Purification and Characterization of Human Placental Aromatase Cytochrome P-450*

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Aromatase cytochrome P-450, which catalyzes the conversion of androgens to estrogens, was purified from human placental microsomes. The enzyme was extracted with sodium cholate, fractionated by ammonium sulfate precipitation, and subjected to column chromatography in the presence of its substrate, androstenedione, and the nonionic detergent, Nonidet P-40. The preparation exhibits a single major band when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and has a specific content of 11.5 nmol of P-450/mg of protein. The purified enzyme displays spectroscopic properties typical of the ferric and ferrous forms of cytochrome P-450. Full enzymatic activity can be reconstituted with rabbit liver microsomal cytochrome P-450 reductase and Nonidet P-40. Purified aromatase cytochrome P-450 displays catalytic characteristics similar to the enzyme in intact microsomes in the aromatization of androstenedione, 19-hydroxysterol androstenedione, and 19-oxoandrostenedione. Testosterone and 16α-hydroxytestosterone are aromatized at maximal rates similar to androstenedione, and all substrates exhibit relative affinities corresponding to those observed in microsomes. We have raised rabbit antibodies to the purified enzyme which show considerable specificity and sensitivity on immunoblots.

The direct biological conversion of radiolabeled androgens to estrogens was first demonstrated in the mid-1950s (1-3). Subsequently, Ryan (4) reported the enzymatic aromatization of androgens to estrogens by a system consisting of human placental microsomes, NADPH, and oxygen, which is consistent with the involvement of cytochrome P-450 in catalyzing the reaction. By using inhibitors of this class of enzymes, spectroscopic evidence, and inhibition of aromatization by an antibody raised against cytochrome P-450 reductase, Thompson and Siiteri (5) showed that cytochrome P-450 was involved in this catalysis; moreover, they determined that 2 mol each of NADPH and O2 are consumed for each mol of estrogen formed (6). The aromatization reaction is thought to proceed as depicted in Fig. 1. The initial steps involve two sequential hydroxylations at carbon 19 to yield 19-hydroxyandrostenedione (7, 8) and 19-dihydroxyandrostenedione (9, 10) (which may dehydrate to the 19-aldehyde). The details of the third step remain obscure; different laboratories have proposed that either 1,9-hydroxylation (11, 12), 23-hydroxylation (13, 14), or 19-peroxidation (15) would form an unstable intermediate which would decompose nonenzymatically to yield estrogen.

Aromatase cytochrome P-450 (P-450arom)1 has received much attention because of this interesting reaction mechanism and the role of estrogens in endocrine physiology and estrogen-dependent diseases. Despite its importance, however, P-450arom is not as well characterized as other steroid hydroxylases. An active purified preparation of P-450arom is essential for definitive studies on the mechanism of aromatization and chemical and biophysical characterization of the enzyme.

Several laboratories have reported attempts at the isolation of P-450arom, with results reflecting the instability and intractability toward purification of this membrane-bound protein. Based on its apparent molecular mass of 55 kDa, reported herein and by others (16, 17), the expected specific content for homogeneous P-450arom is 18.2 nmol of P-450/mg of protein. In an early study, Osawa and Higashiyama (18) purified P-450arom from human placenta to a specific content of 2.3 nmol of P-450/mg of protein; however, the activity of the preparation was not specified. Tan and Muto (19) described the partial purification of P-450arom to a specific content of 4.2 nmol of P-450/mg of protein. The enzyme was active, with a turnover number of 4.8 min⁻¹ for androstenedione aromatization, but it was electrophoretically inhomogeneous, in accord with the 23% purity indicated by the specific content. Nakajin et al. (17) recently described the purification of human placental P-450arom to apparent electrophoretic homogeneity. However, this preparation had a specific content of only 2.7 nmol of P-450/mg of protein, exhibited a major proportion of denatured material in the ferrous-carbon monoxide complex, and had an anomalously high Kₘ value of 30 µM for androstenedione. The preparation was nevertheless suitable for chemical analysis, and Chen et al. (20) obtained partial amino acid sequence data from this material. In a different approach toward studying P-450arom, Mendelson et al. (16) partially purified the enzyme to 2 nmol of P-450/mg of protein, subjected the preparation to SDS-polyacrylamide gel electrophoresis, and raised polyclonal and monoclonal antibodies to P-450arom excised from the gels.

In this paper we describe a new isolation procedure for P-450arom. The method yields highly purified, active P-450arom with a specific content of 11.5 nmol of P-450/mg of protein. We have found this preparation suitable for spectroscopic and kinetic studies, which we report in this paper. We have also obtained rabbit antibodies to this preparation of P-450arom.

1 The abbreviations used are: P-450arom, cytochrome P-450 which catalyzes the aromatization of androgens to estrogens; SDS, sodium dodecyl sulfate.

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Phenyl-Sepharose column and washed with 150 ml of equilibration buffer. P-450arom was eluted with 500 ml of pH 7.16 buffer containing 300 mM NaCl and 0.2% (w/v) sodium cholate. Fractions containing P-450arom were pooled and dialyzed against 2 changes of 1 l of pH 7.4 buffer for a total of 15 h. A 2.2 × 26-cm column of DE52 (Whatman) was equilibrated with pH 7.4 buffer. The dialyzed phenyl-Sepharose pool was applied to the DE52 column; P-450arom was weakly bound to the column, eluting gradually with 500 ml of the equilibration buffer. Fractions containing P-450arom were pooled and applied to a 2.5 × 5-cm column of hydroxylapatite (Bio-Rad) previously equilibrated with pH 7.4 buffer. The column was washed with 10 ml of 10 mM buffer, pH 7.4, followed by 50 ml of 50 mM buffer, pH 7.4. P-450arom was eluted with a linear gradient (200 ml) in which the sodium phosphate buffer concentration was increased from 50 to 150 mM, pH 7.4. Fractions containing P-450arom were pooled and dialyzed overnight against 1 liter of 10 mM sodium phosphate, pH 7.4, containing 50% (w/v) glycerol. Detergent-free P-450arom was prepared as above, except that the wash and elution of the hydroxylapatite column were performed in the absence of Nonidet P-40. Both preparations are stable for weeks when stored at −20 °C.

**Isolation of Other Enzymes—Cytochrome P-450 reductase** was purified from phenobarbital-induced rabbit liver microsomes by the procedure of Yasukochi and Masters (21), as modified by Johnson et al. (22). The preparation had a specific activity of 11 μmol of cytochrome c reduced/min/mg of protein and displayed a single major band when subjected to SDS-polyacrylamide gel electrophoresis. Rabbit liver cytochrome b5 was purified by a modification of the procedure of Strittmatter et al. (23). The cytochrome was obtained as a by-product during the DE52 chromatography of P-450 reductase. Fractions containing cytochrome b5 were pooled and dialyzed twice against 1.5 liters of 10 mM Tris acetate, 0.9 mM EDTA, pH 8.1. The cytochrome was applied to a DE52 column equilibrated with the same buffer, washed, and eluted with a linear gradient of 0–500 mM NaCl in equilibration buffer containing 0.25% deoxycholate. Cytochrome b5 was further purified by G-75 chromatography, and deoxycholate and NaCl were removed by G-25 chromatography. The final preparation exhibited an A₄₅₀/A₃₅₅ ratio of 2.53.

**Reconstitution and Measurement of Aromatase Activity—Aromatase activity** was reconstituted with 0.003% (w/v) Nonidet P-40 and 100 nm rabbit liver P-450 reductase. These standard assay conditions also included 50 mM sodium phosphate buffer, pH 7.2, substrate, 0.3 mM dithiothreitol, 2.5 mM glucose-6-phosphate, and 0.25 unit/ml glucose-6-phosphate dehydrogenase in a total volume of 1 ml. Reactions were initiated with 20 μM NADPH and incubated at 37 °C. All assays were performed in duplicate.

The conversion of androstenedione to estrone was quantitated by the release of tritium from [1,2,3-3H]androstenedione (25). Conversion of testosterone to estradiol was also quantitated by tritium release in the same fashion. Conversion of androstenedione, 19-hydroxyandrostenedione, and 19-oxoandrostenedione to estrone was measured by radioimmunoassay as previously described (25). Conversion of 16α-hydroxytestosterone to estradiol was measured by radioimmunoassay in the presence of 0.3% bovine serum albumin (66 KDa), phospholase b (97.4 KDa), and β-galactosidase (116 KDa). Immunoblotting was performed by the procedure of Towbin et al. (27); samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose membranes were incubated overnight with a 1:200 dilution of an IgG-enriched fraction of rabbit antisera directed against P-450arom. Immunoreactive proteins were visualized by incubation with 125I-labeled Protein A (ICN, Irvine, CA) followed by autoradiography.

**Antibodies to P-450arom**—Antibodies were raised in a New Zealand White rabbit using the custom antisem production service of Bethyl Laboratories (Montgomery, TX). A rabbit was immunized twice in 1 week with 50 μg of purified P-450arom in complete Freund’s adjuvant. The rabbit was bled 5, 7, and 8 weeks thereafter. The antisera was titered by direct application of P-450arom to nitrocellulose, incubation with immune or preimmune serum, and visualization of bound antibody by incubation with horseradish peroxidase-linked goat anti-rabbit IgG antibody (Sigma). All bleedings showed a positive titer, and the antiserum was pooled and an IgG-enriched fraction was obtained by ammonium sulfate fractionation followed by DE52 chromatography (28).
Other Methods—For spectral studies, P-450arom was diluted into 50 mM sodium phosphate buffer, pH 7.2. Spectra were recorded at ambient temperature in a Cary 17D spectrophotometer interfaced to a Zenith Z-100 computer adapted for data acquisition by On-Line Instruments (Jefferson, GA).

Cytochrome P-450 concentrations were determined by carbon monoxide difference spectra (29) using \( \Delta A_{430-380} = 91 \) (mM-cm) \(^{-1} \). These samples were equilibrated with 50 \( \mu \)M 19-norandrostenedione, which, unlike androstenedione, allows carbon monoxide to ligate the heme iron of P-450arom (30). Difference spectroscopy was also used to determine specific P-450arom concentration. A shift in the Soret absorption band of the enzyme is induced by the high-affinity ligand (19R)-10-thiiranyl-4-estrene-3,17-dione (31, 36) and yields \( \Delta A_{430-380} = 126 \) (mM-cm) \(^{-1} \).

Protein concentrations were determined by the method of Lowry et al. (32) after precipitation of protein with trichloroacetic acid in the presence of deoxycholate (33). Bovine serum albumin, also subjected to the precipitation procedure, was used as a standard.

Other Materials—Miconazole was obtained from Sigma. 9-Hydroxy-a-naphthoflavone (34) was generously provided by Dr. Stephen Nesnow of the U. S. Environmental Protection Agency, Research Triangle Park, NC. 19-Oxo-androstenedione was obtained from Makor theriads (Jerusalem, Israel). 22-Amino-23,24-bisnor-5-cholene-3-ol was synthesized as previously described (35). (19R)-10-Thiiranyl-4-estrene-3,17-dione (36) was generously provided by Dr. Cecil H. Robinson of the Johns Hopkins University School of Medicine, Baltimore, MD. Radiolabeled steroids were purchased from New England Nuclear; tritiated androstenedione and testosterone were purified by high pressure liquid chromatography (25). The remaining nonlabeled steroids were from Steraloids (Wilton, NH).

RESULTS

Purification of P-450arom—P-450arom was isolated from human placental microsomes by cholate extraction, ammonium sulfate fractionation, and column chromatography. The columns were eluted in the presence of Nonidet P-40 and involved hydrophobic chromatography on phenyl-Sepharose and ion exchange chromatography on DE52 and hydroxyapatite. All buffers contained 2 \( \mu \)M androstenedione. The continued presence of substrate was found to be necessary for the stability of P-450arom; this recently has been reported by Tan and Muto (19) as well. Fig. 2 shows the elution profiles for the three chromatography columns. \( A_{360} \) was not monitored due to the high absorbance of Nonidet P-40. In pilot studies, the elution of P-450arom was followed by assaying for androstenedione aromatization, and we saw no indication for the chromatographic separation of multiple forms of P-450arom. In later preparations, the enzyme was identified by the characteristic high spin Soret absorption band (37). The spectrum which has a Soret peak at 393 nm, with this, if not a higher, degree of purity.

Table I summarizes the purification procedure, which, starting with frozen microsomes, requires less than 1 week. Aromatase activity was measured by tritium release from androstenedione, and all samples were reconstituted with rabbit liver P-450 reductase. Addition of exogenous P-450 reductase is required for full activity after cholate extraction. Even with intact microsomes, supplementation with rabbit P-450 reductase results in a 1.5-2-fold increase in aromatase activity, substantiating the previous suggestion that P-450 reductase may be partially rate-limiting in microsomal assays (37). Levels of aromatase activity and P-450arom parallel another at each step of the purification procedure, indicating that the majority of the aromatase activity in microsomes resides in the protein isolated. The overall yields of P-450arom and aromatase activity are 9 and 12%, respectively. The difference between the two recoveries reflects the somewhat higher turnover number of purified P-450arom relative to microsomes.

Fig. 3 shows an SDS-polyacrylamide gel in which microsomes and three amounts of purified P-450arom were subjected to electrophoresis and staining with Coomassie Blue. The preparation displays a single major band which exhibits an apparent molecular mass of 55 kDa. Assuming one P-450 chromophore/apoprotein molecule, the theoretical specific content for a homogeneous sample is 18.2 nmol of P-450/mg of protein. This preparation contained 11.5 nmol of P-450/mg of protein, indicating 63% purity, and Fig. 3 is consistent with this, if not a higher, degree of purity.

Spectroscopic Properties of P-450arom—The absorption spectra of several complexes of the ferric form of P-450arom are presented in Fig. 4. The androstenedione complex of the enzyme exhibits a Soret maximum at 393 nm and a charge-transfer band at 642 nm, strongly suggestive of high spin \( S = 5/2 \) character (38). The spectrum which has a Soret peak at 417 nm was generated by addition of 9-hydroxy-\( \alpha \)-naph-
Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min/mg protein)</th>
<th>Total P-450arom content (nmol)</th>
<th>Specific content (nmol/mg protein)</th>
<th>P-450arom yield (%)</th>
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<tr>
<td>Microsomes</td>
<td>4958</td>
<td>915</td>
<td>0.18</td>
<td>255</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>Cholate extract</td>
<td>3796</td>
<td>626</td>
<td>0.16</td>
<td>251</td>
<td>0.07</td>
<td>98</td>
</tr>
<tr>
<td>Ammonium sulfate, 35-55%</td>
<td>834</td>
<td>349</td>
<td>0.37</td>
<td>156</td>
<td>0.17</td>
<td>61</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>125</td>
<td>304</td>
<td>2.4</td>
<td>107</td>
<td>0.86</td>
<td>42</td>
</tr>
<tr>
<td>DE52</td>
<td>7.6</td>
<td>152</td>
<td>20</td>
<td>39</td>
<td>5.1</td>
<td>15</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>1.95</td>
<td>111</td>
<td>57</td>
<td>22</td>
<td>11.5</td>
<td>9</td>
</tr>
</tbody>
</table>

* Samples were reconstituted with 100 nM rabbit liver P-450 reductase and 0.003% Nonidet P-40 as described under “Experimental Procedures.”

P-450arom concentration was determined by difference spectra induced by (19R)-10-thiiranyl-4-estrene-3,17-dione as described under “Experimental Procedures.”

Aromatase activity equal to that observed in microsomes supplemented with P-450 reductase can be obtained by reconstituting purified P-450arom with Nonidet P-40 and rabbit liver P-450 reductase. Table II shows the contribution of each component of the reconstitution system to aromatase activity. Cytochrome b5 stimulates the activity of certain cytochromes P-450 (46), but from Table II it is evident that the addition of 200 nM rabbit liver cytochrome b5 to the P-450arom system inhibits activity thioflavone, a potent inhibitor of P-450arom. This ligand induces a difference spectrum indicative of a shift from high spin to low spin when added to the substrate-saturated enzyme (39), and the location of the Soret maximum and the α (570 nm) and β (537 nm) bands in the absolute spectrum are typical of low spin, oxygen-ligated P-450 (40, 41). The spectrum with the Soret peak at 424 nm was generated by the addition of the P-450arom inhibitor, miconazole, which elicits difference spectra with human placental microsomes indicative of nitrogen coordination to the heme iron (42). The location of the Soret maximum, α shoulder (575 nm), and β band (542 nm) of the miconazole complex of P-450arom are characteristic of low spin, nitrogen-ligated cytochrome P-450 (40, 41).

The spectral shifts produced by the addition of these ligands are consistent with the presence of a single cytochrome P-450 species. However, cholesterol side chain cleavage cytochrome P-450 (P-450scc) is found in placental mitochondria (43, 44) and is a potential contaminant of the P-450arom preparation. We tested for the presence of this enzyme spectroscopically by addition of 22-amino-23,24-bisnor-5-cholene-3-β-ol, a high-affinity ligand for placental P-450scc which elicits characteristic spectra indicative of nitrogen coordination to the heme iron (45). This ligand did not elicit a spectral shift with P-450arom, implying less than 1% contamination by this cytochrome P-450.

Investigation of the UV absorption of P-450arom required isolation of the enzyme in the absence of Nonidet P-40. For this purpose, detergent was removed at the hydroxylapatite step of the purification, as described under “Experimental Procedures.” With this procedure, the yield was approximately one-half of that obtained in the presence of Nonidet P-40, and the specific content was reduced slightly to 10 nmol of P-450/mg of protein. Fig. 5 shows a UV and Soret absorption spectrum of the high spin androstenedione complex of this preparation, which exhibits an A280/A393 ratio of 1.53. Also shown in Fig. 5 is a carbon monoxide difference spectrum of the ferrous enzyme. This spectrum is characteristic of P-450 enzymes, and exhibits a maximum at 448.5 nm. The slight shoulder at 420 nm indicates the presence of only a small amount of denatured P-450arom.

Catalytic Characterization of P-450arom—Aromatase activity equal to that observed in microsomes supplemented with P-450 reductase can be obtained by reconstituting purified P-450arom with Nonidet P-40 and rabbit liver P-450 reductase. Table II shows the contribution of each component of the reconstitution system to aromatase activity. Cytochrome b5 stimulates the activity of certain cytochromes P-450 (46), but from Table II it is evident that the addition of 200 nM rabbit liver cytochrome b5 to the P-450arom system inhibits activity...
by 79%. This indicates that cytochrome b₅ can interact with the reconstitution system, but we have not explored this phenomenon further. Because a number of purified cytochromes P-450 require phospholipid for full reconstitution of activity, we substituted sonicated egg phosphatidylcholine for Nonidet P-40; this yielded virtually the same activity as omitting an amphiphile altogether, indicating no requirement for added phosphatidylcholine in this system. Activity is almost nil with the omission of hepatic P-450 reductase, indicating that endogenous placental P-450 reductase has been almost completely removed during purification. This is consistent with the lack of a band corresponding to the apparent molecular weight of placental P-450 reductase (79 kDa (47)) in Fig. 3.

Fig. 6 shows the P-450 dependence and time course of androstenedione aromatization. The complete system contained 250 nM [18,28-³H]androstenedione, 100 nM P-450 reductase, 0.003% Nonidet P-40, and 2 nM P-450arom in 1 ml and was assayed as described under “Experimental Procedures.” NP-40, Nonidet P-40; PC, egg phosphatidylcholine.

<table>
<thead>
<tr>
<th>Activity</th>
<th>pmol estone/min</th>
<th>Percent of complete system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>6.91</td>
<td>100</td>
</tr>
<tr>
<td>+200 nm cytochrome b₅</td>
<td>1.42</td>
<td>21</td>
</tr>
<tr>
<td>+30 µg/ml PC, -NP-40</td>
<td>3.00</td>
<td>43</td>
</tr>
<tr>
<td>-NP-40</td>
<td>2.87</td>
<td>42</td>
</tr>
<tr>
<td>-P-450 reductase</td>
<td>0.03</td>
<td>0.4</td>
</tr>
<tr>
<td>-P-450arom</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

Fig. 7 shows a determination of the Kₐ value for rabbit liver microsomal cytochrome P-450 reductase. Aromatase activity was reconstituted using 250 nM [18,28-³H]androstenedione and 2 nM purified P-450arom and liver P-450 reductase.

To determine the effect of solubilization and purification of P-450arom on its steroid binding and catalytic properties, the kinetics of the reconstituted enzyme were compared with those of microsomes. Figs. 8 and 9 show Lineweaver-Burk plots for the metabolism of androstenedione and the intermediates in the aromatization reaction, 19-hydroxyandrostenedione, and 19-oxoandrostenedione. With both microsomes (Fig. 8) and purified P-450arom (Fig. 9), the intermediates exhibit similar specificity constants (Vₘᵢₓ/Kₐ) relative to androstenedione. The specificity constants for 19-hydroxyandrostenedione are 46 and 43% of those for androstenedione with microsomes and purified P-450arom, respectively; likewise, the specificity constants for 19-oxoandrostenedione are 33 and 30% of those for androstenedione in the two systems. The molecular basis for the decreased affinity of the oxygenated intermediates is not known, but presumably the increase in Vₘᵢₓ is due to the decrease in the number of reaction cycles required for aromatization. The absolute affinities of each substrate in the reconstituted system are somewhat lower than those observed in microsomes; this effect has been previously described for a crude preparation of detergent-extracted aromatase (48) and may result from the increase in the volume of the hydrophobic phase into which the substrates can partition. These kinetic data show that the catalytic properties of P-450arom were not significantly altered by the purification process and are consistent with the isolation of a single enzyme species which catalyzes all three steps of androstenedione aromatization.

For each substrate in the experiment shown in Figs. 8 and
9, estrone formation was measured by radioimmunoassay (open circles). Androstenedione aromatization in the reconstituted system was also assayed by tritium release from [1,2,3H]androstenedione (Fig. 9, closed circles). Calculation of estrone formation from tritiated androstenedione was based on the manufacturer's information that 80% of the tritium was distributed in the 1β and 2β positions (determined by tritium NMR) and assumed stereospecific loss from these positions. The close correlation between these two assay methods is consistent with 1β,2β proton loss (12, 49, 50) but is in disagreement with the recent suggestion that the 1α proton is eliminated during aromatization (51).

We also investigated the kinetics of the aromatization of testosterone and 16α-hydroxytestosterone; the $K_m$ and $V_{max}$ values for these substrates are listed in Table III in addition to those for androstenedione. The relative affinities of each substrate are the same as observed in microsomes, with 16α-hydroxytestosterone exhibiting a characteristic low affinity (52). The $V_{max}$ values for each substrate are very similar to one another, which suggests that the same enzyme species is responsible for the aromatization of all three steroids.

Antibodies to P-450arom—A rabbit, injected with purified P-450arom, produced antibodies directed to the enzyme. Fig. 10 shows an autoradiogram of an immunoblot experiment with this polyclonal antibody. The lanes compare microsomes, a sample from an early step in the P-450arom purification (the phenyl-Sepharose pool), and increasing amounts of purified P-450arom. The antiserum appears to be quite specific, detecting one major band corresponding to P-450arom. The minor band at 63 kDa observed in microsomes is probably coincidental cross-reactivity, since it is not present in the purified preparation used for immunization. The two minor bands seen with highest amount of P-450arom correspond in apparent molecular mass to approximately 105 and 150 kDa.

When the length of autoradiographic exposure was increased from 2 to 18 h, additional bands of lesser intensity were detected in microsomes; however, no additional bands were detected in the purified P-450arom preparation (not shown). To test the sensitivity of the antiserum, P-450arom was directly applied to nitrocellulose using a "slot blot" apparatus. In this manner, 1 ng of the enzyme could be reliably detected (not shown).

**DISCUSSION**

We have purified aromatase cytochrome P-450 to a specific content of 11.5 nmol of P-450/mg of protein, a value which represents 63% purity based on the apparent molecular mass of 55 kDa for the protein. By the criterion of SDS-polyacrylamide gel electrophoresis (Fig. 3), the preparation contains no major contaminants and appears to be significantly more homogeneous than 63%. It is not known whether this discrepancy arises from the presence of a number of minor protein...
contaminants, partial loss of heme during purification, or an overestimation of protein by the method used. The P-450arom preparation is active and amenable to kinetic and spectroscopic studies, described in this report. In addition, we have raised a polyclonal antibody to the enzyme which should be useful in molecular cloning as well as studying tissue localization and the regulation of enzyme levels.

To obtain maximal aromatase activity, we reconstituted purified P-450arom with Nonidet P-46 and cytochrome P-450 reductase. This yields a turnover number (5.0 min⁻¹) which exceeds that observed in P-450 reductase-supplemented microsomes under the same conditions (3.6 min⁻¹). These turnover numbers are typical of cytochromes P-450 and indicate that solubilization and purification of P-450arom has not adversely affected its catalytic activity and, in addition, that the reconstitution conditions are probably near optimal. The efficacy of rabbit P-450 reductase and its high affinity (Kₐ = 20 nM) may reflect a conservation of electron transfer domains between P-450arom and liver cytochromes P-450. This efficient coupling between P-450arom and reductase does not appear to have a specific lipid requirement, since high activity is attained with detergent micelles. When 200 nM cytochrome b₅ is added to the system, aromatase activity is decreased. The mechanism of this inhibition is not known, and we have not yet examined the effects of specific lipids or the dependence of this effect on the ratios of the proteins involved.

The purified preparation of P-450arom catalyzes all three steps of androgen aromatization as evidenced by tritium release from [1,2,3H]androstenedione and radioimmunoassay for estrone. The kinetics of the aromatization of androstenedione and the intermediates 19-hydroxyandrostenedione and 19-oxoandrostenedione parallel those observed in microsomes, suggesting that the enzyme is not altered by the purification procedure. These results imply that either a single enzyme is responsible for the entire reaction sequence, or two or more very similar species were copurified in a constant ratio. Using placental microsomes, Kelley et al. (53) have presented data consistent with our findings, i.e. a single common binding site for androstenedione, 19-hydroxyandrostenedione, and 19-oxoandrostenedione (53). Fishman and Goto (55), on the other hand, observed slight differences in the potency of 19-oxoandrostenedione toward inhibition of accumulation of the products of each of the three steps of the aromatization reaction and have postulated the existence of a separate catalytic site for the third step of the reaction. As pointed out by Fevold (55), this type of experiment is difficult to interpret because the nonlabeled steroid can act as a trap and dilute the measured isotope; it is also simultaneously metabolized as a competitive substrate.

Androstenedione, testosterone, and 16α-hydroxytestosterone are all aromatized by purified P-450arom. The values of Vₘ₉₉, the observed Vmax values similar to one another, and the relative Kₐ values correspond to those observed in microsomes; this suggests that we have isolated a single enzyme species which catalyzes the three steps of A ring aromatization at a fixed maximal rate, independent of differences in D ring substitution. If P-450arom is composed of multiple forms, they must be very closely related, since we observed no chromatographic, electrophoretic, spectroscopic, or kinetic evidence for heterogeneity. Elucidation of any of P-450arom multiplicity may have to await further protein sequencing or molecular cloning of aromatase.

The absorption spectrum of purified P-450arom exhibits ligand-induced shifts which are characteristic of cytochromes P-450. It has been demonstrated, by combining cytochrome P-450 or model porphyrin complexes with a variety of ligands, that these shifts arise from differences in ligation of the sixth coordination site of the heme iron (38, 40, 41). The distinctive spectral characteristics of ferric P-450arom suggest that when androstenedione is bound to the enzyme the sixth coordination site of the heme iron is vacant, whereas 9-hydroxy-α-naphthoflavone and miconazole binding produce spectra suggestive of oxygen and nitrogen ligation, respectively. The ferrous carbon monoxide complex of P-450arom has a spectrum typical of cytochromes P-450. These results are in agreement with observations of the enzyme in situ in microsomes (5, 39, 42) and indicate that these properties of P-450arom have not been significantly altered by purification. As described in the following paper, spectroscopic studies with this preparation have been useful for "mapping" the distance and orientation between the substrate binding site and the heme iron of P-450arom (31).

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REFERENCES

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