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KINETIC PROPERTIES OF AROMATASE MUTANTS Pro308Phe, Asp309Asn, AND Asp309Ala AND THEIR INTERACTIONS WITH AROMATASE INHIBITORS

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Summary—Mutant forms of aromatase cytochrome *P*-450 bearing modifications of amino acid residues Pro308 and Asp309 and expressed in transfected Chinese hamster ovary cells were subjected to kinetic analysis and inhibition studies. The K_m for androstendione for expressed wild type (11.0 ± 0.3 nM SEM, $n = 3$) increased 4-, 25- and 31-fold for mutants Pro308Phe, Asp309Asn and Asp309Ala, respectively. There were significant differences in sensitivity among wild type and mutants to highly selective inhibitors of estrogen biosynthesis. 4-Hydroxyandrostenedione (4-OHA) a strong inhibitor of wild type aromatase activity ($IC_{50} = 21$ nM and $K_i = 10$ nM), was even more effective against mutant Pro308Phe ($IC_{50} = 13$ nM and $K_i = 2.8$ nM), but inhibition of mutants Asp309Asn and Asp309Ala was considerably less ($IC_{50} = 345$ and 330 nM and $K_i = 55$ and 79 nM, respectively). Expressed wild type aromatase and Pro308Phe aromatase were strongly inhibited by CGS 16949A ($IC_{50} = 4.0$ and 4.6 nM, respectively) whereas mutants Asp309Asn and Asp309Ala were markedly less sensitive ($IC_{50} = 140$ and 150 nM, respectively). CGS 18320B produced similar inhibition. Kinetic analyses produced $K_i = 0.4$ nM for CGS 16949A inhibition of wild type versus 1.1, 37 and 58 nM, respectively, against Pro308Phe, Asp309Asn and Asp309Ala. The results demonstrate significant changes in function resulting from single amino acid modifications of the aromatase enzyme. Our data indicate that mutation in Asp309 creates a major distortion in the substrate binding site, rendering the enzyme much less efficient for androstenedione aromatization. The substitution of Pro308 with Phe produces weaker affinity for androstenedione in the substrate pocket, but this alteration favors 4-OHA binding. Similarly, mutant Pro308Phe exhibits a slightly greater sensitivity to inhibition by CGS 18320B than does the wild type. These results indicate that residues Pro308 and Asp309 play critical roles in determining substrate specificity and catalytic capability in aromatase.

INTRODUCTION

Estrogen biosynthesis is catalyzed by an enzyme complex located in the endoplasmic reticulum of cells from a number of estrogen-producing tissues, principally the ovarian granulosa in premenopausal women, the placenta in pregnant women and adipose tissue in post-menopausal women. The heme protein termed aromatase cytochrome *P*-450, in concert with a flavoprotein, NADPH–cytochrome *P*-450 reductase, catalyzes the three monooxygenation steps necessary for the bioconversion of C_{19}-androgens to aromatic C_{18}-estrogens. Even though the steroid reaction pathway and stereochemistry of aromatization have been studied extensively by numerous investigators, we are just beginning to understand the precise mechanism from the standpoint of the molecular structure of aromatase cytochrome *P*-450 and the indentification of active site residues involved in substrate binding and catalysis [1–3].

Detailed structural analysis and characterization of aromatase have for many years been precluded by its instability and intractability to purification due to its particulate nature. However, recent attempts at characterization of aromatase structure have been greatly aided by the isolation of human placental aromatase cDNA and its insertion into expression vectors [4, 5]. Subsequently, the generation of mutant forms of aromatase by site-directed mutagenesis has made possible the evaluation of amino acid residues in aromatase that may play key roles in substrate specificity and enzyme activity [1–3]. In this approach, computer

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modeling was initially used to align segments of homologous amino acid sequences in aromatase cytochrome *P*-450 with those of cytochrome *P*-450cam for which the three-dimensional polypeptide conformation of the active site has been resolved by X-ray crystallography [6]. The putative substrate binding pocket of aromatase cytochrome *P*-450, enclosed by the heme-binding region and hydrophobic I-helix, was determined from the alignment with cytochrome *P*-450cam and comparison with other steroidogenic cytochrome *P*-450s. The sterically best-fit orientation of the androstenedione molecule produced a three-dimensional structure with critical contact points located within a segment between residues 302 to 312 of the I-helix [2, 3].

Introduction of specific base changes by site-directed mutagenesis yielded mutant forms of aromatase. Based on changes in kinetic parameters following modification, residues determined to be important for substrate binding and catalysis include Glu302, Pro308, Asp309 and Ser312 [1–3]. These studies of the structure–function relationship were performed in cell culture. Aromatase activity measured by such "in cell" assays is subject to a number of modulating factors including endogenous steroid content, co-factor requirements and competing metabolic pathways. In the present study, we report the kinetic properties of mutant aromatases [1, 2] with emphasis on amino acid substitutions Pro308Phe, Asp309Asn and Asp309Ala. Microsomal preparations rather than intact cells were used as enzyme source. The effects of specific amino acid modifications within the active site region of aromatase were studied in terms of changes in kinetic parameters and sensitivity to aromatase-specific inhibitors. The results are compared to those obtained by the "in cell" assay.

EXPERIMENTAL

Chemicals and cell culture reagents

Androstenedione, progesterone, estrone and estradiol were purchased from Steraloids, Inc. (Wilton, NH), [1β-^{3}H]androstenedione from Dupont, New England Nuclear (Boston, MA) and [4-^{14}C]estrone, and [4-^{14}C]estradiol from Amersham (Arlington Heights, IL). Culture media, serum, trypsin and antibiotics were obtained from Gibco (Grand Island, NY). Bovine serum albumin (BSA), EDTA, dithiothreitol (DTT) and reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma (St Louis, MO). Micro BCA kit for protein determination was purchased from Pierce (Rockford, IL). All other reagents were of analytical grade. Aromatase inhibitors 4-hydroxyandrostenedione (4-OHA), aminoglutethimide (AG), 4-(5,6,7,8-tetrahydroimidazo[1,5a]pyridin-5-yl)benzonitrile hydrochloride (CGS 16949A) and bis-(*p*-cyanophenyl)imidazo-1-yl methane hemisuccinate (CGS 18320B) were gifts from Ciba-Geigy Ltd (Basel, Switzerland) and Ciba-Geigy Corp. (Summit, NJ). 6α-Bromoandrostenedione and 6β-bromoandrostenedione were synthesized by bromination of androstenedione with *N*-bromosuccinimide [7].

Aromatase expression, transfection and mutagenesis

The construction of aromatase expression plasmid (pH β-Aro) containing human placental aromatase cDNA, transfection into host Chinese hamster ovary (CHO) cells by a stable expression method and the generation of site-specific mutant aromatases were described previously [1, 2, 5]. The mutants studied were: Pro308Phe, Asp309Asn and Asp309Ala. CHO cells expressing wild type aromatase or transfected with a control plasmid were also investigated.

Cell culture and preparation of microsomes

CHO cells were grown in Dulbecco's Modified Eagle Medium–Nutrient Mixture F-12 (Ham)1:1 (D-MEM/F-12) medium containing 10% fetal bovine serum, 0.6 mg Geneticin/ml, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), 1 mM D-MEM sodium pyruvate, 0.1 mM D-MEM non-essential amino acids, 100 U penicillin G/ml, 100 μg streptomycin sulfate/ml and 0.25 μg amphotericin B/ml. Cells were harvested by trypsinization and stored at −80°C until use. Approximately 2×10^9 cells were thawed and resuspended in 67 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.24 M sucrose and 0.5 mM DTT. The cells were disrupted by sonication in a Branson Sonifier, Cell Disruptor 200 (Danbury, CT). Microsomes were prepared as described previously [8]. The final microsomal pellet was suspended in 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.5 mM DTT and stored at −80°C.

Aromatase and other assays

Aromatase assays were based on [^{3}H]water release (1β elimination mechanism, with 75%

^3H release into water) from [1β-^3H]androstenedione (8.8×10^7 dpm per μg) [9–11]. Microsomal preparations were used as the enzyme source. Substrate concentrations were: 150 nM for wild type and mutant Pro308Phe microsomes, 300 nM for mutant Asp309Asn microsomes, and 750 nM for mutant Asp309Ala and control plasmid-transfected CHO cell microsomes, according to their previously determined K_m values. An ethanolic solution of the substrate plus 10 μM progesterone (to inhibit [^3H]water release via the 5α-reductase pathway) [12, 13] was dried under nitrogen in the presence of 20 μl propylene glycol. Subsequent additions were: 67 mM potassium phosphate buffer, pH 7.4, 0.1% BSA, and microsomes, 5 (wild type) to 170 μg (mutant Asp309Ala) protein, depending on specific activity. Control samples contained BSA in amounts equivalent to protein content of the microsomes. An incubation mixture in which NADPH co-factor was omitted also served as negative control. Following a 10 min pre-incubation at room temperature, the reaction was started by the addition of NADPH (1.2 mM final concentration). The total volume was 0.5 ml and the incubation was performed in a shaking water bath at 37°C. The reaction was terminated by the addition of 0.5 ml of 5% trichloroacetic acid. After charcoal absorption and filtration through cotton-plugged pipet, [^3H]water release was quantitated by liquid scintillation counting with Ultima Gold cocktail in a Packard Instruments Model 4640 Spectrophotometer. Protein determinations were carried out using Micro BCA Protein Assay Reagent with BSA as a standard.

Product isolation method

Verification of [^3H]water assay was performed on microsomes prepared from CHO cells transfected with wild type aromatase cDNA. The tritiated water method [9–11] uses [1β-^3H]-androstenedione (75% 1β and 25% 1α) as substrate and quantitates 1β elimination, leaving the 1α tritium intact for product isolation identification. Microsomes (1.05 mg protein) were pre-incubated with [1β-^3H]androstenedione (1.18×10^7 dpm ^3H, 96 nM), bovine liver NADPH–cytochrome *P*-450 reductase (27 μg), and 0.25% BSA in 1.8 ml of 67 mM potassium phosphate buffer, pH 7.4, containing 0.005% Emulgen at room temperature for 10 min. The incubation was initiated by the addition of NADPH (2 mg/0.2 ml) and continued for 5, 10 and 20 min at 37°C. The incubation was terminated and the metabolites extracted by the addition of ethyl acetate–hexane (1:1) containing authentic standard steroids and [4-^{14}C]estrone and [4-^{14}C]estradiol as tracers. The organic extracts were evaporated and residue subjected to two-dimensional thin layer chromatography (TLC): 1st, cyclohexane–ethyl acetate (2:1, developed 3 times) and 2nd, chloroform-methanol (95:5). Following another TLC in benzene–ethanol (9:1), products were recrystallized to constant $^3H/^{14}C$ ratio with authentic steroid and quantitated by double label isotope counting. The aqueous phase of the extracted incubation was shaken with 5% charcoal suspension for 30 min, filtered through a cotton plug, then counted for ^3H release into water.

RESULTS

Validation of expressed aromatase activity

Microsomes prepared from CHO cells transfected with only the expression vector exhibited virtually no aromatase activity. The level of expressed wild type aromatase in microsomal preparations (770–1050 fmol · min^{-1} · mg protein^{-1}) was 3- to 4-fold lower than that measured in intact cells in culture by the [^3H]water method [5]. The [^3H]water assay in the present system was validated by the product isolation methods. As shown in Fig. 1, wild type microsomal aromatase activity measured by [^3H]water release showed a correlation with estrogen formation. The enzyme exhibited normal Michaelis–Menten kinetics with K_m values of 9.3–12.2 nM for androstenedione. The turnover rate (defined as moles of estrogen synthesized per min per mol of aromatase cytochrome *P*-450) of wild type microsomes

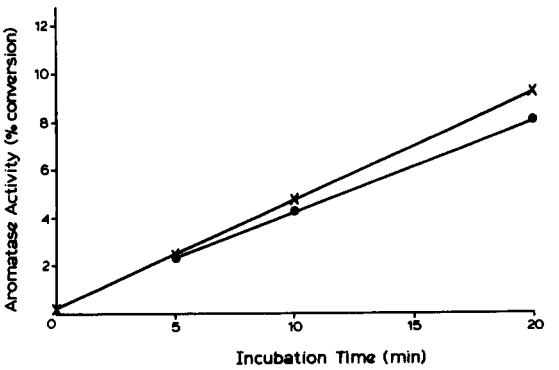

Fig. 1. Comparison of aromatase activity determined by the [^3H]water release assay and product isolation method. Wild type microsomes were incubated with [1β-^3H]androstenedione and processed as described in Experimental. [^3H]Water released (●), isolated estrogens (estrone plus estradiol) (×).

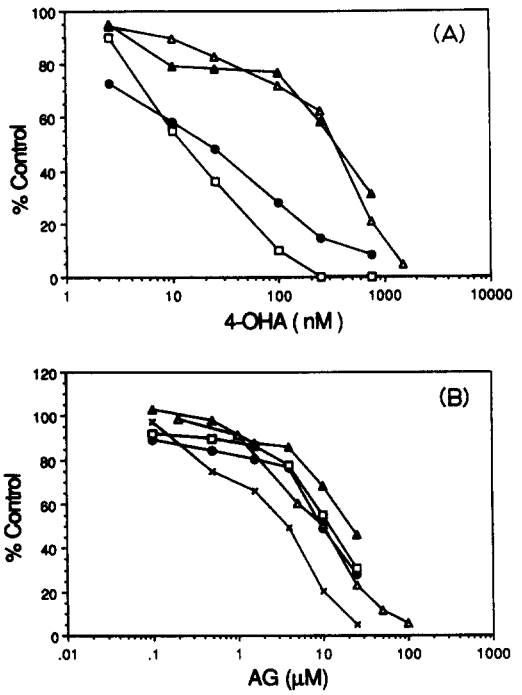

Fig. 2. Inhibition of expressed aromatase by 4-OHA (A) and AG (B). Wild type (●), Pro308Phe (□), Asp309Asn (▲), Asp309Ala (△), Asp309Ala assayed in medium containing rate-limiting concentration (43% K_m value) of androstenedione (×).

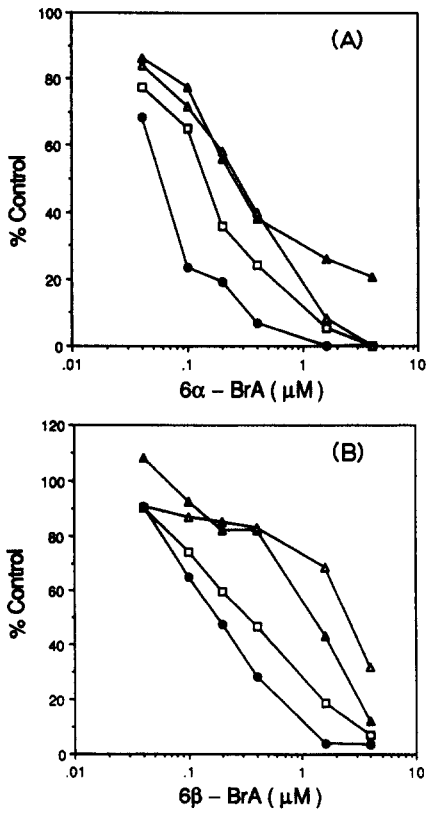

Fig. 3. Inhibition of expressed aromatase by 6α-BrA (A) and 6β-BrA (B). Wild type (●), Pro308Phe (□), Asp309Asn (▲), Asp309Ala (△).

was 1.77 min^{-1}. Thus, despite its relatively low activity, the expressed aromatase retained catalytic efficiency of the same order as that reported for placental microsomes [8].

Response to selective inhibitors of aromatase

Inhibition profiles for wild type and mutant aromatases were compared using known inhibitors of aromatase activity. The inhibitory effects of 4-OHA and AG are shown in Fig. 2. Mutant Pro308Phe aromatase was slightly more sensitive to 4-OHA than was the wild type enzyme and both Asp309Asn and Asp309Ala were significantly less sensitive [Fig. 2(A)]. At a concentration of 4-OHA (21 nM) which produced 50% inhibition of wild type aromatase, Asp309Asn aromatase was inhibited 20% and Asp309Ala aromatase showed 16% inhibition. In the inhibition patterns against AG shown in Fig. 2(B), mutant Asp309Ala is represented by two curves. When initially determined in standard incubations containing 150 nM androstenedione as substrate, Asp309Ala aromatase was more sensitive to AG as compared to wild type. Upon our subsequent discovery that the K_m for mutant Asp309Ala aromatase is unusually high (in the range of 330–370 nM compared to 9–12 nM for wild type aromatase and more than 2-fold greater than the 150 nM substrate concentration used), the inhibitory effect of AG was re-assessed in the presence of 750 nM androstenedione. The overall results show that the inhibitory response of mutant aromatase does not differ significantly from wild type, except for Asp309Asn which was slightly less sensitive. All mutant forms were inhibited less than the wild type enzyme by both 6α-BrA and 6β-BrA, as shown in Fig. 3. In particular, inhibition of Asp309Ala by 6β-BrA differed

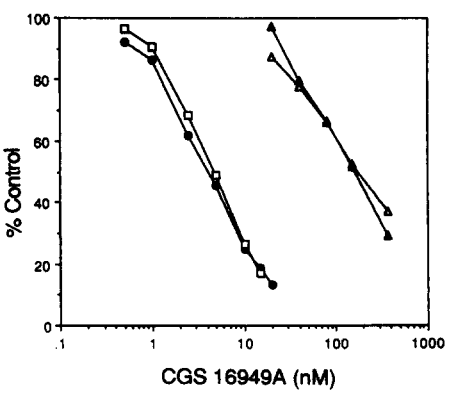

Fig. 4. Inhibition of expressed aromatase by CGS 16949A. Wild type (●), Pro308Phe (□), Asp309Asn (▲), Asp309Ala (△).

from the wild type by at least one order of magnitude.

A highly potent and specific aromatase inhibitor, CGS 16949A, was also evaluated and the results shown in Fig. 4. The profiles show a clear difference between the inhibition of wild type aromatase and aromatase containing modified amino acids at residue 309 i.e. Asp309Asn and Asp309Ala. Mutant Pro308Phe responded almost identically with the wild type. The approximate IC_{50} value for CGS 16949A inhibition against the expressed wild type aromatase was 4 nM, in agreement with the value of 5 nM previously obtained against human placental microsomes [14]. In contrast, the corresponding values for mutants Asp309Asn and Asp309Ala were 140 and 150 nM, respectively. At a 20 nM concentration, CGS 16949A produced 90% inhibition of expressed wild type aromatase but little or no inhibition was observed against the Asp309 modified mutant aromatase. A second imidazole derivative, CGS 18320B, reported to be more potent and more selective for inhibiting estrogen biosynthesis than CGS 16949A, gave similar results (profile not shown).

Table 1 presents a summary of the IC_{50} values of the tested aromatase inhibitors. Compared to the wild type, the IC_{50} of 4-OHA inhibition was 16 times greater for mutants Asp309Asn and Asp309Ala but 38% less for mutant Pro308Phe. Increases in IC_{50} values were characteristic of 6α and 6β-BrA inhibition of all mutant forms of aromatase tested, particularly Asp309Asn and Asp309Ala against 6β-BrA (7- and 13-fold increase over wild type, respectively). The inhibition by 6α-BrA, a competitive inhibitor, was similar for Asp309Asn and Asp309Ala whereas Asp309Asn was almost twice as sensitive as Asp309Ala to inhibition by 6β-BrA, a mechanism-based irreversible inhibitor [15]. In the case of AG inhibition, Asp309Ala exhibited increased sensitivity ($IC_{50} = 3.6\ \mu M$, not shown in Table 1) but only under conditions in which the androstenedione concentration was limiting (approx. 43% of K_m, see Fig. 2). In the presence of excess androstenedione (750 nM), the Asp309Ala aromatase was no longer more sensitive than wild type enzyme ($IC_{50} = 10.0$ vs $9.7\ \mu M$). All mutant aromatases were similar to wild type except for Asp309Asn for which there was an approximate doubling of the IC_{50}. Inhibition of mutant Pro308Phe aromatase by CGS 16949A was not significantly different from the wild type ($IC_{50} = 4.6$ and 4.0 nM, respectively). On the other hand, the substitution of Asp309 residue with Asn or Ala caused 35- and 37.5-fold decreases, respectively, in inhibitory potency of CGS 16949A. Against the wild type aromatase, CGS 18320B was slightly more effective than CGS 16949A ($IC_{50} = 2.6$ vs 4.0 nM). The relative potency of CGS 18320B was also 18% greater against mutant Pro308Phe than it was against wild type. Like CGS 16949A, CGS 18320B was a weak inhibitor of mutants Asp309Asn and Asp309Ala with IC_{50} values that are 48 and 127 times greater than the wild type control.

Inhibition kinetics of expressed mutant aromatase

As in the inhibition experiments, kinetic evaluation of expressed wild type and mutant aromatase was performed using [1β-^{3}H]androstenedione as substrate and microsomes from the transfected CHO cells as enzyme source. Figures 5 and 6 show representative kinetic analyses exhibiting changes resulting from site-directed mutagenesis. Figure 5 shows typical inhibition kinetics by 4-OHA of expressed wild type aromatase and mutant Asp309Ala aromatase. For the expressed wild type aromatase [Fig. 5(A)], the K_m for androstenedione was 11 nM and inhibition by 4-OHA was competitive with an apparent K_i of 10 nM. For Asp309Ala aromatase [Fig. 5(B)], the K_m was 345 nM and its V_{max} was 57 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1}, considerably lower than the V_{max} of 924 fmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of the wild type aromatase [Fig. 5(A)]. The inhibition of Asp309Ala by 4-OHA was competitive with an apparent K_i of 79 nM. The kinetics of inhibition by CGS 16949A are shown in Fig. 6 for mutant Pro308Phe and Asp309Asn aromatase. Pro308Phe exhibited a dose response to CGS 16949A similar to that of the wild type.

Table 1. IC_{50} of aromatase inhibitors against androstenedione aromatization by wild type and mutant aromatases

Expressed aromatase	4-OHA (nM)	AG (μM)	6α-BrA (nM)	6β-BrA (nM)	CGS 16949A (nM)	CGS 18320B (nM)
Wild type	21	9.7	60	180	4.0	2.6
Pro308Phe	13	12.0	145	340	4.6	2.2
Asp309Ala	330	10.0	260	2370	150	330
Asp309Asn	345	21.2	255	1250	140	125

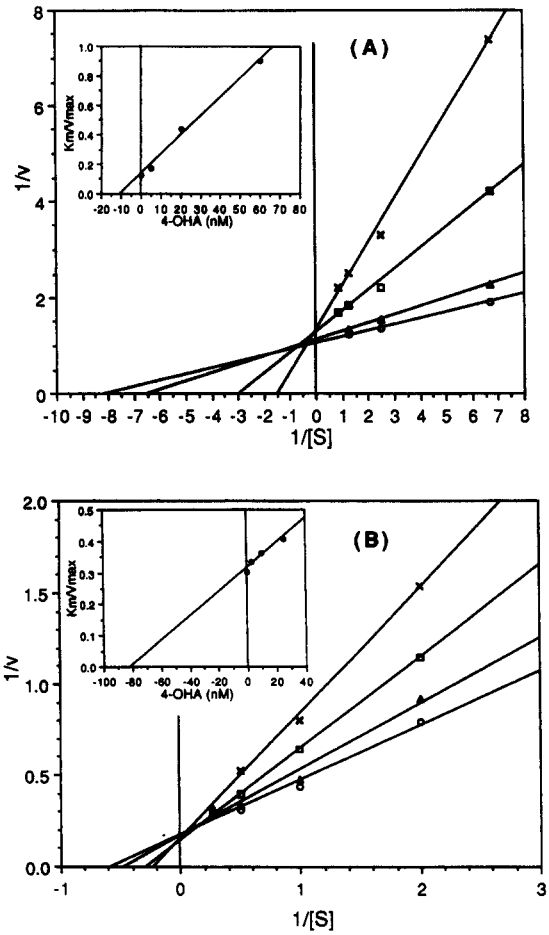

Fig. 5. Kinetics of inhibition of wild type (A) and Asp309Ala (B) aromatase by 4-OHA. Double reciprocal plots are shown with derivations of K_i in inset. Inhibitor concentrations were 0 nM (○), 5 nm (▲), 20 nM (□), and 60 nM (×) in (A) and 0 nM (○), 2.5 nM (▲), 10 nM (□) and 25 nM (×) in (B).

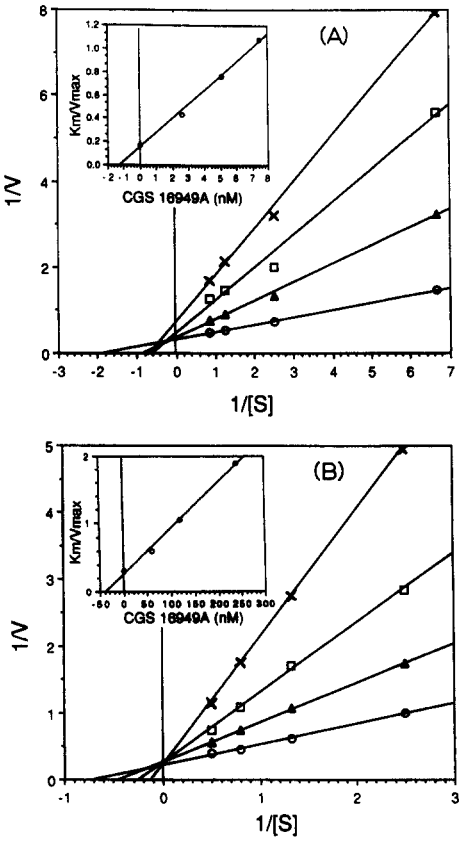

Fig. 6. Kinetics of inhibition of Pro308Phe (A) and Asp309Asn (B) aromatase by CGS 16949A. Double reciprocal plots are shown with derivations of K_i in inset. Inhibitor concentrations were 0 nM (○), 2.6 nM (▲), 5.1 nM (□) and 7.5 nM (×) in (A) and 0 nM (○), 60 nM (▲), 120 nM (□) and 240 nM (×) in (B).

Asp309Asn exhibited a dose response to CGS 16949A similar to that of Asp309Ala (Fig. 4). The K_m for androstenedione aromatization exhibited by Pro308Phe was 47 nM. Inhibition by CGS 16949A was competitive, with an apparent K_i of 1.1 nM. Corresponding values for Asp309Asn were K_m of 279 nM and an apparent K_i of 37 nM.

A summary of the kinetic properties is presented in Table 2. The K_m and V_{max} values for androstenedione aromatization indicate that substitution of aspartate residue at 309 in the aromatase P-450 has a profound effect on substrate affinity and enzyme activity. In particular, mutant Asp309Ala exhibited a 31-fold higher K_m than the wild type value of 11.0 nM, while its activity, reflected by V_{max}, was only 6% of that of wild type. Mutant Pro308Phe showed a smaller change in K_m (approximately 4-fold increase) but its V_{max} was only 9% compared to the wild type. However, Pro308Phe exhibited a 61% increase in sensitivity to inhibition by 4-OHA in terms of IC_{50} (13 vs 21 nM, Table 1) and a 3.9-fold increase in terms of K_i (2.8 vs 10.0 nM, Table 2). Relative to their respective K_m, K_i/K_m ratio of 0.06 for Pro308Phe and 0.91 for wild type represent a significant difference in the inhibitory effect of 4-OHA. A comparison of Asp309Asn and Asp309Ala indicates that substitution of Ala for Asp at residue 309 produced a less functional aromatase for androstenedione aromatization (V_{max} of 57 fmol · min^{-1} · mg^{-1}) than did the replacement of Asp with Asn (V_{max} of 198 fmol · min^{-1} · mg^{-1}). In terms of IC_{50}, Asp309Ala and Asp309Asn exhibited similar degrees of sensitivity to 4-OHA and CGS 16949A (Table 1). In terms of K_i, however, 4-OHA and CGS 16949A were weaker inhibitors of Asp309Ala than of Asp309Asn. The K_i for CGS 16949A inhibition was also greater against Asp309Ala than against Asp309Asn. The K_i for the AG inhibition of Asp309Ala (4.5 μM) was

Table 2. Kinetic parameters for wild type and mutant aromatases

Expressed aromatase	K_m for A[a] (nM)	V_{max} (fmol · min^{-1} · mg^{-1})	K_i for 4-OHA (nM)	K_i for AG (μM)	K_i for CGS 16949A (nM)
Wild type	11.0 ± 0.3[b]	924 ± 67[b]	10.0	1.3	0.4
Pro308Phe	47 ± 3	87 ± 5	2.8	2.7	1.1
Asp309Asn	279 ± 6	198 ± 10	55	20.0	37
Asp309Ala	345 ± 7	57 ± 2	79	4.5	58

[a]A = androstenedione.
[b]All values for K_m and V_{max} represent mean ± SEM for 3 determinations.

smaller than the K_i for AG against Asp309Asn (20.0 μM). Relative to their respective K_m, K_i/K_m values of 0.07 for Asp309Asn and 0.013 for Asp309Ala, compared to 0.12 for wild type, suggest that the interaction of AG with the catalytic site is affected by Ala substitution of Asp309 more profoundly than by Asn replacement. The altered inhibitory responses observed in these experiments indicate that the single amino acid substitutions in aromatase result in significant changes in the way the enzyme functions and inhibitors interact.

DISCUSSION

Despite recent successes in the purification of aromatase to homogeneity, its hydrophobic nature has precluded crystallization for three-dimensional structural determination by X-ray crystallography. The only successful resolution of a cytochrome *P*-450 three-dimensional structure reported to date has been for the soluble cytochrome *P*-450cam [6]. For the analysis of structure–function relationship, several groups have recently used computer modeling of three-dimensional conformation based on the cytochrome *P*-450cam structure, together with site-directed mutagenesis [1–3]. In these studies, single amino acid-substituted mutant forms of expressed aromatase were probed in terms of altered kinetic properties.

The investigation reported herein has been performed using microsomal preparations of the CHO cells transfected with the expression plasmid containing human placental aromatase cDNA. The K_m value for androstenedione for expressed wild type aromatase activity contained in microsomal preparations was 9.3–12.2 nM (Table 2), which was comparable to previously reported values of 10 nM for human placental microsomal aromatase and 12 nM for immunoaffinity purified and reconstituted aromatase [8]. The K_m values for wild type aromatase previously measured using whole cultured cells were 40–60 nM [1, 2]. Whereas culture-derived K_m values for androstenedione for expressed mutant Asp309Asn and Asp309Ala aromatases were 31 and 23 nM, respectively [2], the corresponding microsomal activities measured in the present study gave a range of K_m values of 266–294 and 333–394 nM, respectively. Thus, there appear to be major differences in the kinetic properties when aromatase activity is assayed in cell culture or in microsomal preparations. In an earlier study using the cell culture assay [5], it was observed that the wild type enzyme expressed in three different host cells responded differently to aromatase inhibitors. These observations, taken together with discrepancies with our *in vitro* K_m values, are consistent with intracellular modulation of the enzyme-substrate or *P*-450 reductase–aromatase interactions. Results have been obtained indicating that the rate of product formation can be altered varying the *P*-450 reductase/aromatase ratio (unpublished data). *In vitro* assays using microsomal preparations have the advantage of permitting investigation of direct enzyme-substrate–inhibitor interactions without the influence of endogenous substances or *P*-450 reductase. On the other hand, the "in cell" assay may better approximate the *in vivo* situation.

Based on computer modeling data [1–3], amino acid residues Pro308 and Phe309 in aromatase are situated in the bend of the α-helix adjacent to the heme prosthetic group and are part of the substrate binding site. Kinetic properties of expressed aromatase in intact cells in culture indicated that, despite having less than one-tenth of the wild type activity, mutant Pro308Phe has a lower K_m (40.0 nM) than the wild type (50.2 nM), and is more sensitive to 4-OHA inhibition than the wild type [1]. The present results based on microsomal aromatase activity confirm that Pro308Phe is more sensitive to 4-OHA. However, in contrast to "in cell" findings, the K_m for aromatization of androstenedione increased in Pro308Phe (47 ± 3 nM SEM compared to the wild type 11.0 ± 0.3 nM SEM) (Table 2). These current observations indicate that the altered conformation adopted by Pro308Phe has a weaker affinity for androstenedione

and yet is 3.5-fold more sensitive to 4-OHA inhibition. The Pro308-induced bend in the α-helix is presumably required for an optimal fit of androstenedione. A distortion of the geometry of this bend by the substitution of Pro with Phe appears to favor 4-OHA binding.

The present experiments have revealed major differences in kinetic parameters between Asp309-altered mutant aromatases and the wild type enzyme, which the "in cell" method [2] did not show. The K_m values of 30.9 and 23.2 nM previously reported for Asp309Asn and Asp309Ala, respectively, were lower than the K_m of 61.5 nM for the wild type [2] and differ from the "*in vitro*" values presented herein by one order of magnitude. Large decreases of V_{max} values for Asp309Asn and Asp309Ala were observed in both types of assay. The substantial losses in enzyme activity in Asp309 mutants is more consistent with markedly increased K_m values measured in our microsomal preparations and is difficult to reconcile with the apparent decrease in K_m values obtained in culture.

CGS 16949A is a highly potent and selective inhibitor of aromatase [14, 16, 17]. Our data (Fig. 4 and Tables 1 and 2) show that the sensitivity of the expressed wild type aromatase to this inhibitor was similar to that determined for human placental aromatase with respect to IC_{50} (4.0 vs 5 nM) [14] and K_i (0.4 vs 0.35 nM) [17]. As indicated in Fig. 4 and Table 1 mutant Pro308Phe was unchanged from wild type, but substitution of Asp309 with either Asn or Ala produced drastic changes in the response to CGS 16949A. The IC_{50} values against Asp309Asn and Asp309Ala reflected 35- and 37.5-fold increases, respectively, over the wild type control aromatase. This difference, together with the marked differences between the wild type and these Pro309 mutant aromatases with respect to their K_ms and K_is for CGS 16949A inhibition (Table 2), clearly demonstrates significant functional changes resulting from these single amino acid substitutions.

Amino acid residue Asp251 in cytochrome *P*-450cam, considered to be equivalent to Asp309 in aromatase [1, 3], forms ion pairs with Lys178 and Arg186 to stabilize the oxygen pocket [6]. If the extrapolation from cytochrome *P*-450cam to aromatase proves valid, it is reasonable to assume that Asp309 fulfills a similar role in aromatase cytochrome *P*-450. Our findings that the substitution of Asp309 with Asn or Ala produces a 25- and 31-fold increase in K_m and marked reductions in activity can be explained by the elimination or impairment of polar interaction with basic group(s) contributing to the stability of substrate pocket. Consistent with this interpretation is the finding of a moderate difference between the inhibitory effects of 4-OHA, a substrate analog, on Asp309Asn (K_i of 55 nM) and Asp309Ala (K_i of 79 nM) (Table 2). The large increase in K_m and the reduced ability to inhibit mutants Asp309Asn and Asp309Ala shown here by the steroid analogs (4-OHA, 6α-BrA, 6β-BrA) are consistent with a major distortion of the substrate binding site. In addition, the relative ineffectiveness of the CGS compounds against Asp309 mutants could reflect a major change in the orientation of the active site in relation to the heme prosthetic group. Since the wild type and mutant aromatase are expressions of placental cDNA, the possibility remains that the stereochemical consideration of the substrate binding site may not be directly extrapolatable to ovarian or adipose aromatase.

Assays in cell culture indicated that mutant Asp309Ala was significantly more sensitive than wild type to inhibition by AG [2]. The *in vitro* measurements, conducted in the present study using microsomal preparations, showed that Asp309Ala was indeed more sensitive but only in medium containing low substrate concentrations. Although the K_i values for AG against Asp309Asn and Asp309Ala are significantly higher than that for the wild type enzyme, at saturating substrate concentrations, no difference was observed in IC_{50} values between Asp309Ala and the wild type [Fig. 2(B) and Table 1]. These observations suggest that AG inhibits aromatase in a complicated fashion.

This study analyzed the effect of specific amino acid substitutions in the active site of aromatase cytochrome *P*-450 in terms of changes in kinetic parameters and responsiveness to specific aromatase inhibitors. The present analysis demonstrates the importance of the use of isolated enzyme systems for deriving kinetic data. Measurements of kinetic parameters in cell culture are readily subject to modulation by intracellular factors, which in turn have important bearing on the interpretation of structure– function relationship. As these studies show, different results may be obtained depending on whether the assay is performed using "in cell" or *in vitro* microsomal preparations. It seems appropriate that both assays be utilized to evaluate the inhibitor–aromatase interaction *in vitro* and its potential to produce biological effects *in vivo*.

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