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PARTIAL RESOLUTION OF THE PLACENTAL MICROSOMAL AROMATASE COMPLEX*

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SUMMARY

Incubation of placental microsomes with digitonin under defined conditions results in a solubilization of 15–20% of the total aromatase activity. The aromatase activity remains in the non-turbid supernatant after prolonged centrifugation at 148,000 *g*, exhaustive dialysis, and column chromatography on Bio-Gel P-10. The particulate and solubilized enzymes are indistinguishable in that the apparent K_m for both androstenedione and NADPH are similar. Also, the K_m for NADPH of the aromatase is approximately the same as the K_m of NADPH-cytochrome *c* reductase in both the microsomal and solubilized enzyme. Cytochrome P-450 recovered in the digitonin supernatant retained its native spectral characteristics as well as its substrate specificity.

The solubilized aromatase can be purified about five-fold (20% recovery). Under these conditions, cytochrome P-450 can be purified about three to five-fold (15% recovery) and NADPH-cytochrome *c* reductase about 30-fold (100% recovery). Cytochrome P-450 and the reductase could be partially resolved into two fractions by batchwise adsorption on DEAE-cellulose. The unadsorbed fraction contained cytochrome P-450 while that obtained by extraction with 1.0 M KCl contained the reductase and a trace of cytochrome P-450. Both of these fractions contained minimal aromatase activity. When these fractions were recombined, however, the rate of aromatization was increased by about two-fold. This observation is consistent with a requirement for both cytochrome P-450 and NADPH-cytochrome *c* reductase for aromatization of androstenedione.

INTRODUCTION

The aromatization of C-19 steroids by placental microsomes to yield C-18 estrogens is catalyzed by the aromatase enzyme complex which is known to require NADPH and O_2[1]. Although cytochrome P-450 has been demonstrated in placental microsomes, the role of this hemoprotein in aromatization has been questioned since the reaction is not inhibited by carbon monoxide[2–4]. However, the following lines of evidence recently obtained in this laboratory strongly suggest that cytochrome P-450 participates in aromatization: (1) the stoichiometry of aromatization of androstenedione is 30_2:3NADPH:1 estrone; (2) known cytochrome P-450 inhibitors such as aminoglutethimide and SKF-525A inhibit aromatization; (3) aromatization requires microsomal NADPH-cytochrome *c* reductase; (4) placental microsomal cytochrome P-450 demonstrates absolute binding specificity for aromatase substrates and inhibitors; and finally, (5) 19-nortestosterone, the aromatization of which is CO-sensitive, is a competitive inhibitor of both the aromatization and cytochrome P-450 binding of androstenedione[5–7].

In order to provide unequivocal evidence for involvement of cytochrome P-450 in aromatization, solubilization of the aromatase complex has been attempted. A number of workers used various cholate bile salts or lipolytic digestion to resolve the liver microsomal cytochrome P-450 hydroxylase or the adrenal mitochondrial cytochrome P-450 hydroxylase[8–12]. Similar procedures have been used in attempts to extract the placental aromatase; however, yields have been poor and little purification has been achieved (unpublished observations). More recently, NADPH-cytochrome *c* reductase and NADPH-testosterone 5α-reductase have been extracted in good yield from prostatic microsomes by treatment with digitonin [13]. Therefore, this mild treatment, which combines detergent and cholesterol-specific properties, has been examined as a means of solubilizing the microsomal aromatase.

EXPERIMENTAL

Cytochrome P-450 was estimated by CO difference spectrophotometry using an Aminco-Chance dual wavelength/split beam spectrophotometer [14]. NADPH-cytochrome *c* reductase was assayed according to the procedure described by Masters *et al.* [15]. The ^3H_2O assay for aromatase activity [16] was

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modified to enable the determination of initial velocity using low concentrations of protein and androstenedione. Routinely, 1 mg/ml of microsomal or solubilized protein was incubated with 1 μM androstenedione and an NADPH generating system consisting of 0.1 mM NADPH, 1.0 mM glucose-6-phosphate, and about 1 IU/ml of glucose-6-phosphate dehydrogenase. All reactions were carried out at 37° in 0.005 M potassium phosphate buffer (pH 7.4). Under these conditions, maximum velocity was maintained for longer than 20 min.

Microsomes were prepared as described earlier [16]. Prior to digitonin extraction, microsomes were sedimented at 148,000 *g* in 0.005 M potassium phosphate buffer (pH 7.4) containing 30% glycerol (PG). The use of high salt buffers was avoided since the stability of solubilized NADPH cytochrome *c* reductase was adversely affected by high salt concentrations. Digitonin was obtained from Nutritional Biochemicals Co. and was recrystallized from ethanol prior to use.

Digitonin extractions were carried out as follows: microsomal pellets were resuspended by sonication in PG containing digitonin and 10^{-3} M dithioerythritol (DTE). The resulting suspensions were incubated at 0° for varying time periods prior to centrifugation at 148,000 *g* for 1 h.

RESULTS

Within 5 min after the addition of digitonin, aromatase activity could be detected in the 148,000 *g* supernatant (Table 1). Recovery of activity was not increased by incubation with digitonin for more than 30 min, while solubilization of microsomal protein did increase with time. Moreover, a loss of activity in the supernatant was observed when microsomes were incubated with digitonin for 90 min prior to centrifugation.

In preliminary experiments, recovery of aromatase activity was low. However, as shown in Table 2, the addition of glycerol and DTE to the extraction buffer greatly increased the activity whereas the addition of EDTA had no effect. The observed increase in activity is presumed to be due to stabilization of the enzymes since neither the aromatase or NADPH-cytochrome *c* reductase activity of microsomes was increased when assayed in the presence of glycerol or DTE.

Table 1. Digitonin extraction of placental microsomes

Exposure to digitonin, min	Aromatase in supernatant, nmol/min	mg Protein in supernatant
0	0.030	0.9
5	0.070	5.6
30	0.070	5.8
60	0.065	6.0
90	0.047	6.5

Microsomes (11 mg protein) were extracted with 22 mg digitonin in 5.0 ml. After centrifugation for 1 h at 148,000 *g*, 1.0 ml of the supernatant was assayed for aromatase activity using the ^3H_2O assay.

Table 2. Aromatase activity of the digitonin supernatant prepared in the presence of various compounds

Additions	Concentration	nmol/min/ml
None	—	0.007
EDTA	10^{-4} M	0.007
DTE	10^{-3} M	0.013
Glycerol	30%	0.019

Microsomes were extracted with 2 mg digitonin per mg protein. Aromatase activity in intact microsomes was 0.070 nmol/min/ml.

Recovery of any given component of the aromatase system in the digitonin supernatant varied to some degree with the ratio of digitonin to protein. As shown in Fig. 1, recovery of aromatase and NADPH-cytochrome *c* reductase activity was maximum at a ratio of 1 mg digitonin/mg protein, while cytochrome P-450 and protein were recovered maximally at 2–3 mg digitonin/mg protein. Using 2 mg digitonin per mg microsomal protein, cytochrome P-450 could be purified three fold, aromatase activity five fold, and NADPH-cytochrome *c* reductase about 30 fold, as seen in Table 3.

As with all detergent extracted enzymes, it was necessary to determine that the aromatase was in solution in the glycerol-containing buffer, rather than in suspension. Centrifugation of the digitonin extract for 8 h at 148,000 *g* resulted in a loss of only 9% of the total aromatase activity from the supernatant as compared with 7% loss of activity which was incurred upon standing for 8 h at 4°. In order to determine if the aromatase activity would remain in solution after removal of digitonin, the extract was exhaustively dialysed. After dialysis against PG containing 10^{-4} M DTE for 24 h followed by centrifugation for 1 h at 148,000 *g*, no observable precipitate formed and

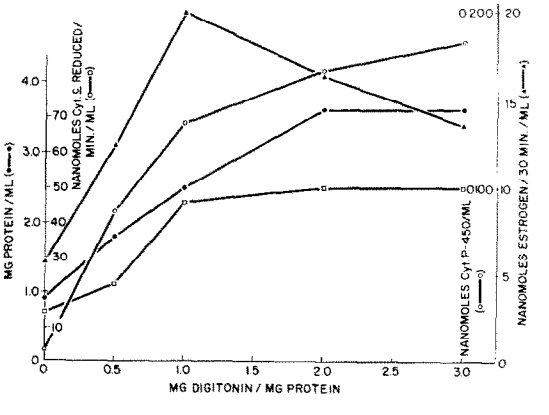

Fig. 1. Solubilization of placental microsomal components in the presence of various concentrations of digitonin. Extraction mixtures contained 60 mg microsomal protein in 5.0 ml PG. Following centrifugation for 1 h at 148,000 *g*, various parameters measured are expressed per ml of supernatant.

Table 3. Purification of microsomal enzymes by digitonin solubilization

	Protein, mg/ml	P-450, nmol/mg	Aromatase, nmol/min/mg	NADPH-cytochrome *c* reductase, nmol/min/mg
Microsomes	22	0.050	0.114	14
Digitonin extract	0.9 (4%)*	0.142 (12%)	0.530 (19%)	386 (126%)

* Numbers in parenthesis express % recovery.

90% of the aromatase activity remained in solution in the dialysed extract. In addition, when an aliquot of the solubilized enzyme was passed through a Bio-Gel P-10 column, 100 ± 5% of the initial aromatase activity was excluded in a non-turbid solution while digitonin was retained. Since the aromatase was active and did not precipitate when the detergent was removed, it appeared that this preparation might indeed be solubilized.

In order to compare the enzymatic properties of the membrane bound and extracted preparations, the metabolites of androstenedione formed by the solubilized preparation were examined. Tritiated androstenedione was incubated with the solubilized enzymes in the presence of an NADPH generating system and the reaction products separated using Celite gradient chromatography [17] and then purified by thin layer chromatography on silica gel G. Using this technique, testosterone, estrone, estradiol, 19-hydroxyandrostenedione and 19-oxoandrostenedione were isolated in yields comparable to those obtained when using microsomes. These steroids accounted for 102% of the total tritium recovered from the column. No unusual metabolites were detected, which suggests that the solubilized aromatase retained its normal specificity.

In the experiment described above, the extent of aromatization of androstenedione was determined by ^3H_2O release to be 18.6%, while isolation of estrone and estradiol indicated that 20% of the substrate had been aromatized. This established the validity of the ^3H_2O assay which was then used to determine various kinetic properties of the solubilized enzymes and compare them with the kinetic properties of the microsomal enzymes. A double reciprocal plot of the rate of aromatization vs the concentration of androstenedione indicated that 7 μM androstenedione was required for half-maximum velocity as compared with 5 μM in intact microsomes (Table 4). These results suggest that the affinity of the solubilized aromatase for androstenedione was similar to that of the native enzyme. Likewise, the K_m for NADPH in intact microsomes (12 μM) was comparable to that of the solubilized aromatase (15 μM). Also shown in Table 4, the K_m for NADPH of the solubilized NADPH-cytochrome *c* reductase was essentially the same as the K_m of the microsomal reductase. The similarity in the kinetic constants of the microsomal and the solubilized enzymes indicated that the digitonin extraction process did not alter the binding affinity or catalytic activity. This conclusion was further substantiated by the observation that the solubilized cytochrome P-450 demonstrated a typical Type I binding spectrum with androstenedione, as shown in Fig. 2. Furthermore, as in the native enzyme [6], binding of estrone could not be demonstrated, which indicates that the specificity of cytochrome P-450 had not been altered.

The ability to prepare soluble aromatase, which retained many of its native binding and kinetic properties, encouraged attempts to resolve the enzyme complex into its various components. The aromatase could be partially resolved by DEAE-cellulose chromatography into a fraction which was eluted at low ionic strength and contained a mixture of hemoproteins and another fraction containing NADPH-cytochrome *c* reductase which was eluted at high ionic strength (about 1.0 M KCl). However, chromatography of the aromatase on DEAE-cellulose resulted in irreversible loss of cytochrome P-450, concomitant with cytochrome P-420 formation. In an effort to decrease the length of time required for separation, batchwise extraction was attempted. Accordingly, DEAE-cellulose, previously equilibrated with PG, was added to the solubilized enzyme preparation. After mixing, DEAE-cellulose was sedimented by centrifugation, and the supernatant assayed for NADPH-cytochrome *c* reductase activity. In this manner, the solution was titrated with DEAE-cellulose until all the reductase was adsorbed. The supernatant (Fraction 1) was decanted and assayed for cytochrome P-450 content. The DEAE-cellulose pellet was resuspended in PG, resedimented by centrifugation, and the supernatant, containing any residual cytochrome P-450, discarded.The pellet was then extracted with PG containing 1.0 M KCl which eluted NADPH-cytochrome *c* reductase (Fraction 2). In a typical experiment of this type, 0.032 μM cytochrome P-450

Table 4. Kinetic constants of the solubilized enzyme complex

	Microsomes, μM	Solubilized, μM
K_m NADPH for aromatization	12	15
K_m NADPH for cytochrome *c* reductase	14	15
K_m Androstenedione	5	7

All kinetic parameters for the aromatase were determined in the presence of 3.0 mg of either microsomal or solubilized protein. The K_m for NADPH-cytochrome *c* reductase was determined in the presence of 0.3 mg protein.

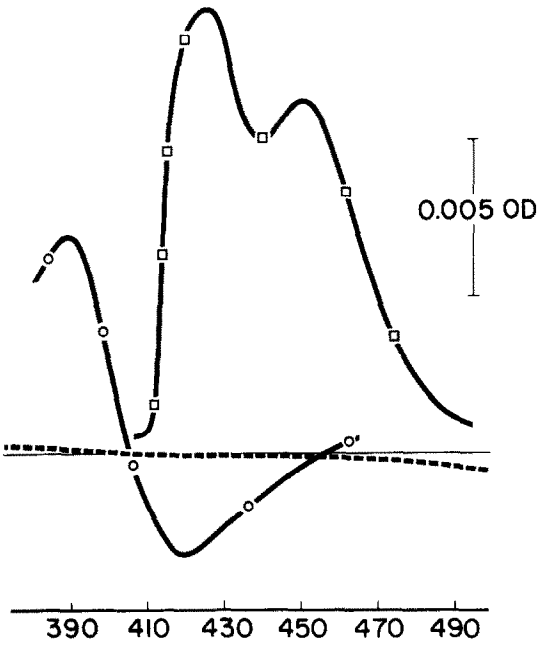

Fig. 2. Difference spectra of solubilized cytochrome P-450. The difference spectrum of the reduced CO-cytochrome P-450 minus reduced cytochrome P-450 (–□–) is shown as well as the Type I difference spectrum produced by the addition of 15 μM androstenedione (–○–). Estrone (20 μM) produced no detectable difference spectrum (– – –). Protein concentration was 1.0 mg/ml in all experiments.

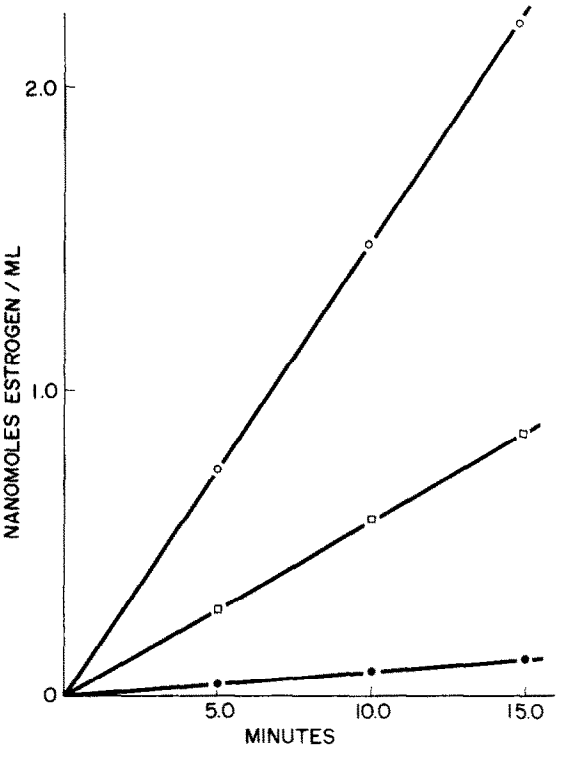

Fig. 3. Aromatase activity following fractionation of the solubilized enzyme by batchwise extraction with DEAE-cellulose. Aromatase activity was determined as follows: 0.4 ml Fraction 1 (–●–), 0.4 ml Fraction 2 (–□–) or 0.4 ml each of Fraction 1 and Fraction 2 (–○–) were assayed in a final volume of 1.0 ml PG containing an NADPH generating system and 17.5 μM androstenedione.

and no detectable reductase was recovered in Fraction 1 while Fraction 2 contained 0.006 μM cytochrome P-450 and reduced 13.6 nmol of cytochrome *c*/min/ml. The aromatase activity of these fractions was determined and is shown in Fig. 3. Fraction 1, containing no reductase, had very little aromatase activity. Fraction 2, contained NADPH-cytochrome *c* reductase and a trace of cytochrome P-450, and was capable of aromatizing androstenedione at a low rate. However, when these fractions were combined, the aromatase activity was approximately two-fold greater than the sum of the activity in the separate fractions. These results confirm and extend our previous findings which suggested that NADPH-cytochrome *c* reductase and cytochrome P-450 participate in aromatization.

DISCUSSION

The studies described above indicate that the aromatase complex can be solubilized by the use of digitonin. Such preparations lose activity rapidly, but can be partially stabilized by the addition of glycerol and DTE. Unpublished experiments using cholate or other bile salts resulted in soluble preparations which could not be stabilized by this means. Aromatase activity in the extracted preparation could not be removed from solution by prolonged centrifugation at 148,000 ***g***, nor did removal of excess digitonin by gel filtration chromatography or dialysis result in precipitation of the enzyme activity. The kinetic properties of the digitonin preparation are similar to those of the native enzyme, and chromatographic separation of reaction products revealed no substantial differences in distribution of products and intermediates between the extracted enzymes and the microsomal form. Furthermore, androstenedione was bound to solubilized cytochrome P-450 in a Type I manner, suggesting that this hemoprotein retained its native state and was not saturated or altered by detergent. Although the solubilized enzyme proved refractory to purification beyond three or four-fold, cytochrome P-450 could be separated from the solubilized NADPH-cytochrome *c* reductase by batchwise extraction with DEAE cellulose. While both of these fractions had detectable aromatase activity, when both fractions were recombined, activity was approximately doubled providing direct evidence for the requirement for both cytochrome P-450 and NADPH-cytochrome *c* reductase.

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