_p = 1.37 \mu M$; Ref. 50). The length of this substituent is such that the glutarimide ring must lie about 2–3Å below the position occupied by ring A of a steroid substrate (Fig. 6D). The small size of this inhibitor limits the interactions possible with the relatively large active site of the enzyme, accounting for its relatively high IC$_{50}$ value (Table 2). However, the same characteristic of the inhibitor reduces its sensitivity to changes in the active site brought about by mutations, and reductions in the IC$_{50}$ values for certain mutants may be as much a result of improved binding of the inhibitor to an active site of reduced volume as of impaired binding of the bulkier substrates. Evidence that AG does indeed occupy portions of the active site not used by the substrates, i.e., that the glutarimide is not a perfect mimic of a steroid A ring, comes from the observation that certain mutations, e.g., F235L, I474Y, I474W, and I474M, that appear to have little effect on the binding of the substrate to the active site (on the basis of their $K_m$ values) would appear to induce tighter binding of AG (on the basis of reduced IC$_{50}$ values). The enhanced inhibitory activity of AG against the mutants T310S or D309A may be associated with the removal of a sterically clash between the threonine methyl group and the other to occupy a region of space close to that occupied by the chlorophenyl and cyanophenyl groups of vorozole and CGS 20267, respectively. However, this orientation results in a rather different positioning of the triazole substituent with respect to the heme than for the other two inhibitors (Fig. 6F and G). An alternative orientation (Fig. 6H) provides a better overlap with the other nonsteroidal inhibitors, but provides no mimicry of steroidal C- and D-rings. In these orientations, all three inhibitors protrude into a region below the A ring of a steroid substrate in the direction of Asp-309 (Fig. 6H). In accordance with their observed sensitivity to the mutation D309A, we hypothesize that this change to a smaller nonpolar residue results in the collapse of the active site cavity in this region, hindering the binding of these inhibitors. The fact that ICI D1033 shows the greatest sensitivity to this mutation leads us to prefer the second alternative orientation of this inhibitor in the active site; as in the case of the three inhibitors, it places the greatest bulk in this region. Unlike 7α-APTADD, the sensitivity of these nonsteroidal inhibitors to mutations at Ile-474 shows a dependence only on bulk, and again supports the view that these inhibitors do not position one of their aromatic substituents in the extra hydrophobic pocket, but that mutations which partially fill it help to improve general van der Waals interactions between the active site and the inhibitors. All three inhibitors are oriented to have an aromatic ring perpendicular to the A ring of a steroid and in part above it (Fig. 6H). The potential for clashes between the edge of this ring and the methyl group of Thr-310 may explain, as for AG, the sensitivity of the mutant T310S for these inhibitors.

Among the three inhibitors, CGS 20267 and vorozole inhibit the wild-type aromatase with similar IC$_{50}$ values, agreeing with our model that these compounds bind to the active site in similar fashions. Inevitably, in view of its rather different structure, the binding of ICI D1033 is certainly different from the other two inhibitors, resulting in a larger IC$_{50}$ value for its inhibition of the wild-type aromatase. However, its profile of activity against the panel of mutant enzymes bears a close resemblance to that of the other two inhibitors.

Although our recent computer modeling of the structure of aromatase based on the X-ray structure of cytochrome P450b53m3 turned out to be problematic, the recent set of site-directed mutagenesis experiments has generated additional information concerning the active site and proposed hydrophobic pocket of the enzyme. Furthermore, even though several important regions of aromatase have been recognized by both our computer modeling and that recently published by Graham-Lorence et al. (24), a few disagreements still exist between the two models from the two groups. Additional mutagenesis experiments will be performed to evaluate the differences. On the other hand, we believe that our inhibition profile studies are very important. For the first time, we have provided a molecular basis regarding how seven aromatase inhibitors with different structures bind to the active site of aromatase. It is most interesting to find that the models generated based on our enzyme mutagenesis studies agree well with those generated based on the conformational analysis of the molecular structures of inhibitors (16, 53).

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