Articles

Purification of Human Placental Aromatase Cytochrome P-450 with Monoclonal Antibody and Its Characterization†

Nobutaka Yoshida‡ and Yoshio Osawa*

Endocrine Biochemistry Department, Medical Foundation of Buffalo Research Institute, Buffalo, New York 14203

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ABSTRACT: A simple and efficient method is described for the purification of microsomal aromatase cytochrome P-450 from human placenta. The enzyme was solubilized with Emulgen 913 and sodium cholate and subjected to chromatography on a column of Sepharose 4B coupled with a specific monoclonal antibody, followed by hydroxyapatite column chromatography. The specific cytochrome P-450 content of purified aromatase was 13.1 (12–14.8) nmol/mg of protein. Aromatase assays were carried out with reconstituted systems of bovine liver P-450 reductase and dilauroyl-L-α-phosphatidylcholine with [1β-^{3}H,4-^{14}C]-androstenedione as substrate. The specific activity of purified aromatase was 65.0 (50.6–74.3) nmol·min^{-1}·(mg of protein)$^{-1}$ or a turnover rate of 5.0 (4.3–5.9) min^{-1}. The total recovery of purified aromatase activity was 32.2%, and P-450 recovery was 17.6%. The K_m of immunoaffinity-purified aromatase was 12, 210, 41, and 2830 nM for androstenedione, 16α-hydroxyandrostenedione, testosterone, and 16α-hydroxytestosterone, respectively. The very high K_m value for 16α-hydroxytestosterone aromatization gives a reasonable indication that estriol is not the directly aromatized product in the fetoplacental unit of human pregnancy. The aromatase P-450 was subjected to SDS–polyacrylamide gel electrophoresis in increasing quantities. Silver stain detection techniques indicated a single band having a molecular mass of 55 kDa with greater than 97% purity. The stability analysis showed a half-life of over 4 years on storage at −80 °C.

Since Bagget (1956) first reported the conversion of androgen to estrogen by placental tissue, it has been demonstrated by Ryan (Ryan, 1959; Meigs & Ryan, 1968, 1971) and Thompson and Siiteri (1974a,b) that placental aromatase is a form of cytochrome P-450 which exhibits characteristics that are different from those of other P-450s. We attempted to purify aromatase so that it can be studied in greater detail. Our early studies resulted in a method of solubilization which was limited by a low recovery of aromatase activity (Bellino & Osawa, 1977). Pasanen and Pelkonen (1981) reported the purification of the P-450 fraction from placental microsomes but did not include the aromatase specific activity. Recently, Tan and Muto (1985, 1986) determined that androstenedione is necessary to stabilize aromatase activity and published an aromatase specific activity of 20 nmol·min^{-1}·(mg of protein)$^{-1}$ but were unable to eliminate a contaminant. Nakajin et al. (1986) reported a homogeneous aromatase preparation, but it was unstable, with a half-life of 2.5 days and K_m of 25 μM for androstenedione. Hagerman (1987) used substrate affinity column chromatography to obtain purified aromatase, specific activity 23 nmol·min^{-1}·(mg of protein)$^{-1}$, but the recovery was very low, only 1.2% of initial aromatase activity. More recently, Kellis and Vickery (1987) reported the purification of aromatase with a specific aromatase activity of 57 nmol·min^{-1}·(mg of protein)$^{-1}$ and 11.5 nmol of P-450/mg of protein and claimed that the purified P-450 was the only P-450 in placental microsomes. Mendelson et al. (1985) isolated a denatured 55-kDa protein by SDS–PAGE separation, produced a monoclonal antibody to it, and purified a P-450 using the monoclonal antibody column. However, they showed very poor aromatase activity (0.004 nmol·min^{-1}·mg^{-1}) in spite of the fact that the P-450 was shown without a P-420 peak. Harada (1988) reported the purification of aromatase by means of conventional methods; however, his preparation had a molecular mass of 51 000 daltons and had no measurable P-450 content. These conflicting results indicate the necessity for chemically pure, catalytically active aromatase cytochrome P-450 in a stable form.

Using the catalytically suppressive anti-human placental aromatase cyt P-450 monoclonal antibody (MAb3-2C2) produced in our laboratory (Washida et al., 1985; Kitawaki et al., 1989), we prepared the antibody-coupled Sepharose 4B column which was used to efficiently isolate the aromatase P-450 from the partially purified placental 900*g* pellets (Osawa et al., 1987a) and placental microsomes (Osawa et al., 1987b) for preliminary studies. We further developed and refined the procedures, establishing a method to yield very high aromatase specific activity and P-450 specific content with unchanged substrate affinity directly from solubilized placental microsomes.

Experimental Procedures

Materials. [1β-^{3}H]Androstenedione was purchased from Du Pont (New England Nuclear Products, Boston, MA) and [4-^{14}C]androstenedione from Amersham (Arlington Heights, IL). [1β-^{3}H,4-^{14}C]Testosterone was obtained by reduction of [1β-^{3}H,4-^{14}C]androstenedione with sodium borohydride. [1β-^{3}H]-16α-Hydroxyandrostenedione and [1β-^{3}H,4-^{14}C]-16α-hydroxytestosterone were obtained by hydroxylation of [1β-^{3}H]androstenedione and [1β-^{3}H,4-^{14}C]testosterone, re-

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* To whom correspondence should be addressed at the Endocrine Biochemistry Department, Medical Foundation of Buffalo Research Institute, 73 High St., Buffalo, New York 14203-1196.

‡ Postdoctoral Research Fellow, on leave from the Department of Obstetrics and Gynecology, Okayama University Medical School, Okayama, Japan.

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spectively, by male rat liver microsomes (Waxman et al., 1983; Morgan et al., 1985). These radioactive steroids were purified by repeated thin-layer chromatography (TLC) (Waxman et al., 1983). Androstenedione was purchased from Steraloids, Inc. (Wilton, NH). Dilauroyl-L-α-phosphatidylcholine, NADPH, dithiothreitol (DTT), EDTA, sodium cholate, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Affi-Gel Protein A MAPS II Kit and hydroxyapatite were purchased from Bio-Rad (Richmond, CA). CNBr-activated Sepharose 4B and G-25 and molecular weight standards for electrophoresis were obtained from Pharmacia (Piscataway, NJ). Emulgen 913, a high molecular weight polymer [poly(oxyethylene) nonylphenyl ether] which has a hydrophile–lipophile balance of 14.5 (Imai & Sato, 1974), was a generous gift from Kao Chemicals (Tokyo, Japan). All other reagents were of analytical grade.

Preparation of Placental Microsomes. Human placentas were obtained after delivery at local hospitals and placed on ice. The following procedures were carried out at 0–5 °C. The cotyledon tissue was severed from the chorionic plate, cut into small pieces, and rinsed thoroughly with 67 mM potassium phosphate buffer (pH 7.4) containing 1% KCl. The tissue was homogenized three times for 30 s in a Waring blender with 67 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and 0.5 mM DTT (2 g wet tissue/mL of buffer). The homogenate was centrifuged at 12000*g* for 35 min, and the supernatant was centrifuged at 125000*g* for 60 min. The resulting pellet was resuspended in 67 mM potassium phosphate buffer (pH 7.4) containing 20% gycerol, 0.1 mM EDTA, and 0.5 mM DTT and recentrifuged at 125000*g* for 45 min. The washed microsomes were resuspended in 67 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol, and 0.5 mM DTT and were stored at −80 °C.

Preparation of Monoclonal Antibodies and Immunoadsorbent. Hybridoma cells were produced by fusion of NS-1 melanoma cells to mouse spleen cells and selected and cloned as previously described (Washida et al., 1985; Kitawaki et al., 1989). The monoclonal antibody (MAb3-2C2) was produced in mouse peritoneal ascites fluid by inoculating the mouse intraperitoneally with hybridoma cells. The MAb3-2C2 in ascites fluid was purified by MAPS II Kit, after which SDS–polyacrylamide gel electrophoresis showed it to be quite pure. The monoclonal antibody (12 mg of IgG/mL of matrix) was coupled to CNBr-activated Sepharose 4B in 0.1 M sodium carbonate and 0.5 M sodium chloride (pH 8.3, coupling buffer) by shaking for 2 h at room temperature and standing overnight at 4 °C. The resin was then washed with 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl and allowed to stand overnight in this buffer to block the activated site. The following day the resin was washed with 10 mM phosphate buffer (pH 7.4) containing 4.0 M sodium chloride, followed by 0.2 M glycine buffer (pH 2.8) and 0.1 M Tris-HCl buffer (pH 8.0). The immunoaffinity resin was stored at 4 °C in 10 mM Tris-HCl (pH 8.0) containing 0.15 M sodium chloride and 0.02% sodium azide.

Solubilization and Isolation of Aromatase. The washed microsomal suspension (approximately 600 mg of protein) was thawed in an ice water bath and added to 300 mL of 10 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.5 μM androstenedione, and 20% glycerol. The mixture was centrifuged at 145000*g* for 50 min, and the resulting pellet was resuspended in 50 mL of the same buffer. To this stirred suspension were added 10% Emulgen 913 and 10% sodium cholate solutions to a final concentration of 0.15% each. The mixture was stirred at 4 °C for an additional 30 min and centrifuged at 145000*g* for 50 min. In some cases, we used a 0.45% concentration of each detergent for solubilization and diluted the mixture 3-fold, to 0.15%, before centrifugation. We did not observe any significant difference between the two methods. The supernatant was allowed to pass slowly overnight at 5 °C through a preequilibrated column of MAb3-2C2–Sepharose 4B (1.6 i.d. × 2.0 cm). The column was then washed with 90 mL of 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.5 μM androstenedione, 20% glycerol, and 0.15% Emulgen 913 (equilibration buffer) at a flow rate of 1.0 mL/min, followed by 20 mL of equilibration buffer containing 0.5 M sodium chloride. The immunopurified aromatase P-450 was eluted with a linear gradient in which the concentration of sodium chloride in the equilibration buffer was increased from 0.5 to 4.0 M (40 mL, 1 mL/min), followed by washing with 4.0 M NaCl (20 mL). The fractions from the 2.0–4.0 M eluate (40 mL) were combined and applied to a Sephadex G-25 column (2.5 i.d. × 90 cm) preequilibrated with equilibration buffer containing 0.5 mM DTT and eluted with the same buffer. The desalted protein fraction was then applied overnight on a hydroxyapatite column (1.6 i.d. × 1.0 cm) preequilibrated with the same buffer, and the column was washed with the equilibration buffer (45 mL, 0.5 mL/min). The aromatase P-450 was eluted with a linear gradient with a potassium phosphate concentration of 10–200 mM in the equilibration buffer containing 0.5 mM DTT. Absorbance at 280 nm showed a single protein peak at approximately 70 mM phosphate. This area was pooled and stored at −80 °C.

It was necessary to remove Emulgen 913 in order to measure the oxidized spectrum and to determine the effect of Emulgen concentration on aromatase activity. Therefore, after reducing the phosphate concentration to less than 10 mM, the purified aromatase was applied to a hydroxyapatite column. The column was washed with 10 mM potassium phosphate buffer containing 20% glycerol, 0.1 mM EDTA, 0.5 μM androstenedione, and 0.5 mM DTT, and the aromatase was eluted with 200 mM phosphate buffer containing glycerol, EDTA, androstenedione, and DTT.

Preparation of Reductase. Cytochrome P-450 reductase was purified from bovine liver microsomes according to Yasukochi and Masters (1976) with some modifications (Osawa et al., 1981; Bellino, 1982). Reductase was measured as NADPH–cytochrome *c* reductase activity. The microsomal fractions were extracted by addition of 0.15% Emulgen 913 with stirring for 30 min, followed by centrifugation at 145000*g* for 50 min. The supernatant was applied to a DEAE-cellulose (DE-52) column (5.0 i.d. × 10 cm) preequilibrated with 10 mM potassium phosphate buffer containing 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, and 0.15% Emulgen 913. The column was washed with 0.1 M potassium chloride in the equilibration buffer. The reductase-rich fractions were eluted with 0.3 M potassium chloride in the equilibration buffer and were desalted on a Sephadex G-25 column (2.5 i.d. × 90 cm) with the equilibration buffer. The activity-rich fractions were applied to a 2′,5′-ADP–Sepharose 4B column (1.6 i.d. × 15 cm) preequilibrated with the equilibration buffer. The column was washed with the same buffer and eluted with the same buffer containing 2 mM 5′-AMP. A Sephadex G-25 column (2.5 i.d. × 90 cm) was used to remove 5′-AMP from the resulting reductase fraction. This material was applied to a DEAE-cellulose column (1.6 i.d. × 2.0 cm) which was then washed with equilibration buffer, equilibration buffer without Emulgen 913, and 0.1 M Tris-HCl buffer (pH 7.0) in the equilibration buffer without Emulgen 913. The reductase was

Table I: Purification of Aromatase Cytochrome P-450[a]

	total protein (mg)	aromatase specific activity[b] (nmol·min^{-1}·mg^{-1})	aromatase total activity (nmol/min)	cytochrome P-450 specific content[c] (nmol/mg)	cytochrome P-450 total (nmol)	turnover rate of estrogen biosynthesis (min^{-1})
microsomes	678 ± 102	0.174 ± 0.027	122 ± 29	0.0701 ± 0.0241	46.1 ± 18.4	2.96 ± 1.51
microsomal extract	385 ± 88	0.438 ± 0.073	167 ± 30	0.121 ± 0.034	46.9 ± 20.1	3.84 ± 1.06
immunoaffinity column	1.28 ± 0.16	48.0 ± 17.7	59.6 ± 16.6	8.42 ± 2.50	10.7 ± 2.3	5.39 ± 1.59
hydroxyapatite column	0.613 ± 0.117	65.0 ± 12.7	39.3 ± 8.5	13.1 ± 1.5	8.10 ± 2.22	4.97 ± 0.89

[a] The values represent the mean ± SD of three preparations. [b] Aromatase activity was measured with 120 nM bovine liver P-450 reductase and 16 μM dilauroyl-L-α-phosphatidylcholine as described under Experimental Procedures. Specific activity of purified aromatase was 50.6–74.3 nmol·min^{-1}·mg^{-1}. [c] The P-450 content was based on $\Delta\epsilon$ 450–490 = 91 mM^{-1}·cm^{-1}. The peak at 448 nm for microsomes was measured after equilibration with 0.0375% Emulgen 913, 0.125 μM androstenedione, and 49 μM 19-norandrostenedione. Specific content of purified aromatase P-450 was 12.0–14.8 nmol/mg.

then eluted with a linear gradient of Tris-HCl buffer of 0.1–0.3 M concentration. Specific activity of the purified reductase was ~16–20 μmol of cytochrome *c* reduced·min^{-1}·(mg of protein)$^{-1}$.

Aromatase Assays. The standard incubation contained 16 μM dilauroyl-L-α-phosphatidylcholine, 120 nM bovine cytochrome P-450 reductase, 10% glycerol, and substrate in a final volume of 1.0 mL of 100 mM potassium phosphate buffer (pH 7.4) containing 0.00075% Emulgen 913. Incubations were carried out in air at 37 °C in a shaking water bath. After addition of 5 μL of purified aromatase P-450 preparation, the incubation was initiated by addition of 600 nmol of NADPH in 0.1 mL of 100 mM phosphate buffer. The conversion of androstenedione was measured by the [^{3}H]water method using [1β-^{3}H,4-^{14}C]androstenedione as described previously (Osawa et al., 1989; Osawa & Spaeth, 1971). We have confirmed that the cross-reconstituted aromatase produces estrogens by the same stereospecific 1β-hydrogen elimination mechanism as in placental microsomes (Osawa et al., 1987c). Kinetic studies of aromatase were conducted by use of [1β-^{3}H]-androstenedione (5–86 nM), [1β-^{3}H,4-^{14}C]testosterone (15–678 nM), [1β-^{3}H]-16α-hydroxyandrostenedione (25–1600 nM), or [1β-^{3}H,4-^{14}C]-16α-hydroxytestosterone (890–14200 nM) as substrate. The K_m and V_{max} values were analyzed by Lineweaver–Burk plots of the data.

Cytochrome P-450. Absorbance spectra were determined with either a Varian Instruments Cary 2290 or an SLM Aminco DW-2C recording spectrometer. Measurements of cytochrome P-450 were carried out at room temperature in 100 mM potassium phosphate buffer containing 0.1 mM EDTA, 20% glycerol, 0.125 μM androstenedione, 0.125 mM DTT, and 49 μM 19-norandrostenedione. The spectra were recorded both by the CO reduced minus CO according to the method of Greim (1970) and by the reduced CO minus reduced according to the method of Omura and Sato (1964a,b) with the addition of few grains of sodium dithionite as a reducing agent. The specific P-450 content was determined with a molar extinction difference of 91 mM^{-1}·cm^{-1} between 450 and 490 nm, and the specific P-420 content was determined with a molar extinction difference of 111 mM^{-1}·cm^{-1}.

Other Procedures. SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) using silver stain for detection. Protein concentrations were determined by the modified method of Lowry et al. (1951) after precipitation of the protein by trichloroacetic acid (Bensadoun & Weinstein, 1976). Bovine serum albumin was used as standard. Radioactivity was determined on a Packard Model 4640 liquid scintillation spectrophotometer.

Results

Solubilization of Microsomes. The effectiveness of Emulgen 913 and sodium cholate, at 0.15% concentration, as detergents for extracting aromatase activity from the microsomal membrane was compared against protein concentration. The total aromatase activity extracted in the supernatant of placental microsomes is shown in Figure 1. Less than 10% of the initial activity was found in the supernatant solubilized by using sodium cholate alone. However, we found that over 100% of the starting activity was extracted in the supernatant when solubilized with a mixture of Emulgen 913 plus sodium cholate at a lower concentration of microsomal protein (Suhara et al., 1987). We did not see an appreciable difference in solubilization with 0.15% and 0.2% detergent concentration or with 0.45% detergent concentration followed by 3-fold dilution. From these results we selected 3 mg of microsomal protein/mL at 0.15% each of Emulgen 913 and sodium cholate as a suitable condition of solubilization.

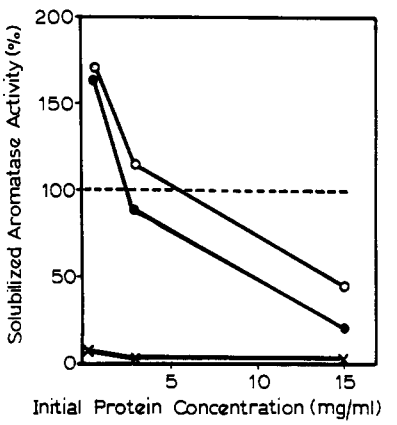

FIGURE 1: Effect of protein concentration for solubilization of aromatase: Microsomes were solubilized with 0.1% detergent at various protein concentrations for 30 min and then centrifuged at 145000*g* for 50 min. Initial microsomal aromatase activity was taken as 100% (assayed under reconstitution conditions). Detergents used were Emulgen 913 + sodium cholate (○), Emulgen 913 (●), and sodium cholate (×).

Purification of Aromatase. The aromatase P-450 specific monoclonal antibody affinity column was instrumental in removing nonspecific, nonbinding material including other cytochrome P-450 from the solubilized microsomes. Elution of aromatase P-450 from the column by varying the pH of the medium to either acidic or basic resulted in inactivation of the enzyme. A high salt gradient in equilibration buffer eluted aromatase P-450 in catalytically active form. After immediate desalting by passing through a Sephadex G-25 column, the aromatase fraction was applied to a hydroxyapatite column for the concentration of protein and elimination of minor contaminants. Only one peak at approximately 70 mM phosphate concentration was observed on gradient elution. Table I summarizes the results of each step of the purification procedure. The aromatase activity was recovered with a 32% overall yield and 370-fold purification, having a specific activity of 65 (50.6–74.3) nmol·min^{-1}·(mg of protein)$^{-1}$ and a specific

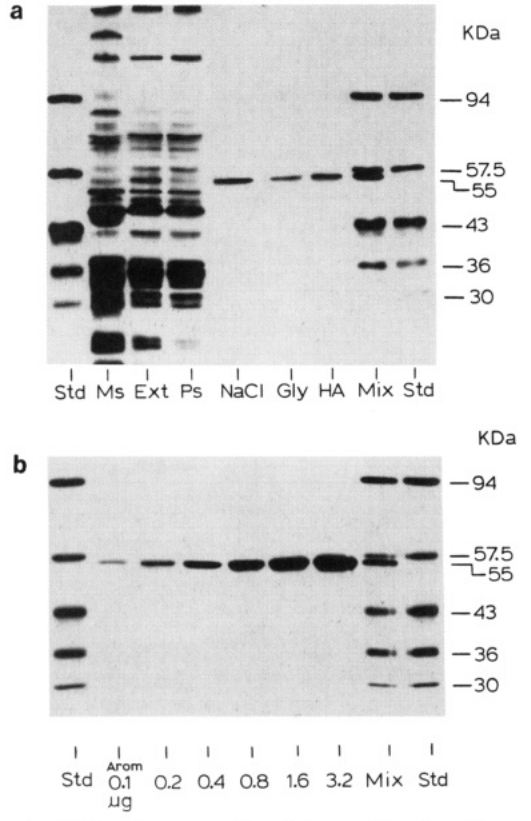

FIGURE 2: SDS–polyacrylamide gel electrophoresis of immunoaffinity-purified aromatase detected by silver stain. (a) Std = standard proteins: carbonic anhydrase, 30 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; ovalbumin, 43 kDa; catalase, 57.5 kDa; and phosphorylase *b*, 94 kDa. Ms = microsomes (102 μg of protein). Ext = Emulgen–cholate extract (59.4 μg of protein). Ps = immunoaffinity chromatography pass-through fraction (52.4 μg of protein). NaCl = immunoaffinity chromatography sodium chloride (2.0–4.0 M) eluant (0.25 μg of protein). Gly = immunoaffinity chromatography 0.2 M glycine (pH 2.8) eluant (0.2 μg of protein). HA = purified aromatase P-450 hydroxyapatite chromatography eluant (0.22 μg of protein). Mix = standard proteins plus purified aromatase P-450 (HA, 0.22 μg of protein). (b) Std = standard proteins as in (a). Arom 0.1 μg = purified aromatase P-450, hydroxyapatite eluant (0.1 μg of protein). 0.2 = same (0.2 μg of protein). 0.4 = same (0.4 μg of protein). 0.8 = same (0.8 μg of protein). 1.6 = same (1.6 μg of protein). 3.2 = same (3.2 μg of protein). Mix = standard proteins plus purified aromatase P-450 (0.2 μg of protein).

P-450 content of 13.1 (12.0–14.8) nmol·(mg protein)$^{-1}$. Figure 2a shows the SDS–polyacrylamide gel electrophoresis in which microsomes, microsomal extract, the pass-through fraction, and purified aromatase were subjected to silver staining. The molecular size of the aromatase P-450 was determined to be 55 kDa.

The gel showed that the 55-kDa protein (aromatase P-450) was enhanced after extraction with the detergents and was mostly adsorbed by the immunoaffinity resin. After elution of the immunoaffinity column with 10 mM phosphate buffer containing 4.0 M sodium chloride, the column was washed with 0.2 M glycine buffer (pH 2.8). The 0.2 M glycine fraction had the same 55-kDa protein band as the sodium chloride eluate but contained very low aromatase activity (0.157–0.208 nmol·min^{-1}·mg^{-1}). Purity of the aromatase P-450 preparation was analyzed by SDS–PAGE when 0.1–3.2-μg samples of aromatase P-450 were applied to the same gel plate. As shown in Figure 2b no detectable contamination was observed even at the highest concentration, indicating a homogeneity of greater than 97% pure.

Stability of Aromatase. Analysis of aromatase activity over an extended period showed that the purified aromatase P-450 was very stable, with a half-life of greater than 4 years when stored at −80 °C in 70 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 20% glycerol, 0.5 μM androstenedione, 0.5 mM DTT, and 0.15% Emulgen 913.

Table II: Comparison of the K_m and the V_{max}[a]

substrate	K_m (nM) microsomes	K_m (nM) purified aromatase	V_{max} (nmol·min^{-1}·mg^{-1})[b] microsomes	V_{max} (nmol·min^{-1}·mg^{-1})[b] purified aromatase
androstenedione	10	12	0.33	71
16α-hydroxy-androstenedione	94	210	0.17	40
testosterone	39	41	0.18	66
16α-hydroxytesto-sterone	3000	2830	0.24	5.6

[a] Kinetic analysis was conducted as described under Experimental Procedures. [b] The K_m and V_{max} were analyzed by Lineweaver–Burk plots.

Table III: Reconstitution of Aromatase Activity[a,b]

omission from complete system	pmol of estrone/min	% activity as compared to complete system
none	4.79	100
phosphatidylcholine	2.98	62
Emulgen 913 (0.00075%)[c]	3.17	66
Emulgen 913[c] + phosphatidylcholine	1.85	39
reductase	0.00	
aromatase P-450	0.00	
NADPH	0.00	

[a] The complete system (see footnote *b*) contains 0.00075% Emulgen, 16 μM dilauroyl-L-α-phosphatidylcholine, 100 nM cytochrome P-450 reductase, and 600 μM NADPH, as described under Experimental Procedures. [b] A 0.005-mL aliquot of purified aromatase P-450 preparation was used as standard incubation. [c] Emulgen was eliminated by use of a hydroxyapatite column in order to evaluate its effect on purified aromatase.

Characterization of Reconstituted Aromatase. Examination of the effects of aromatase P-450 concentration and incubation time indicated that the aromatase specific activity was constant within 50–300 ng of P-450/mL incubation volume, and within a 10-min incubation time (data not shown). The effect of reductase concentration was also examined, since other reports (Kellis & Vickery, 1987; Hagerman & Kautsky, 1982; Bellino & Osawa, 1980) indicated a need for at least 100 nM reductase per assay of aromatase. We found that 120 nM reductase concentration, as described under Experimental Procedures, gives the most stable and desirable aromatase activity under our reconstitution conditions. Kinetic analysis of androgen aromatization using androstenedione, testosterone, 16α-hydroxyandrostenedione, or 16α-hydroxytestosterone as substrate was conducted on both placental microsomes and purified aromatase, and the results are summarized in Table II. The immunoaffinity-purified P-450 cross-reconstituted aromatase gave an apparent K_m of 12 nM for androstenedione, 41 nM for testosterone, 126 nM for 16α-hydroxyandrostenedione, and 2830 nM for 16α-hydroxytestosterone. The very high K_m for 16α-hydroxytestosterone indicates that under competitive aromatization among androgens there is no direct formation of estriol.

Aromatase preparations missing individual components were assayed and compared to the complete system. As shown in Table III, reductase, cyt P-450, and NADPH were essential for the activity. Removal of either phosphatidylcholine or Emulgen 913 resulted in a loss of approximately one-third of the activity, and elimination of both substances resulted in a loss of approximately two-thirds of the activity. To exhibit

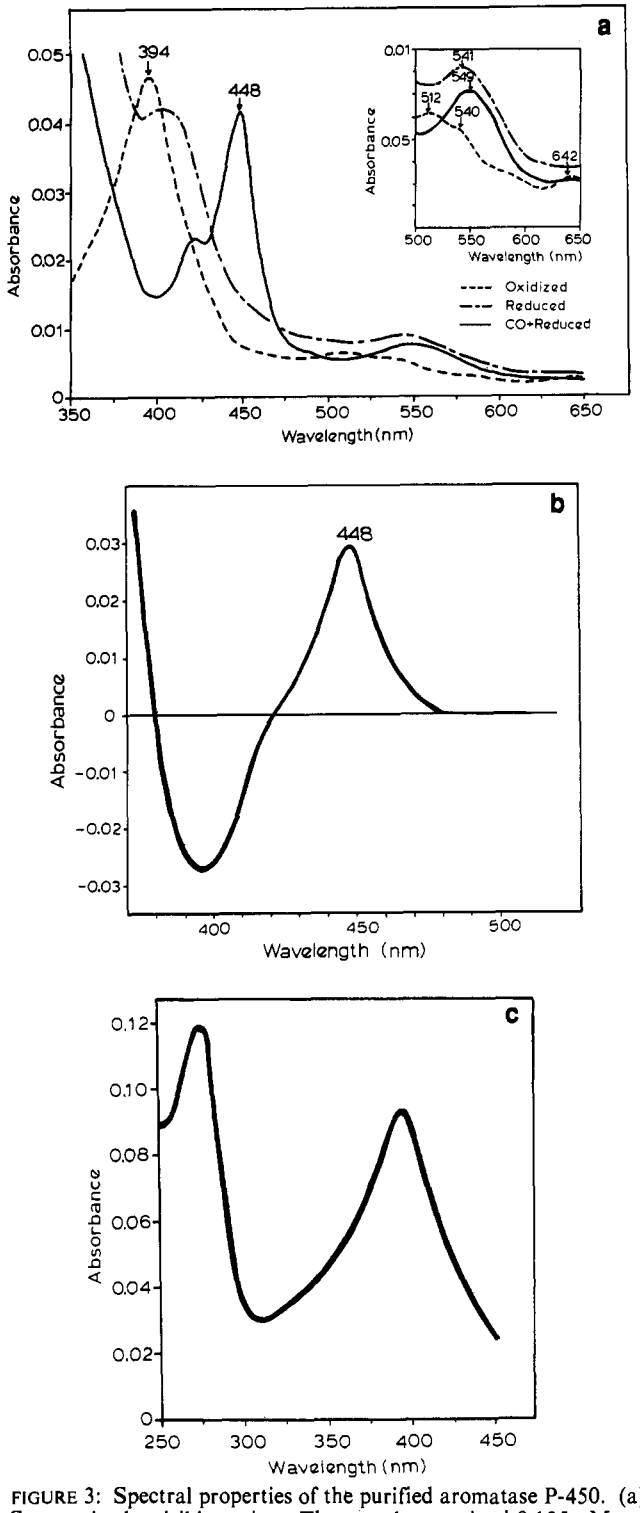

FIGURE 3: Spectral properties of the purified aromatase P-450. (a) Spectra in the visible region. The sample contained 0.125 μM androstenedione, 49 μM 19-norandrostenedione, and 0.0375% Emulgen 913. The specific P-450 content of the sample was 12.0 nmol/mg of protein, and the protein concentration was 41 μg/mL. (b) CO reduced minus CO difference spectrum of the purified aromatase P-450. The specific P-450 content is 14.8 nmol/mg, and protein concentration is 22 μg/mL. (c) Oxidized spectrum in the ultraviolet–visible region. The sample contained 0.5 μM androstenedione in the absence of Emulgen 913. The ratio of A_{275}/A_{394} was found to be 1.27.

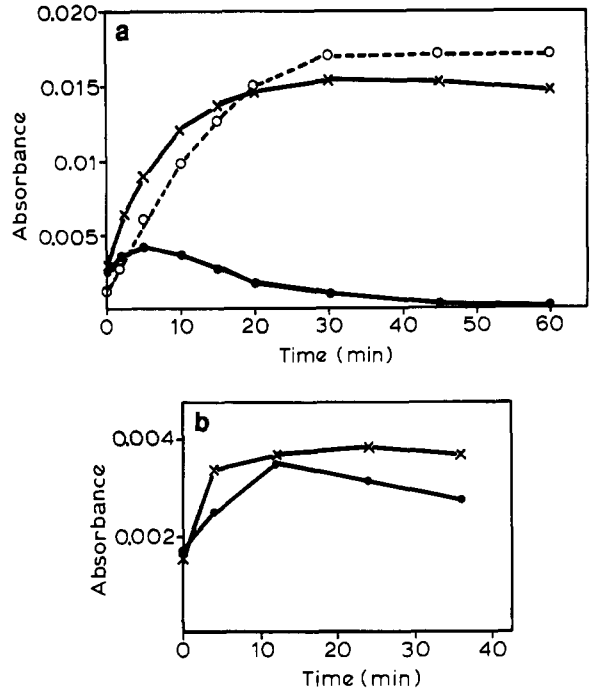

FIGURE 4: CO difference spectra with and without 19-norandrostenedione. (a) Purified aromatase P-450 containing 0.17 μM androstenedione, 0.05% Emulgen 913, 20% glycerol, and with (×, P-450-490) or without (●, P-450-490; ○, P-420-490) 49 μM 19-norandrostenedione. (b) Microsomal fraction containing 0.125 μM androstenedione, 0.0375% Emulgen 913, and 20% glycerol, with (×) or without (●) 49 μM 19-norandrostenedione (P-450-490).

full activity, purified aromatase P-450 requires proper amounts of cytochrome P-450 reductase, NADPH, phospholipid, and detergent.

Absorption Spectra of Aromatase. The absolute spectra of immunoaffinity-purified aromatase P-450 are shown in Figure 3a. In the oxidized state, the Soret maximum of the aromatase P-450–androstenedione complex is at 394 nm and shows a minor maximum at 512 and 642 nm with a shoulder at 540 nm. These results demonstrate a typical "type I" spectrum. After reduction of the aromatase by dithionite, the minor maximum shifted to a minor peak of 541 nm. Subsequent bubbling of CO in the reduced aromatase caused the Soret peak to shift to 448 nm and a minor maximum to 549 nm. Figure 3b shows the CO-reduced difference spectra (CO-reduced minus CO), with the maximum at 448 nm. The P-450 content was 14.8 nmol/mg of protein, which is 81.4% of the expected value (18.2 nmol/mg) as calculated from the molecular weight of pure heme protein. The ultraviolet spectrum and the Soret maximum were measured after removal of Emulgen 913 since the Emulgen 913 has an absorption maximum at 280 nm. The results are shown in Figure 3c. The aromatase–androstenedione complex had the same Soret maximum of 394 nm, and the absorption maximum of ultraviolet region was at 275 nm. The A_{275}/A_{394} ratio (protein vs heme) was 1.27.

The purified aromatase P-450 is very unstable in the absence of 19-norandrostenedione and shifts completely to a P-420 peak within 30 min after addition of dithionite, as shown in Figure 4a. This phenomenon is not observed with microsomes, as indicated in Figure 4b. We therefore measured the aromatase P-450 content after the addition of 49 μM 19-norandrostenedione. The specific content of P-450 in the purified preparation was 13.1 (12.0–14.8) nmol/mg, and the turnover number of estrogen biosynthesis was 5.0 (4.2–5.9) min^{-1}. The overall recovery rate of P-450 was 17.6%, retaining 32.2% of the total initial aromatase activity.

Discussion

An efficient immunoaffinity chromatography method was developed to isolate aromatase cytochrome P-450 from human

Table IV: Comparison of Methods and Results of the P-450 Purification from Human Placental Microsomes

	Mendelson et al. (1985)	Tan and Muto (1986)	Nakajin et al. (1986)	Hagerman (1987)	Kellis and Vickery (1987)	Harada (1988)	Yoshida and Osawa
androstenedione (μM)	20	10		100	2	30	0.5
detergent	1.2% cholate	1% Chaps	0.6% cholate, 0.35% Em 913[a]	1% cholate	1% cholate	0.4% cholate	0.15% cholate, 0.15% Em 913
solubilized aromatase activity (%)		70.6		51.2	68.4	98	137
purification method	phen-Seph, Bio-SM2, DE-cell., immun	AH-Seph, ConA-Seph, HA, AH-Seph	DE-cell., HA, octyl-agar, HA	DE-tris, dialyze, test.-agar	$(NH_4)_2SO_4$ ppt, dialyze, phen-Seph, dialyze, DE-cell., HA	OA-Seph, dialyze, HA, dialyze, Mono S, HA-HCA, dialyze, Mono Q, HA-HCA	immun, G-25, HA
purified aromatase SA (nmol·min^{-1}·mg^{-1})	0.004	19.9	0.9	23.3	57.0	103	65.0
aromatase yield (%)		9.1	0.9	1.2	12.1	14	32.2
protein yield (%)		0.065	0.15	0.0064	0.039	0.3	0.09
cytP-450 content (nmol/mg)		4.2	0.07	0.67	11.5	0	13.1
P-450 yield (%)		2.9		0.10	8.6	0	17.3
turnover (min^{-1})		4.8	13.3	35.0	5.0		5.0
K_m (androstenedione) (nM)			30000	1.4	60	100	12
molecular mass (KDa)	55	55	55	50	55	51	55

[a] Abbreviations used are as follows: Em 913 = Emulgen 913, phen-Seph = phenyl-Sepharose column; Bio-SM2 = Bio-Rad SM2 column; DE-cell. = DEAE-cellulose; Immun = immunoaffinity column; AH-Seph = AH-Sepharose column; ConA-Seph = ConA–Sepharose column; HA = hydroxyapatite column; octyl-agar = octylaminoagarose column; DE-tris = DEAE-trisacrylamide column; test-agar = testosterone–agarose substrate affinity column; OA-Seph = octylamino-Sepharose 4B column; HA-HCA = hydroxyapatite HCA column; $(NH_4)_2SO_4$ ppt = ammonium sulfate precipitation method; and SA = specific activity of aromatase.

placental microsomes. The choice of a monoclonal antibody which interacts only with the catalytically active form of aromatase P-450 (Kitawaki et al., 1989) and dissociation of the interaction by high salt with little damage to the catalytic activity served to isolate the P-450 with full catalytic capability. The entire procedure takes only 3 days, starting from microsomes, to obtain a concentrated stabilized solution with a half-life of over 4 years on storage. The major influencing factors and results of aromatase P-450 isolation from human placental microsomes as published in the literature (Tan & Muto, 1986; Nakajin et al., 1986; Hagerman, 1987; Kellis & Vickery, 1987; Mendelson et al., 1985; Harada, 1988) are compared with those of this report in Table IV. Investigators who used only sodium cholate as detergent for solubilization of microsomal aromatase tended to obtain lower recovery rates of aromatase activity (Hagerman, 1987; Kellis & Vickery, 1987; Mendelson et al., 1985). We found that a detergent combination of Emulgen 913 and sodium cholate in the presence of androstenedione solubilizes aromatase quantitatively and in catalytically active form. As shown in Table III, a low concentration of Emulgen and phosphatidylcholine gave the most active aromatase system.

Since earlier studies (Thompson & Siiteri, 1974b; Zachariah & Juchau, 1975) suggested that all of the measurable P-450 in placental microsomes belonged to aromatase, the purification of aromatase has been attempted repeatedly with a goal of increasing the specific content of P-450. Initially, the placental P-450 was purified by an ammonium sulfate precipitation method (Hodgson & Jachau, 1977) following phenyl-Sepharose and DEAE column chromatography (Pasanen & Pelkonen, 1981). These methods raised the cytochrome P-450 content but not the specific activity of aromatization. Recently, androstenedione was added to stabilize aromatase activity, as has been reported in the purification of another P-450 (Takemori et al., 1975; Yu et al., 1974). Tan and Muto (1985) prepared a ConA–Sepharose column for purification of aromatase and suggested that aromatase was a glycoprotein, but could not resolve it to a single protein (Table IV). Subsequently, substrate affinity chromatography was used by Hagerman (1987) for purification, which succeeded in raising the specific aromatase activity. However, addition of androstenedione was necessary as a stabilizer for aromatase. With this addition, the recovery of pure aromatase protein decreased to less than 0.0064% as compared to 0.09% with our method. Most recently, Kellis and Vickery (1987) reported good results using the conventional method for purification of human placental cytochrome P-450 with increased aromatase specific activity.

Immunopurification methods for cytochrome P-450 have been reported (Friedman et al., 1983; Cheng et al., 1984); however, the catalytically competent enzyme was not isolated. Mendelson et al. (1987) reported a 55-kDa protein specific monoclonal antibody which did not suppress aromatase activity. They used the monoclonal antibody based immunoaffinity column for purification of placental aromatase cytochrome P-450 and eluted with NaCl (2 M) and with glycine buffer (0.2 M, pH 3.0). The purified enzyme showed, however, extremely low specific activity (0.004 nmol·min^{-1}·mg^{-1}) of aromatase in both fractions. They observed a clear cytochrome P-450 peak in the glycine buffer eluant. We, on the other hand, did not find a cytochrome P-450 peak in the glycine buffer eluant.

In the past, aromatase has been reported by most groups to have a molecular mass of 55 000 daltons. Harada (1988) reported a 51 000-dalton protein which showed high aromatase activity (103 nmol·min^{-1}·mg^{-1}) and no measurable P-450 peak. Evaluation of his data is complicated by the fact that he used only a UV detection method to determine aromatase specific activity. Although we have isolated a 50 000-dalton protein which exhibited a P-450 peak and showed high benzphetamine demethylase activity (154 nmol·min^{-1}·mg^{-1}; Osawa et al., 1988) with no aromatase activity, we assume that it is not identical with that of Harada.

We were able to obtain highly active immunoaffinity-purified aromatase P-450 which showed a molecular mass of 55 kDa upon SDS–polyacrylamide gel electrophoresis and did not give any indication of contamination, even when a 32-fold higher amount of protein was analyzed. The cytochrome P-450 peak was clearly observed having no P-420 peak. However, when the immunoaffinity column was further eluted with 0.2 M glycine, the eluate showed a cytochrome P-420 peak (4.2 nmol/mg) with no P-450 peak and a molecular size of 55 kDa, and the aromatase activity (0.18 nmol·min^{-1}·mg^{-1}) was markedly decreased. This fraction is considered to be

inactivated aromatase P-450. From these results alone, one could not eliminate the possibility that a cytochrome P-450 other than aromatase could overlay the aromatase band at 55 kDa on SDS gel electrophoresis. To eliminate this possibility, an immunoaffinity purification method using other aromatase P-450 specific epitopes might be necessary. At this stage, we are relying on the specificity of the activity-suppressing monoclonal antibody, MAb3-2C2, to aromatase P-450.

When the purified and reconstituted aromatase was incubated with [19-$^3H_3$,4-^{14}C]androstenedione and the products were subjected to 2D TLC followed by autoradiography, the major product was estrone, with some formation of 1β- and 2β-hydroxyandrostenedione as the metabolic switching products of aromatase (Osawa et al., 1987b). When placental microsomes are incubated under similar conditions, these compounds plus many unidentified products are detected, indicating the presence of other androgen-metabolizing enzymes in the microsomes. Therefore, the K_m values of immunoaffinity-purified aromatase for androstenedione, testosterone, 16α-hydroxyandrostenedione, and 16α-hydroxytestosterone were determined and compared with those obtained for microsomes. The results showed, as in Table II, that immunoaffinity-purified aromatase P-450 retained the characteristics of the natural membrane-bound form of aromatase. These results were quite different from the reports of others which showed a rise in the K_m when they solubilized and purified aromatase activity (Nakajin et al., 1986; Chikhaoui et al., 1985).

Following separation of hydroxysteroid dehydrogenase and other contaminating enzymes from aromatase, the kinetic study showed a clear picture of 16α-hydroxytestosterone aromatization. The V_{max} was found to be 1 order of magnitude less than that for other natural substrates, and the K_m was 1–2 orders of magnitude higher than that for other androgens. This indicates that aromatization of 16α-hydroxytestosterone is practically negligible in the presence of other androgens in the fetoplacental unit and therefore estriol is not the direct product of placental aromatization. It has been established that, in estrogen formation during human pregnancy, the phenolic pathway (16α-hydroxylation of estrogens) is a minor pathway and the neutral pathway (aromatization of 16α-hydroxylated androgens) is dominant (Diczfalusy & Mancuso, 1969). In this neutral pathway there are two possible routes, namely, aromatization of 16α-hydroxytestosterone directly to estriol or aromatization of 16α-hydroxyandrostenedione to 16α-hydroxyestrone, followed by reduction of the 17-ketone to estriol. From the kinetic data of the purified aromatase presented in Table II, we deduce that almost all of the estriol production in human pregnancy must come from the aromatization of 16α-hydroxyandrostenedione followed by the reduction of 16α-hydroxyestrone.

In previous studies we separated solubilized placental aromatase into two active aromatase forms by column chromatography. The major form, aromatase II, was unable to produce estriol, and the minor form, aromatase I, maintained estriol formation from 16α-hydroxytestosterone (Osawa et al., 1982). The difference may have been derived from complex formation in aromatase I with non-aromatase enzyme systems catalyzing oxidation of 16α-hydroxytestosterone to 16α-hydroxyandrostenedione and reduction of 16α-hydroxyestrone to estriol.

Juchau et al. (1976) reported that the effect of androstenedione on the binding of carbon monoxide is due to an allosteric change in the apoprotein of aromatase P-450 and that the displacement of carbon monoxide from NADPH-reduced (not dithionite-reduced) human placental microsomal P-450 by androstenedione was reversed by 19-norandrostenedione (Zachariah & Juchau, 1975; Juchau & Zachariah, 1975; Juchau et al., 1976). Kellis and Vickery (1987) claimed that androstenedione did not allow carbon monoxide to ligate the heme iron of aromatase P-450, and they assayed cytochrome P-450 using the shift of the Soret adsorption band. With these methods the turnover ratio of aromatase P-450 should be the same in each purification step, yet it fluctuated from 2.2 to 5.0. They also claimed that aromatase P-450 is the only P-450 of placental microsomes. In our studies when the purified aromatase P-450 was measured with androstenedione and without 19-norandrostenedione, the P-450 seemed to be able to ligate the carbon monoxide (CO), but after reduction by dithionite the 450-nm peak quickly shifted to 420 nm. On the other hand, when the purified aromatase P-450 was measured with 49 μM 19-norandrostenedione, the 450-nm peak was stable for at least 30 min. These phenomena were not observed in the P-450 assay of the crude fractions. The reason for this is still obscure. To ensure the stability of the 450-nm peak, we assayed all of the P-450s of this series in the presence of 49 μM 19-norandrostenedione. The turnover rate of biosynthesis was increased in the immunopurified fraction and decreased in the immunoaffinity column pass-through fraction (data not shown), which indicates the existence in placental microsomes of P-450 other than aromatase P-450. Indeed, we have recently isolated another P-450 from the pass-through fraction of this immunoaffinity chromatography which shows no aromatase activity but has high benzphetamine demethylase activity (Osawa et al., 1988).

Kellis and Vickery (1987) also showed a shift of the absolute P-450 spectrum after addition of cyanide with androstenedione (Kellis et al., 1987), but this phenomenon was not shown by carbon monoxide with 19-norandrostenedione. Moreover, they reported that the ratio of absorbance A_{280}/A_{394} (protein/heme) of pure aromatase P-450 was 1.53, while our result with pure aromatase P-450 was 1.27, which indicates a further improvement of the heme protein purity. Our purification methods of human placental aromatase resulted in higher purity and recovery rate of the P-450 with far better stability of catalytic activity by means of a faster and simpler procedure.

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References

Bagget, B., Engel, L. L., Savard, K., & Dorfman, R. I. (1956) *J. Biol. Chem. 221*, 931–941.

Bellino, F. L. (1982) *J. Steroid Biochem. 17*, 261–270.

Bellino, F. L., & Osawa, Y. (1977) *J. Steroid Biochem. 9*, 219–228.

Bellino, F. L., & Osawa, Y. (1980) *J. Steroid Biochem. 13*, 339–344.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Cheng, K. C., Gelboin, H. V., Song, B. J., Park, S. S., & Friedman, F. K. (1984) *J. Biol. Chem. 259*, 12279–12284.

Chikhaoui, Y., Chitour, F., Boussioux, A., Nicolas, J., Descomps, B., & Crastes de Paulet, A. (1985) *J. Steroid Biochem. 22*, 727–731.

Diczfalusy, E., & Mancuso, S. (1969) in *Foetus and Placenta* (Klopper, A., & Diczfalusy, E., Eds.) pp 191–248, Blackwell

Scientific Publications, Oxford and Edinburgh.
Friedman, F. K., Robinson, R. C., Park, S. S., & Gelboin, H. V. (1983) *Biochem. Biophys. Res. Commun. 116*, 859–865.
Greim, H. (1970) *Naunyn-Schmiedeberg's Arch. 266*, 260–275.
Hagerman, D. D. (1987) *J. Biol. Chem. 262*, 2398–2400.
Hagerman, D. D., & Kautsky, M. P. (1982) *Fed. Proc., 41*, 883.
Harada, N. (1988) *J. Biochem. 103*, 106–113.
Hodgson, E., & Juchau, M. R. (1977) *J. Steroid Biochem. 8*, 669–675.
Imai, Y., & Sato, R. (1974) *J. Biochem. 75*, 689–697.
Juchau, M. R., & Zachariah, P. K. (1975) *Biochem. Biophys. Res. Commun. 65*, 1026–1032.
Juchau, M. R., Mirkin, D. L., & Zachariah, P. K. (1976) *Chem.-Biol. Interact. 15*, 337–347.
Kellis, J. T., & Vickery, L. E. (1987) *J. Biol. Chem. 262*, 4413–4420.
Kellis, J. T., Childers, W. E., Robinson, C. H., & Vickery, L. E. (1987) *J. Biol. Chem. 262*, 4421–4426.
Kitawaki, J., Yoshida, N., & Osawa, Y. (1989) *Endocrinology 124*, 1417–1423.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Meigs, R. A., & Ryan, K. J. (1968) *Biochim. Biophys. Acta 165*, 476–482.
Meigs, R. A., & Ryan, K. J. (1971) *J. Biol. Chem. 246*, 83–87.
Mendelson, C. R., Wright, E. E., Evans, C. T., Porter, J. C., & Simpson, E. R. (1985) *Arch. Biochem. Biophys. 243*, 480–491.
Morgan, E. T., MacGeoch, C., & Gustafsson, J.-A. (1985) *J. Biol. Chem. 260*, 11895–11898.
Nakajin, S., Shinoda, M., & Hall, P. F. (1986) *Biochem. Biophys. Res. Commun. 134*, 704–710.
Omura, T., & Sato, R. (1964a) *J. Biol. Chem. 239*, 2370–2378.
Omura, T., & Sato, R. (1964b) *J. Biol. Chem. 239*, 2379–2385.
Osawa, Y., & Spaeth, D. G. (1971) *Biochemistry 10*, 66–71.
Osawa, Y., Higashiyama, T., & Nakamura, T. (1981) *J. Steroid Biochem. 15*, 449–452.
Osawa, Y., Tochigi, B., Higashiyama, T., Yarborough, C., Nakamura, T., & Yamamoto, T. (1982) *Cancer Res.* (Suppl.) *42*, 3299s–3306s.
Osawa, Y., Higashiyama, T., Fronckowiak, M., Yoshida, N., & Yarborough, C. (1987a) *J. Steroid Biochem. 27*, 781–789.
Osawa, Y., Yoshida, N., Fronckowiak, M., & Kitawaki, J. (1987b) *Steroids 50*, 11–28.
Osawa, Y., Yoshida, N., Fronckowiak, M., & Higashiyama, T. (1987c) *Steroids 50*, 649–650.
Osawa, Y., Higashiyama, T., & Yoshida, N. (1988) 8th International Congress of Endocrinology, Kyoto, Japan, A-15-19-002.
Pasanen, M., & Pelkonen, O. (1981) *Biochem. Biophys. Res. Commun. 103*, 1310–1317.
Ryan, K. J. (1959) *J. Biol. Chem. 234*, 268–272.
Suhara, K., Fujii, K., Tani, T., & Katagiri, M. (1987) *J. Steroid Biochem. 26*, 113–119.
Takemori, S., Sato, H., Gomi, T., Suhara, K., & Katagiri, M. (1975) *Biochem. Biophys. Res. Commun. 67*, 1151–1157.
Tan, L., & Muto, N. (1985) in *Cytochrome P-450 Biochemistry, Biophysics and Induction* (Vereczkey, L., & Magyar, K., Eds.) pp 461–464, Academiai Kiado, Budapest.
Tan, L., & Muto, N. (1986) *Eur. J. Biochem. 156*, 243–250.
Thompson, E. A., Jr., & Siiteri, P. K. (1974a) *J. Biol. Chem. 249*, 5364–5372.
Thompson, E. A., Jr., & Siiteri, P. K. (1974b) *J. Biol. Chem. 249*, 5373–5378.
Washida, N., Matsui, S., & Osawa, Y. (1985) *Fed. Proc. 44*, 861.
Waxman, D. J., Ko, A., & Walsh, C. (1983) *J. Biol. Chem. 258*, 11937–11947.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.
Yu, C. A., Gunsalus, I. C., Katagiri, M., Suhara, K., & Takemori, S. (1974) *J. Biol. Chem. 249*, 94–101.
Zachariah, P. K., & Juchau, M. R. (1975) *Life Sci. 16*, 1689–1692.