J. Steroid Biochem. Molec. Biol. Vol. 39, No. 3, pp. 381–394, 1991
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0960-0760/91 \$3.00 + 0.00
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HUMAN PLACENTAL ESTROGEN SYNTHETASE (AROMATASE). EFFECT OF ENVIRONMENT ON THE KINETICS OF PROTEIN–PROTEIN AND SUBSTRATE–PROTEIN INTERACTIONS AND THE PRODUCTION OF 19-OXYGENATED ANDROGEN INTERMEDIATES IN THE PURIFIED RECONSTITUTED CYTOCHROME *P*450 ENZYME SYSTEM

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(Received 20 February 1991)

Summary—Estrogen synthetase (aromatase) catalyzes the conversion of androgen into estrogen via two hydroxylations at C_{19} and a subsequent $C_{19\text{-}10}$ lyase reaction. We report here the results of a reconstitution study using a highly purified aromatase cytochrome *P*450 monooxygenase enzyme system, with both protein components (cytochrome *P*450 and NADPH-cytochrome *P*450 reductase) obtained from human term placental microsomes. By varying one of the components (amounts of cytochrome *P*450, NADPH-cytochrome *P*450 reductase, or androgen substrate) as the other two were held constant in four different environments (phospholipid, non-ionic detergent, mixture of phospholipid and non-ionic detergent and buffer alone), we obtained evidence supporting the following conclusions. The reconstituted enzyme is more active and the protein components exhibit much lower apparent K_m values in the detergent and/or lipid environment compared with buffer alone. Although the apparent K_m and V_{max} values for each aromatase protein component differ significantly in most cases with the particular limiting component and environment, the catalytic efficiency (K_{cat}/K_m) was independent of the limiting protein component and varied with the environment only (highest in the lipid–detergent mixture and lowest in lipid alone). When the concentration of androgen substrate (androstenedione or testosterone) was varied at constant amounts of the aromatase protein components (NADPH-cytochrome *P*450 reductase saturating), the K_m was lower and the V_{max} was higher for adrostenedione. The specificity constant (V_{max}/K_m) was a function of the reconstitution environment (highest in lipid alone and lowest in detergent alone) and was, on average, about 4-fold higher for androstenedione in a particular environment.

The extent of production of 19-oxygenated androgen intermediates (19-hydroxy and 19-oxo androstenedione) was examined at three different levels of aromatase cytochrome *P*450 (subsaturating, saturating, super-saturating) relative to the NADPH-cytochrome *P*450 reductase component in the three different hydrophobic environments using androstenedione as substrate. Both 19-oxygenated androgens, each made in comparable amounts relative to control, were isolatable in greatest amounts under cytochrome *P*450 super-saturating conditions in the detergent–lipid mixed environment, and in least amounts under cytochrome *P*450 subsaturating conditions in the lipid-only environment. Based on these data, we propose that 19-oxygenated androgen intermediates are biosynthesized sequentially in a step-wise fashion as the cytochrome *P*450 and NADPH-cytochrome *P*450 reductase form transient complexes, and that the amount of isolatable 19-oxygenated androgen is proportional to the amount of excess cytochrome *P*450 component.

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Abbreviations: DPC, dilauroyl phosphatidylcholine; NP40, nonidet P-40; 2′-AMP, adenosine 2′-monophosphate; TLC, thin-layer chromatogram; DTT, dithiothreitol; BHT, butylated hydroxytoluene; tSF, alph toluenesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; and BCA, bicinchoninic acid.

INTRODUCTION

Estrogen synthetase (aromatase) is the cytochrome $P450$ monooxygenase enzyme system responsible for the conversion of the male sex steroid hormone, androgen, into the female sex steroid hormone, estrogen. Regulated estrogen production is crucial for many normal physiologic processes, including reproduction [1], sexual behavior [2] and implantation in some species [3], and is linked to various pathologies [4–7]. The enzymic mechanism of aromatization is complex: apparently, 3 mol of oxygen and NADPH are utilized in the production of estrogen [8], with two 19-oxygenated androgens as postulated intermediates [9], by a single cytochrome $P450$ [10].

To obtain insight into the intricate enzymic process through which this cytochrome $P450$ monooxygenase system operates, we examined the kinetics of protein–protein and substrate–protein interactions in various hydrophobic membrane-like environments. Extensions of these findings to the placental physiologic environment is more direct compared with hepatic cytochrome $P450$ systems. In contrast to hepatic drug hydroxylating systems exhibiting cytochrome $P450$ multiplicity for a given enzymic process and wide substrate acceptance for a particular cytochrome $P450$ [11], the evidence supports a single form of aromatase cytochrome $P450$ [12, 13] that constitutes the majority of cytochrome $P450$ in placental microsomes [8] and demonstrates high substrate specificity [14] and the ability to carry out the three oxygenation reactions required for androgen aromatization [10].

To achieve our purpose, three types of experiments were conducted. First, we investigated the kinetics of interaction of the two aromatase component proteins purified from human term placental microsomes, cytochrome $P450$ and NADPH-cytochrome $P450$ reductase, to form the active aromatizing system capable of carrying out the required oxygenation process. Second, we examined the kinetics of testosterone and androstenedione aromatization in the aromatase reconstitution system, with NADPH-cytochrome $P450$ reductase present in saturating amounts. Finally, we investigated the influence of differing ratios of the cytochrome $P450$ and NADPH-cytochrome $P450$ reductase on the production of the two 19-oxygenated androgen intermediates (19-hydroxy and 19-oxo androstenedione). These "intermediates" are easily isolated from aromatase assays utilizing crude placental microsomes [9], but no one has yet described their formation in the reconstituted aromatase system using highly purified components. To determine the effect of environment on these properties, all three of these investigations were carried out using three different hydrophobic membrane-like environments. These were (a) DPC, the phospholipid reported to be optimally effective for cytochrome $P450$ reconstitution [15] and expected to most closely mimic the natural membrane environment, (b) the non-ionic detergent NP40 to compare the effect of this hydrophobic phospholipid substitute [15] with the natural phospholipid DPC and (c) a mixture of DPC and NP40 to compare results with each one used separately.

EXPERIMENTAL PROCEDURES

Materials

The following items were obtained from Sigma Chemical Co. (St Louis, MO): all unlabeled steroids, cholic acid, DPC, NP40, 2′-AMP and NADPH. Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) supplied octyl-Sepharose CL-4B, DEAE-Sepharose CL-6B and 2′5′ ADP-Sepharose 4B and HA-Ultrogel was purchased from IBF Biotechnics (Savage, MD). Bio-Gel A-1.5 m was acquired from Bio-Rad Laboratories (Rockville Center, NY). Emulgen 913 came from Kao-Atlas Co. (Tokyo, Japan). Leupeptin and pepstatin were obtained from Chemicon International Co. (Temecula, CA). VWR (Piscataway, NJ) supplied the plastic-backed TLC plates (E.M. kieselgel 60 F_{254}, 0.2 mm thickness). BCA came from Pierce Chemical Co. (Rockford, IL) and tSF from Eastman Kodak (Rochester, NY).

Radiolabeled androgens, [1β-^{3}H]androstenedione (28.2 Ci/mmol); [4-^{14}C]androstenedione (52 mCi/mmol); [1, 2, 6,7-^{3}H]androstenedione (90 Ci/mmol) and [1β, 2β-^{3}H]testosterone (46 Ci/mmol), were purchased from Dupont/New England Nuclear (Boston, MA). All substrates were purified by Zaffaroni paper chromatography [16] before use. [1β-^{3}H, 4-^{14}C]-androstenedione (389 dpm ^{3}H/pmol; ^{3}H/^{14}C = 5.5) was prepared by mixing the two radiolabeled components with unlabeled androstenedione. Other radiolabeled androgen substrates for the aromatase assay were prepared by mixing unlabeled androgen with the radiolabeled androgen to achieve the specific activity (see

below). All estimates of estrogen production based on ^3H_2O isolation (using 1β-^3H, or 1β, 2β-^3H substrates) were corrected for the ^3H distribution at the 1β or 1β, 2β positions, as obtained from the supplier.

Cholic acid was purified by crystallization from a saturated solution in ethanol after treatment with activated charcoal [17].

Purification of aromatase cytochrome P450 and NADPH-cytochrome P450 reductase

Human term placental microsomes were prepared according to a modification of a previously described method [18]. Briefly, washed placental tissue was homogenized in 25 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and then centrifuged (at 800 *g* for 10 min). The supernatant was saved as a source of NADPH-cytochrome *P*450 reductase activity (see below). The pellet was re-homogenized and re-centrifuged (at 800 *g* for 10 min). This second supernatant (containing about twice the cytochrome *P*450 specific content as the first supernatant) was centrifuged (at 15,000 *g* for 25 min), and the resulting supernatant was centrifuged again (at 140,000 *g* for 30 min) to obtain the microsomal fraction. The microsomal pellet was washed in 100 mM tetrasodium pyrophosphate (pH 7.4) containing 1 mM EDTA, 23 μM BHT and 2 μM androstenedione. The final pellet was washed and suspended (at 15–20 mg protein per ml) in Buffer A (40 mM potassium phosphate buffer, 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 23 μM BHT, 2 μM androstenedione, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin and 68 μg/ml tSF, pH 7.4), and stored at −85°C.

The purification protocol for the aromatase cytochrome *P*450 was based on a previously described method [19]. The microsomal preparation from five placentas (113 ml, 1.7 g protein) was thawed, adjusted to 225 ml with deaerated Buffer A and degassed. Cholic acid was dissolved in 2 N NaOH and added dropwise to achieve 1% sodium cholate, with continuous stirring for 30 min at 4°C. The detergent-treated microsomes were centrifuged (at 140,000 *g* for 1 h) and the supernatant was applied to an octyl-Sepharose column (2.5 × 41 cm) equilibrated in Buffer B (1% sodium cholate in Buffer A). The octyl-Sepharose column was washed overnight until the A_{405nm} decreased to baseline. The cytochrome *P*450-containing reddish-brown band was excised from the gel, after removing the gel from the glass column, and packed into another column (1.2 × 13 cm). The cytochrome *P*450 was eluted using a 0–1% Emulgen 913 gradient in Buffer B, at about 0.15% Emulgen 913. The cytochrome *P*450-containing fractions were pooled, dialyzed at 4°C for 17 h against 4 l of Buffer C (10 mM potassium phosphate, 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.05% Emulgen 913 and 2 μM androstenedione, pH 7.4), the equilibration buffer for the DEAE-Sepharose column (1.2 × 18 cm), and applied to the anion exchange column. Two aromatase cytochrome *P*450 pools (based on aromatase reconstitution ability) eluted from this column: one during the isocratic wash and another at about 42 mM potassium phosphate during a 10–500 mM potassium phosphate buffer gradient in Buffer C. Only the latter (bound) pool was processed further for this study by dialyzing it at 4°C for 17 h against 2 l of Buffer D (0.1% Emulgen 913 in Buffer C). The preparation was applied to an HA-Ultrogel column (1.2 × 14 cm) equilibrated in Buffer D. After thoroughly washing the column with Buffer D, the cytochrome *P*450 was eluted using a 10–500 mM potassium phosphate buffer gradient in Buffer D at 42–46 mM potassium phosphate. The rear portion of the HA-Ultrogel-eluted peak contained a slightly greater purity (by SDS–PAGE) and was isolated separately for use in this study. The purity was estimated at >95% by silver-stained [20] SDS—PAGE and the cytochrome *P*450 specific content estimated at 2.8 nmol/mg protein (see comment on aromatase cytochrome *P*450 specific content under *Other assays* in Experimental Procedures). After dialyzing the HA-Ultrogel-purified preparation against 2 l of EDTA-free Buffer D for 17 h, it was applied to a fresh HA-Ultrogel column (2.5 ml gel) equilibrated with EDTA-free Buffer D. The gel was washed extensively with Buffer E (10 mM potassium phosphate, 20% glycerol, 0.1 mM DTT and 2 μM androstenedione, pH 7.4) to remove the detergent before eluting the cytochrome *P*450 with 500 mM potassium phosphate buffer in Buffer E. The cytochrome *P*450-containing fractions were pooled and EDTA was added to 1 mM. Note that this detergent-free preparation containing androstenedione was used for the study of the kinetics of cytochrome *P*450-NADPH-cytochrome *P*450 reductase interactions and of the 19-oxygenated androgen intermediate formation. The kinetics of androgen aromatization was determined using an

androstenedione- and detergent-free preparation obtained by dialyzing the detergent-free preparation against androstenedione-free Buffer E.

NADPH-cytochrome *P*450 reductase was purified from human term placental microsomes by a protocol similar to those already described [21, 22]. The first low-speed supernatant, described above in the preparation of placental microsomes, was used to prepare the microsomal fraction that served as the source of NADPH-cytochrome *P*450 reductase. As with the cytochrome *P*450 purification, this microsome preparation was solubilized with Buffer B and the soluble proteins after ultracentrifugation were applied to a 2′5′ ADP-Sepharose gel column (20 ml) equilibrated in Buffer F (40 mM potassium phosphate, 10% glycerol, 1 mM EDTA, 0.1 mM DTT, 1% sodium cholate, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin and 68 μg/ml tSF, pH 7.4) at 4°C. The column was washed with Buffer F, and the NADPH-cytochrome *P*450 reductase was eluted with 4 mM 2′-AMP in Buffer F. The pooled enzyme was dialyzed for 17 h at 4°C against 2 l of Buffer G (67 mM potassium phosphate, 10% glycerol, 1 mM EDTA and 0.1 mM DDT, pH 7.4), and concentrated to 1.5 ml using the Centricon 30 ultrafiltration device (Amicon, Beverly, MA). The concentrated preparation was applied to a Bio-Gel A-1.5 m (100–200 mesh) column (1 × 96 cm) equilibrated with Buffer G. Fractions containing NADPH-cytochrome c reductase activity were pooled and concentrated to 3 ml. The specific activities of several preparations ranged from 6.5 to 17 μmol/min/mg protein. The purity was estimated at >99% by silver-stained SDS–PAGE.

Reconstitution protocol

Purified aromatase cytochrome *P*450 and NADPH-cytochrome *P*450 reductase were mixed at the concentrations indicated in the figure legends in a small volume (<0.1 ml) with 10–20% glycerol, 67 mM potassium phosphate buffer (pH 7.4), 50 μg bovine serum albumin (BSA), 0.1 mM EDTA and one of the following suspended in 67 mM phosphate buffer (pH 7.4): 10 μg DPC; 0.003% NP40 (based on final assay volume); a mixture of 10 μg DPC and 0.003% NP40; 67 mM phosphate buffer (pH 7.4) alone. After incubation at 21°C for 5 min, the volume was increased to 1 ml by adding 67 mM phosphate buffer (pH 7.4) containing 0.5 mg NADPH and substrate (see below), and the aromatase assay was conducted at 37°C. The choice of 10 μg/ml DPC was based on separate experiments showing that this amount maximally stimulated the reconstituted aromatase (data not shown). The final NP40 concentration (0.003%) was chosen to match the aromatase reconstitution protocol of Kellis and Vickery [12], and was later found to be close to the concentration that gives optimal aromatase activity (about 0.002%). The NP40 concentration dependence for aromatase activity was qualitatively similar to that described for lubrol PX on hepatic pentoxyresorufin *O*-dealkylating activity [23]. The aromatase cytochrome *P*450 and NADPH-cytochrome *P*450 reductase preparations were divided into small aliquots and stored at −85°C. Each experiment used a preparation that was frozen and thawed only once. The resulting aromatase reconstitution activities under comparable assay conditions were highly reproducible.

Aromatase assay

Kinetics of aromatization. For the experiments examining the kinetics of aromatase protein component association into active enzyme, and the kinetics of androgen aromatization, we used the standard ^3H_2O-based aromatase assay [8, 24]. Most of these experiments utilized [1β-^{3}H,4-^{14}C]androstenedione (129 nM) as the substrate. To obtain the kinetics of testosterone aromatization, we used [1β, 2β-^{3}H]testosterone (372 nM; 287 dpm/pmol) as substrate. Other constituents of the assay are given above under *Reconstitution protocol.* The assays, conducted in 2 ml microfuge tubes, were terminated after a 15 min assay by adding a 0.5 ml mixture of activated charcoal slurry (1.2%), dextran (0.6%) and trichloroacetic acid (4%). After vortexing and collection of the dextran-charcoal pellet by centrifugation, an aliquot (0.75 ml) of the supernatant was taken for liquid scintillation counting in 3 ml of Liquiscint and 0.15 ml of additional water. A summary of six separate time course experiments using the reconstituted system with various amounts of the two protein components demonstrated linearity for at least 30 min. We used 15 min assays to insure linearity. Using [^{14}C]androstenedione in the substrate allowed the detection of unbound [^{3}H] steroid in the supernatant. Although ^{14}C dpm was rarely detected in the supernatant, samples in which this occurred were treated further to remove the ^{14}C dpm leaving ^3H_2O as the sole source of ^{3}H dpm.

Isolation of aromatization intermediates. The product isolation method [16] was used for this portion of the study. The substrate was [1, 2, 6, 7-^{3}H]androstenedione (99.4 nM, 9792 dpm/pmol). At the end of the incubation (variable times, to achieve 20–30% aromatization), the assay was terminated using 0.4 ml of ethanol containing 20 μg of each of the following steroids: androstenedione; testosterone; estrone; estradiol-17β; 19-hydroxy androstenedione; and 19-oxo androstenedione. The mixture was extracted with ethyl acetate (3 × 2 ml), and the organic phase was backwashed with water and evaporated. The residue dissolved in methanol was carefully applied to a silica gel TLC containing fluorescent indicator and developed twice in ethyl acetate–cyclohexane (1:1, v/v). The pertinent steroid areas (R_p values: estrone, 0.74; estradiol-17β, 0.51; androstenedione, 0.41; testosterone and 19-oxo androstenedione, 0.27; and 19-hydroxy androstenedione, 0.08) were identified by excitation with ultraviolet light, or by spraying with aqueous sulfuric acid (50%) and heating at 100°C. The steroid areas were then cut from the plastic-backed plates, mixed with liquid scintillation cocktail and counted. The average recovery of ^{3}H dpm after TLC (sum of total recovered ^{3}H dpm divided by ^{3}H dpm in the reconstitution assay) was 82.4 ± 8.3%.

All pertinent steroid areas were well resolved in this solvent system except 19-oxo androstenedione plus testosterone. To verify the absence of testosterone from the aromatase reconstitution assays using the purified protein components and radiolabeled androstenedione, the silica gel area containing 19-oxo androstenedione plus testosterone was scraped from the first TLC plate, eluted with methylene chloride–ethanol (1:1, v/v), and applied to a second TLC system [chloroform–methanol (95:5, v/v)] that adequately resolved these compounds (R_f values: testosterone, 0.48; and 19-oxo androstenedione, 0.71). More than 95% of the radioactivity recovered from the second TLC plate was recovered in the 19-oxo androstenedione area. Subsequent determinations of 19-oxo androstenedione were based on the first TLC separation.

Other assays

Protein was estimated using the micro BCA method [25] or Lowry method. NADPH-cytochrome c (P450) reductase activity was measured using the assay of Strobel and Dignam [26] at 21°C. Cytochrome P450 content was measured by the method of Omura and Sato [27]. Although the aromatase cytochrome P450 specific content we obtained (2.8 nmol/mg) appears low compared with the values reported for this protein by two groups [12, 28], or with values reported for purified hepatic cytochrome P450's in general, this has no bearing on our study for the following reasons. First, Osawa *et al.* [29] report that purified aromatase cytochrome P450 is unstable in the typical Omura and Sato assay. This is supported by (a) all other measurements for this protein using the Omura and Sato assay obtain specific content values for purified aromatase cytochrome P450 [30–33] equal to or smaller than the value we obtained, (b) Tan and Muto [31] reported a low specific content (4.15 nmol/mg) by the Omura and Sato assay and a high specific content (12.7) for the same preparation by direct heme assay, and (c) the two laboratories [12, 28] reporting the higher values (11.5–13 nmol/mg) used a modified Omura and Sato assay which included 19-nor androgen, a condition that we find in preliminary studies stabilizes aromatase cytochrome P450 in the Omura and Sato assay (K. Sethumadhavan, unpublished observations). Second, the reconstituted aromatase specific activity is the principal concern of this study and the value we obtain here (39 nmol/min/mg for androstenedione in the DPC environment) is comparable to that reported (57 nmol/min/mg) by Kellis and Vickery [12].

Estimation of kinetic parameters

To obtain estimates of the kinetic constants and their standard errors (± SE) associated with either the protein–protein interactions involved in the aromatase reconstitution studies, or the substrate–active site interactions, we used EZ-FIT, an interactive microcumputer software package [34]. All of the initial velocity of aromatization data for variable NADPH-cytochrome P450 reductase or androgen concentration were used in the analysis. When the aromatase cytochrome P450 concentration was varied, cytochrome P450 concentrations > 15 nM were excluded from the analysis due to non-Michaelis–Menten type behavior.

The K_m and V_{max} kinetic constants obtained from the two different types of experiments have different physical meanings. For example, in the reconstitution study the K_m is more closely identified with a protein–protein association,

except it reflects only those protein associations that lead to active enzyme formation. To distinguish the kinetic constants associated with protein–protein interactions from the standard Michaelis–Menten type substrate–active site interactions, we use the subscript "p" (e.g. K_{m_p} and V_{max_p}) to designate protein–protein interactions and the subscript "s" (e.g., K_{m_s} and V_{max_s}) to designate substrate–active site interactions. The K_{cat} was determined by dividing the V_{max_p} by the amount of limiting aromatase protein component. The catalytic efficiency is the ratio of K_{cat} to K_{m_p} [35]. The specificity constant is the ratio of V_{max_s} to K_{m_s} [12].

Pairwise statistical comparisons of these data were carried out using Duncan's multiple-range test [36]. Significance was maintained at or above the 99% level. The number of data points analyzed for each kinetics experiment was: androgen substrate—7; NADPH-cytochrome $P450$ reductase limiting—6; aromatase cytochrome $P450$ limiting—8. Each kinetics experiment was conducted two to three times and the data averaged to provide the graphs shown. Each individual assessment of 19-oxygenated androgens was made in triplicate and carried out twice.

RESULTS

Kinetics of association of protein components into active aromatase

To obtain information regarding the interaction of the two aromatase protein components to form active enzyme in several different environments, we determined and analyzed enzyme kinetic constants associated with estrogen biosynthesis in reconstituted aromatase assays under conditions in which, in a particular environment, one aromatase protein component was held constant as the other was increased until saturation was achieved. With aromatase cytochrome $P450$ limiting [Fig. 1(A)], Michaelis–Menten type kinetics were observed. In the inverse situation, i.e. NADPH-cytochrome $P450$ reductase limiting [Fig. 1(B)], the amount of active enzyme increased steadily with Michaelis–Menten-like kinetics at low cytochrome $P450$ concentration (< 15 nM). However, at high cytochrome $P450$ levels (> 27 nM), the aromatase activity decreased substantially with increasing cytochrome $P450$; the extent of this decrease was greater in the detergent environment (with or without lipid) than in the lipid-only environment. In the absence of lipid or detergent, i.e. buffer alone, reconstituted aromatase activity increased linearly with increasing cytochrome $P450$ or NADPH-cytochrome $P450$ reductase amounts over the ranges shown in Fig. 1(A) and (B). However, in buffer alone the activity at the highest level of aromatase component was much lower (approx. 0.22 pmol/min for aromatase cytochrome $P450$ limiting and 0.156 pmol/min for NADPH-cytochrome $P450$ reductase limiting) than the activity observed in the presence of lipid and/or detergent (data not shown).

Michaelis–Menten kinetic constants [Fig. 2(A) and (B)], the K_{cat} (see below), and the catalytic efficiency [Fig. 2(C)] were calculated from the data in Fig. 1(A) and (B) [excluding from the analysis the initial velocity of aromatization for cytochrome $P450$ amounts > 15 nM in Fig. 1(B)]. In comparing the effect of the environment on K_{m_p} [Fig. 2(A)] and V_{max_p} [Fig. 2(B)] with cytochrome $P450$ limiting, both of these values were highest in the lipid-only environment and lowest in the detergent-only environment. When the NADPH-cytochrome $P450$ reductase was limiting, the K_{m_p} was

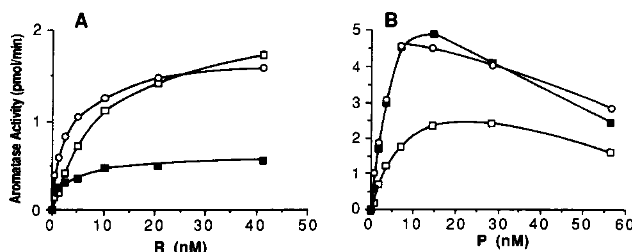


Fig. 1. Initial velocity of aromatization as a function of aromatase protein component concentration. The aromatase reconstitution system was set up as described with (A) 0.94 nM aromatase cytochrome $P450$ and the indicated concentration of NADPH-cytochrome $P450$ reductase (R), or (B) 2.74 nM NADPH-cytochrome $P450$ reductase and the indicated concentration of aromatase cytochrome $P450$ (P). At the end of the incubation period, the initial velocity of estrogen production was determined from the amount of ^3H_2O dpm isolated, as described. The various reconstitution environments were (□) DPC, (○) mixture of DPC plus NP40, and (■) NP40.

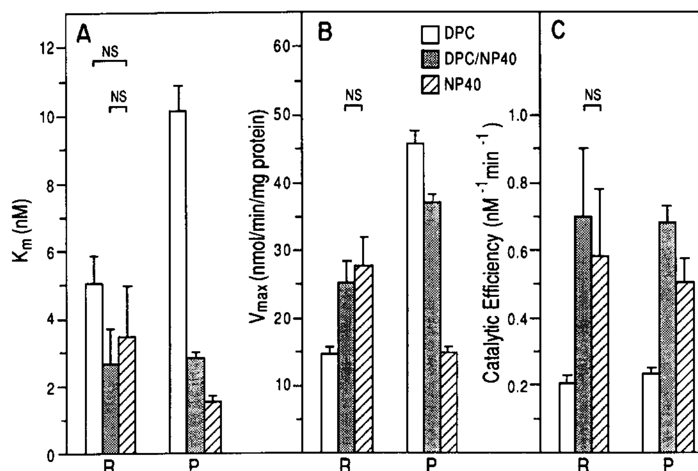


Fig. 2. Kinetic constants of active aromatase formation from purified protein components. The data in Fig. 1 was analyzed as described to obtain estimates of the various kinetic constants: (panel A) K_{m_p}; (panel B) V_{max_p}; (panel C) catalytic efficiency for R—NADPH-cytochrome *P*450 reductase limiting, or P—aromatase cytochrome *P*450 limiting conditions. The key for the various environmental conditions is given in Panel B. The designation near the top of panels A–C labeled "NS" indicates statistically non-significant pair-wise comparisons between the indicated bar graphs. All unmarked pair-wise comparisons between bar graphs are significantly different from one another ($P < 0.01$)

higher in the lipid-only environment than in the other environments; the V_{max_p} was lowest in the lipid-only environment. The K_{cat} values (min^{-1}), in lipid-only, lipid–detergent mixture and detergent-only environments, respectively, were: 2.42 ± 0.06, 1.97 ± 0.16, 0.79 ± 0.02 (for aromatase cytochrome *P*450 limiting); and 1.08 ± 0.06, 1.88 ± 0.21, 2.02 ± 0.28 (for NADPH-cytochrome *P*450 reductase limiting). Although the individual kinetic parameters varied with the identity of the constant component and the environment, the catalytic efficiency [Fig. 2(C)] depended only on the environment and not on the identity of the limiting aromatase protein component. Furthermore, the catalytic efficiency was lowest for the lipid-only environment and highest for the lipid–detergent mixed environment; the value for the mixed environment appears to be similar to arithmetic sums of the value for each individual environment.

Kinetics of androgen aromatization by reconstituted aromatase

To determine the Michaelis–Menten kinetic constants for androgen aromatization, the aromatization system was constituted at a constant, saturating level of the NADPH-cytochrome *P*450 reductase component and the amount of substrate, androstenedione or testosterone, was varied. The initial velocity of aromatization as a function of substrate concentration in the three different environments is shown for testosterone in Fig. 3(A) and for androstenedione in Fig. 3(B). In a particular environment, the K_{m_s} values [Fig. 4(A)] were higher for testosterone and the V_{max_s} values [Fig. 4(B)] were higher for androstenedione. The V_{max_s} values for both substrates were highest in the lipid-only environment and lowest in the detergent-only environment. The K_{m_s} values for testosterone follow the opposite environmental pattern. The K_{m_s} values for androstenedione

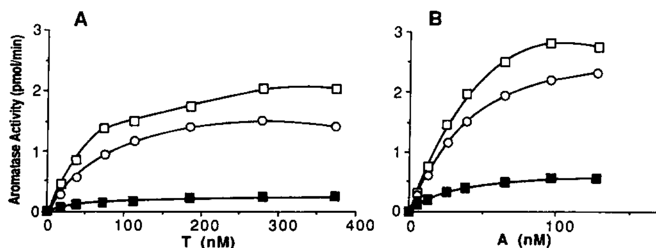


Fig. 3. Initial velocity of aromatization as a function of androgen substrate concentration. At fixed amounts of aromatase cytochrome *P*450 (1.88 nM) and NADPH-cytochrome *P*450 reductase (27.4 nM), the concentration of androgen substrates, testosterone (T, panel A) or androstenedione (A, panel B) were varied as indicated in the aromatase reconstitution assay. Other information is provided in the legend to Fig. 1.

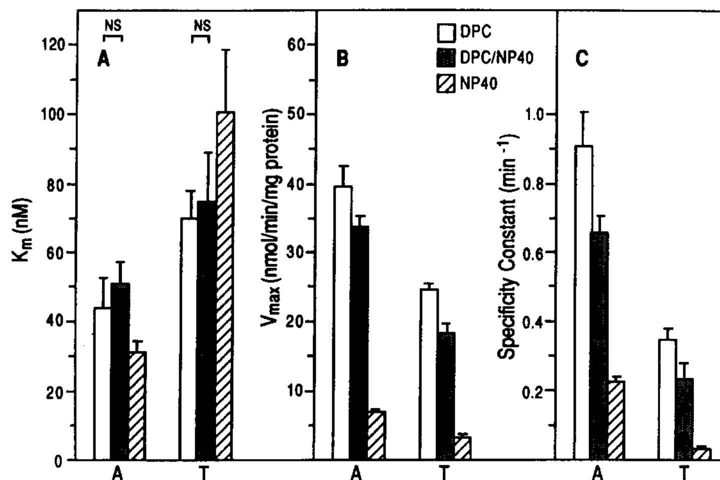


Fig. 4. Kinetic constants of androgen aromatization. The data in Fig. 3 was analyzed as described to obtain estimates of the various kinetic constants: (panel A) K_{m}; (panel B) V_{max}; (panel C) specificity constant for androstenedione (A) or testosterone (T) as substrates. Other information is provided in the legend to Fig. 2.

were highest in the lipid-containing environment and lowest in the detergent-only environment. The specificity constant [Fig. 4(C)] for androstenedione was, on the average, 4-fold greater in a given environment than the specificity constant for testosterone. For both androgens, the specificity constant depended on the environment; it was highest in the lipid environment and lowest in the detergent environment.

Production of 19-oxygenated androgen intermediates at different ratios of the aromatase component proteins

These experiments were designed to explore the ability of the aromatase reconstitution system to produce 19-hydroxy and 19-oxo androstenedione, presumed intermediates of estrogen biosynthesis, as a function of the ratio of the aromatase cytochrome *P*450 to NADPH-cytochrome *P*450 reductase and of the environment. Accordingly, aromatization reactions were carried out under aromatase cytochrome *P*450 subsaturating [0.94 nM cytochrome *P*450, 40 nM NADPH-cytochrome *P*450 reductase in Fig. 1(A)], aromatase cytochrome *P*450 saturating [15 nM cytochrome *P*450 in Fig. 1(B)] and super-saturating [57 nM cytochrome *P*450 in Fig. 1(B)] conditions in the lipid-only, detergent-only, or mixed lipid-detergent environments. Since we wanted to compare the maximum levels of the 19-oxygenated androgen intermediates generated under these various conditions, the best way to conduct these studies would have been to measure maximum levels of the 19-oxygenated androgens formed over time under the various conditions employed. However, the limiting quantities available of these two purified aromatase component proteins did not permit extensive time course studies under the nine different conditions (three different protein ratios and three different environments). Separate time course experiments using placental microsomes (data not shown), as well as published reports of 19-oxygenated androgen and estrogen production with time in placental microsomes [9, 37], showed that maximum 19-oxygenated androgen production occurred as estrogen production increased linearly through 20–40% of the supplied substrate. Therefore, we adjusted the incubation times to achieve reasonably constant levels (20–30%) of estrogen production, so that the levels of isolatable 19-oxygenated androgens measured under the nine different conditions could be compared. This approach was generally successful for eight of the nine combinations. Only in the combination of limiting levels of aromatase cytochrome *P*450 in detergent were we unable to reach 20–30% aromatization since the aromatization reaction terminated prematurely after 20–30 min incubation when the maximum level of estrone produced reached only 10–15% and 85–90% of the substrate remained unaromatized (K. Sethumadhavan, unpublished observations). The measurement of estrone and 19-hydroxy and 19-oxo androstenedione at a particular ratio of aromatase cytochrome *P*450 to NADPH-cytochrome *P*450 reductase in each of the three different environments are shown

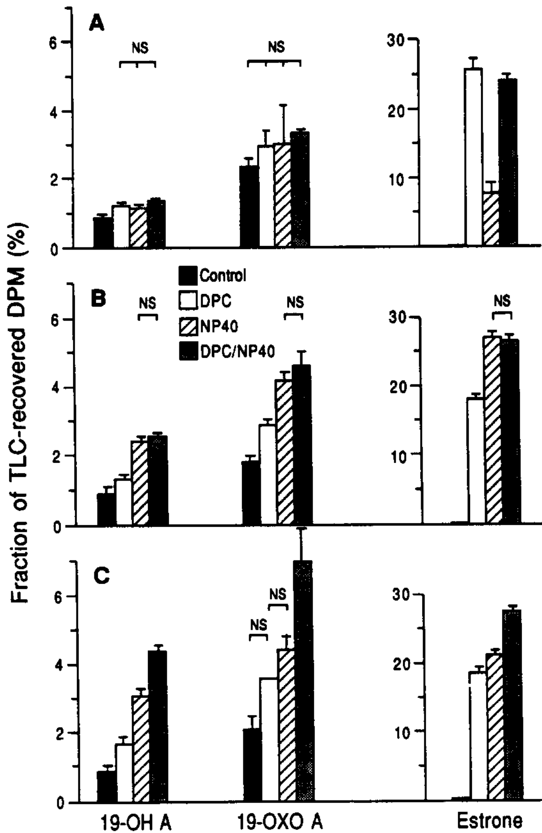


Fig. 5. Aromatization intermediate production in various environments. The aromatization intermediates, 19-hydroxy androstenedione (19-OH A) and 19-oxo androstenedione (19-OXO A), and end product, estrone, were measured in the aromatase reconstitution system as described for (panel A) cytochrome $P450$ subsaturating, (panel B) cytochrome $P450$ saturating, and (panel C) cytochrome $P450$ super-saturating conditions. Control incubations contained either substrate without protein components, or all ingredients but terminated at zero time. The key for the various environmental conditions is given in panel B. The statistical pairwise designation "NS" is as described in the legend for Fig. 2.

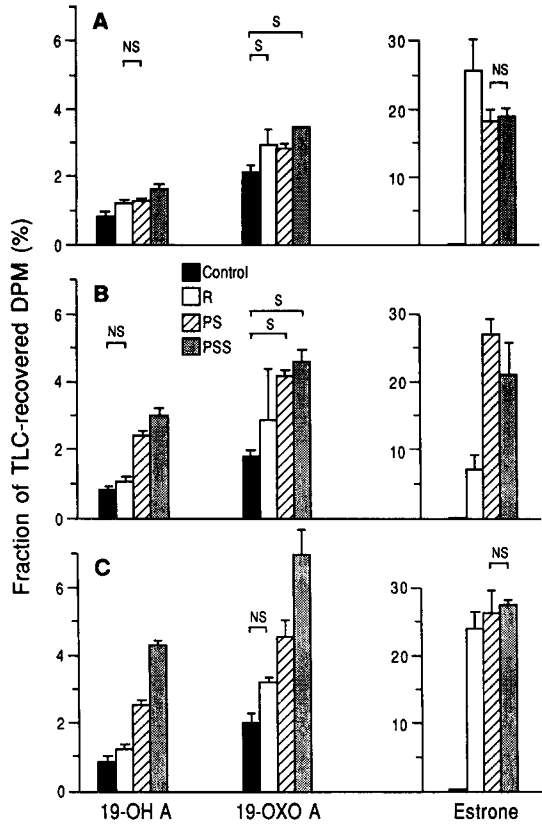


Fig. 6. Aromatization intermediate production in various ratios of aromatase protein components. The data in this figure are taken from Fig. 5: (panel A) DPC; (panel B) NP40; (panel C) DPC plus NP40. The key for the various cytochrome $P450$ to NADPH-cytochrome $P450$ reductase ratios is given in Panel B: R—cytochrome $P450$ subsaturating; PS—cytochrome $P450$ saturating; PSS—cytochrome $P450$ super-saturating. The statistical pair-wise comparison information is slightly different from that given in previous figure legends; in this figure, two sets of four-bar graphs (19-OXO A; panels A and B) show statistically significant ($P < 0.01$) pair-wise comparisons as "S". All other pair-wise comparisons in these two sets are non-significant ($P > 0.01$). The statistical information in the other seven sets of bar graphs is as described in the other figure legends.

in Fig. 5(A)–(C). With aromatase cytochrome $P450$ subsaturating [Fig. 5(A)], 19-hydroxy androstenedione in all three environments was significantly greater than control, but the levels were not significantly different from each other. None of the 19-oxo androstenedione levels were significantly greater than control. With aromatase cytochrome $P450$ at saturation [Fig. 5(B)] or super-saturating [Fig. 5(C)], significantly greater amounts of both of these intermediates were recovered relative to control incubations, although some pair-wise comparisons between environments were not significantly different. Although greater gross amounts of 19-oxo androstenedione were measured, the net amounts of both intermediates (relative to controls) are similar.

The data in Fig. 5(A)–(C) were re-plotted as a function of the ratio of aromatase protein components for a given environment in Fig. 6(A)–(C). When analyzed in this manner, it is apparent that the extent of production of both 19-oxygenated androgen intermediates depended on the ratio of the two aromatase components. The levels of both intermediates were greatest under aromatase $P450$ super-saturating conditions, regardless of the environment in which the reconstitution was conducted.

DISCUSSION

The aromatase component proteins, cytochrome $P450$ and NADPH-cytochrome $P450$

reductase, were obtained in highly purified form from human term placental microsomes. Mixing these protein components in a lipid and/or detergent environment resulted in a highly active aromatizing system. The V_{max_s} for androstenedione with saturating levels of NADPH-cytochrome *P*450 reductase in the lipid-only environment was 46 nmol/min/mg protein, a value comparable to other reports using purified human placental cytochrome *P*450 (range from 23 [30] to 103 [33] nmol/min/mg protein). Using this effective, placental-derived aromatizing system, we provide data here supporting the following conclusions: (a) the K_{m_p}, V_{max_p} and K_{cat} parameters vary depending on the limiting aromatase component and on the environment of the reconstitution system, (b) the catalytic efficiency was independent of the limiting protein component and depended only on the environment (lowest in lipid; highest in lipid plus detergent); (c) as reported for placental microsomes [16, 38], androstenedione was a better substrate than testosterone based on its lower K_{m_s}, higher V_{max_s} and higher specificity constant; (d) the K_{m_s}, V_{max_s} and specificity constant for the aromatization of each androgen was a function of the environment; the V_{max_s} and specificity constant decreased in the order of lipids > lipid + detergent > detergent; (e) although aromatization occurred in an apparently non-hydrophobic environment (buffer alone), a lipid and/or detergent environment was highly stimulatory for aromatase; and (f) 19-oxygenated intermediates were readily formed in this reconstitution system, and the extent of their formation depended primarily on the ratio of the two aromatase components.

Molecular interactions between cytochrome *P*450 and NADPH-cytochrome *P*450 reductase are not well understood. The results of a number of studies suggest that the association between these two proteins follows the law of mass action [39, 40]. In this study, as reported in several other studies using purified mammalian hepatic cytochrome *P*450 systems [23, 39, 41], the affinity of the interaction between the two protein components of this system was estimated by the K_{m_p} value, and the number (or effectiveness) of the complexes formed in converting substrate to product was estimated by the V_{max_p} value. One might anticipate that these two experimentally determined values would be interrelated, i.e. as affinity increases (K_{m_p} decreases), the V_{max_p} would be expected to increase due to a presumed increase in the number (or effectiveness) of the complexes, and vice-versa. Although this relationship appeared to hold under NADPH-cytochrome *P*450 reductase limiting conditions (e.g. changing the environment from lipid to lipid–detergent mixture was associated with an increased affinity and increased maximum velocity), it did not hold for the changing environment when cytochrome *P*450 was limiting. As the environment changed from lipid alone to lipid–detergent mixture and detergent alone, the affinity of the NADPH-cytochrome *P*450 reductase for the cytochrome *P*450 increased, but the effectiveness of the complex (V_{max_p}) decreased. One possible explanation involves interference by the detergent with the electron transport function [42]. Note that all of these studies were conducted with androstenedione present in the aromatase cytochrome *P*450 preparation, a condition expected to increase the association between the two component proteins [43]. Because of the low aromatase activity in the buffer-only environment, extensive experimentation was not carried out. However, one can reasonably conclude based on the linear increase in aromatase activity over the entire range of aromatase component protein that the affinity of each protein component for its partner was considerably lower in the buffer-only environment compared with any of the hydrophobic environments.

Further kinetic analysis of interactions of these two proteins in the various environments included determination of the catalytic constant or turnover number, K_{cat} [39], and the catalytic efficiency, K_{cat}/K_{m_p} [35]. Miwa *et al.* [39] used their observation that the K_{cat} values determined under cytochrome *P*450 and NADPH-cytochrome *P*450 reductase limiting conditions were similar as evidence that these two proteins interact according to the law of mass action. However, our data show similarity of K_{cat} for both limiting proteins only for the lipid–detergent mixed environment. We did observe, however, that the catalytic efficiency, i.e. the catalytic constant divided by the K_{m_p} of the interaction, was independent of the identity of the limiting protein component and depended solely on the environment. The fact that the catalytic efficiency was highest in the lipid-detergent mixed environment and the values in the individual environments when added together approximated the value in the mixed environment suggested that each environment promoted the cytochrome *P*450–NADPH-cytochrome *P*450 reductase catalytic efficiency independently.

Nadler and Strobel [35] estimated the various kinetic values determined here (K_{m_p}, V_{max_p}, K_{cat} and K_{cat}/K_{m_p}) in a hepatic cytochrome $P450$ reconstitution system by independently varying both protein components of the system. Rather than use environment as an independent parameter, they used several different chemical modifications to the NADPH-cytochrome $P450$ reductase component. As we report here, they also find that the K_{m_p} and V_{max_p} values vary independently, the K_{cat} for different limiting protein components were not comparable, and the catalytic efficiency tended to be independent of the limiting protein component but dependent on the particular chemical modification employed. The independence of the K_{m_p} and V_{max_p} measured under cytochrome $P450$ limiting conditions in two different lipid environments was also reported recently by Rietjens *et al.* [23].

The K_{m_p} for reconstituted aromatase in the detergent environment with cytochrome $P450$ limiting, 2 nM, is 10-fold less than that estimated by Kellis and Vickery [12] in the same environment using a heterologous source (rabbit liver) for the NADPH-cytochrome $P450$ reductase component. Other estimates of this parameter in reconstituted hepatic hydroxylation systems for a phospholipid environment range (under cytochrome $P450$ limiting conditions) from 17–95 nM [23, 41]. Our estimate in the phospholipid environment was about 10 nM. In other studies using limiting amounts of NADPH-cytochrome $P450$ reductase component in the phosphatidylcholine environment [35, 43], the K_{m_p} varies from 17–110 nM (compared with 5.1 nM observed here) and, in contrast to our observations, Michaelis–Menten-like kinetics appears to hold at high cytochrome $P450$ amounts.

We have no satisfactory explanation for the decline in the aromatization rate at high cytochrome $P450$ amounts seen in Fig. 1(B). The ability to isolate increased amounts of 19-oxygenated androgen intermediates under this condition seems insufficient to explain the large decrease in initial velocity. Since the maximum amount of cytochrome $P450$ used (57 nM) approximates the amount of substrate (129 nM), we thought the cytochrome $P450$ that is not complexed with NADPH-cytochrome $P450$ reductase might bind sufficient amounts of substrate to lower the effective free substrate concentration as the amount of excess cytochrome $P450$ is increased, thereby decreasing the initial velocity of aromatization. We tested this possibility by using higher substrate amounts for saturating (15 nM) as well as super-saturating (57 nM) aromatase cytochrome $P450$ levels. There was no differential change in aromatization rate (K. Sethumadhavan, unpublished observations), suggesting that substrate availability was not responsible. A third possible explanation comes from the work of Gut *et al.* [44] that increasing the ratio of cytochrome $P450$ to lipid decreases cytochrome $P450$ mobility, presumably due to cytochrome $P450$ aggregation. In our experiments [Fig. 1(B)], the cytochrome $P450$ was increased as the lipid and/or detergent concentration was held constant, possibly leading to increased cytochrome $P450$ aggregation and decreased aromatase activity.

The kinetic parameters of androgen aromatization obtained under normal Michaelis–Menten conditions, i.e. constant enzyme amount and variable substrate amounts, support prior determinations in placental microsomes that androstenedione is a better substrate than testosterone with higher affinity, higher rates of aromatization [16, 38], and greater specificity constant [12]. We show here for the first time that the environment strongly influences all of these parameters. The specificity constant for both androgens is highest in the lipid environment and lowest in the detergent environment. In the detergent environment, the lower K_{m_s} for androstenedione suggests a tighter binding between the substrate and active site but the lower V_{max_s} may be caused by inhibitory effects of the detergent on the enzyme complex or a less effective orientation of the substrate within the active site. A greater lipophilic partition of androstenedione relative to testosterone may explain, at least in part, the greater affinity and reaction velocity of androstenedione.

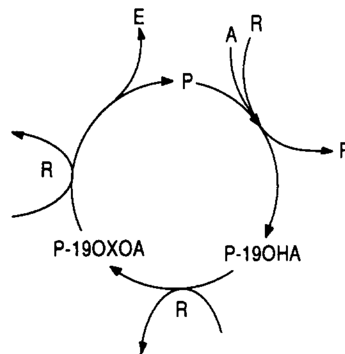
In this report, we described 19-oxygenated androgens as "intermediates" of aromatization. Early investigations of the role of these compounds using crude placental microsomes postulated a step-wise progression of androgen → 19-hydroxy androgen → 19-oxo androgen → estrogen, possibly generated by a multi-enzyme complex in which the product of one enzyme became the substrate for the next. Since most of the 19-hydroxy androgen "intermediates" remained bound during aromatization [45] and 19-oxygenated androgens competitively interfered with androgen binding and androgen aromatization [46], the concept of a multi-

enzyme complex was replaced by the concept of a concerted enzymic process at one [46], or possibly two adjacent [47], active site (s) involving bound transition states. In this view, 19-oxygenated androgens were not intermediates in the traditional sense, but by-products of the transition states. This transition state model accounted only for interactions between the active site and the substrates or intermediates and ignored interactions between the two protein components of the aromatase enzyme system, thereby tacitly assuming a rigid cytochrome *P*450–NADPH-cytochrome *P*450 reductase complex with a lifetime greater than that required for the triple hydroxylation process. The requirement for a single cytochrome *P*450 protein in the complex androgen aromatization process has been confirmed both by reconstitution with purified protein components [12, 30–33] and by cDNA transfection studies of the aromatase cytochrome *P*450 [10].

In contrast to previous studies of 19-oxygenated androgen generation which utilized crude placental microsomes containing a fixed ratio of the two aromatase protein components, we used the flexibility of the reconstitution system to examine the role of protein–protein interactions on aromatization intermediate production by manipulating the ratio of the two aromatase protein components in various environments. Our ability to isolate greater amounts of 19-oxygenated androgens by increasing the amount of the cytochrome *P*450 component relative to the NADPH-cytochrome *P*450 reductase component led us to two general conclusions pertinent to the aromatization process. First, only if cytochrome *P*450–NADPH-cytochrome *P*450 reductase existed as a transient complex would we expect that increasing the amount of cytochrome *P*450 over that required to saturate the NADPH-cytochrome *P*450 reductase would result in increasing production of 19-oxygenated androgens. This concept of a transient interaction between cytochrome *P*450 and NADPH-cytochrome *P*450 reductase has substantial support from studies of hepatic cytochrome *P*450 systems [40, 42, 48–50]. Second, during the period that the cytochrome *P*450 component is not associated with the NADPH-cytochrome *P*450 reductase component, we expect the active site to be free or occupied with substrate or intermediate. In another multiple hydroxylation steroidogenic cytochrome *P*450 system involving a single cytochrome *P*450, the reconstituted C_{21} side chain cleavage system, as in our system, the ratio of hydroxylase activity (responsible for "intermediate" production) decreased relative to lyase activity (responsible for end product) as the ratio of cytochrome *P*450 to NADPH-cytochrome *P*450 reductase decreased [51].

These conclusions prompt us to propose the following model to explain the production of androgen aromatization intermediates in this reconstitution system. Substrate (androgen) binds to the cytochrome *P*450 active site (see Scheme 1) probably as the cytochrome *P*450 interacts with the NADPH-cytochrome *P*450 reductase [43]. In the presence of cofactor, 19-hydroxylation occurs and NADPH-cytochrome *P*450 reductase quickly dissociates, leaving 19-hydroxy androgen bound to the cytochrome *P*450. Another transient complex with the NADPH-cytochrome *P*450 reductase results in the cytochrome *P*450-bound 19-oxo androgen. Following a third transient complex formation, estrogen and uncomplexed cytochrome *P*450 are released to re-initiate the cycle.

In this model there is no need to embrace the concept of concerted enzymic processes with bound transition states. Rather aromatization is a step-wise process in which stable intermediates remain tightly bound to the cytochrome *P*450 active site until association with reductase occurs. An interesting contrast with aromatase is the purified 19-hydroxylase from adrenal mitochondria ($P450_{11\beta}$; [52]) which appears to have a similar 19-hydroxylation sequence as aromatase, but freely releases the product of each 19-hydroxylation so that only one step occurs at a time.



Scheme 1. Proposed model for interaction of aromatase cytochrome *P*450 and NADPH-cytochrome *P*450 reductase leading to generation of aromatization intermediates and estrogen end product. See text for details. P—aromatase cytochrome *P*450, A—androstenedione substrate, R—NADPH-cytochrome *P*450 reductase, 19OHA—19-hydroxy androstenedione, 19OXOA—19-oxo androstenedione and E—estrone.

In addition, we conclude that the environment of the reconstituted multiple monooxygenase system also plays a role in the ratio of final product to recovered hydroxylated "intermediates". Our data show that with aromatase cytochrome *P*450 saturating, the amounts of both 19-oxygenated androgens are significantly different in the lipid-detergent mixed and lipid-only environments. For cytochrome *P*450 at super-saturating levels, both intermediates are significantly different for mixed relative to lipid- or detergent-only environments. The largest amount of intermediate for the cytochrome *P*450 super-saturating condition was isolated in the lipid–detergent environment, the environment which gave the highest catalytic efficiency in the reconstitution study, and the lowest amount was in the lipid-only environment, which demonstrated the lowest catalytic efficiency. It is conceivable that lipophilic partitioning of substrates, intermediates and products of the aromatization reaction may affect dissociation rates to account for the different effect of the various environments observed here.

Acknowledgements—We wish to thank the obstetrical nurses at Millard Fillmore Suburban Hospital for assisting us in procuring term placentas, Carol Culp for secretarial assistance, Jim Stamos for the preparation of the figures, and Dr Om Bahl for providing access to needed equipment. We also appreciate the helpful comments from Drs Om Bahl, Jack Fishman and Juliet Lobo regarding this manuscript. This investigation was supported by Grant HD 16593 from the National Institute of Health.

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