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PURIFICATION OF OESTROGEN SYNTHETASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TWO MEMBRANE-BOUND ENZYMES FROM THE HUMAN PLACENTA

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SUMMARY

Human placental NADPH-cytochrome P-450 reductase, obtained by 2',5'-ADP-Sepharose affinity chromatography, was separated into two reductase-active peaks on a Pharmacia Mono-Q column. By this short, two-step chromatographic procedure, the two reductases were obtained in a homogeneous state with high retention of activity and in over 900-fold purification. Aromatase-reconstituting activity was present only in the higher-molecular-weight reductase (79 000 D), not in the smaller, 70 000 D reductase, which turned out to be a proteolysis product of the former. Both proteins were eluted as a single peak in reversed-phase high-performance liquid chromatography on a Protesil-diphenyl column. Similar results were obtained with bovine hepatic NADPH-cytochrome P-450 reductases. On the other hand, starting from a reductase-free preparation, we have obtained by high-performance ion-exchange chromatography and high-performance size-exclusion chromatography, only partial purification of the aromatase cytochrome P-450, which showed the following values: aromatase activity, 3.995 nmol/min/mg protein (60-fold purification); cytochrome P-450 content, 1.376 nmol/mg protein (23-fold purification); molecular weight, 165 000 D (estimated as an aggregate by size-exclusion chromatography). Although complete purification of the aromatase component has yet to be accomplished, our results suggest that high-performance ion-exchange chromatography on a Mono-Q column is very useful for the purification of acidic, membrane-bound enzymes with good retention of activity.

INTRODUCTION

High-performance ion-exchange chromatography (HPIEC), on particles with pore diameter of 20 nm or larger, has been applied with success in recent years to the analysis of a variety of proteins[1–5]. However, most of these proteins are soluble, *i.e.* of non-particulate nature. The results are less spectacular when one is dealing

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with membrane-bound enzymes, where preservation of biological activity is a prerequisite. These particulate enzymes are amenable to purification by high-performance liquid chromatography (HPLC) only after they have been solubilized. Care must then be taken to keep them in their monomeric form in the artificial lipid matrix, usually by the addition of a suitable detergent to the liquid medium. If the enzyme contains readily oxidizable residues that participate in the catalytic reaction, antioxidants, such as dithiothreitol (DTT) and glycerol, the latter in high concentrations of 20% or more, must also be added. As a consequence, the viscosity of the mobile phase increases considerably and this in turn leads to higher column back-pressures, which in our case are detrimental to maintaining the native conformation of the aromatase. Furthermore, the concentration of the detergent selected must be carefully controlled, as above a certain level all surfactants negatively affect enzyme activity[6]. Because of these obstacles, a general method [such as reversed-phase (RP) HPLC for soluble proteins] that can be applied to the purification of labile, membrane-bound enzymes, still remains an elusive goal.

We have been interested for some time in the enzyme system present in the microsomes of the human placenta that converts the C_{19} androgens into the C_{18} oestrogens. This oestrogen synthetase (or aromatase) complex consists of two components: a flavoprotein, and a cytochrome P-450-type hemoprotein. Both enzymes are membrane-bound. An efficient method for their purification, with good retention of enzyme activity, is the subject of this paper.

EXPERIMENTAL

Materials and methods

The following reagents were purchased from Sigma (St. Louis, MO, U.S.A.): glucose-6-phosphate, G-6-P-dehydrogenase, trypsin, 3-[(cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), DTT, sodium cholate, ethylenediaminetetraacetate (EDTA), equine heart cytochrome *c*, and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular-weight protein kit. Emulgen 913 was a gift from Kao-Atlas (Tokyo, Japan). [1β,2β-^{3}H(N)]4-Androstene-3,17-dione (specific activity 41 Ci/mmol) was obtained from DuPont-Canada (Montreal, Canada), while its non-labeled counterpart was from Steraloids (Wilton, NH, U.S.A.). Glycerol, polyethylene glycol 20 000, Tris, HPLC-grade methanol, 2-propanol, and acetonitrile were from Fisher Scientific (Montreal, Canada). HPLC-grade water was prepared in our laboratory as described earlier[7]. 2',5'-ADP-Sepharose 4B, and the Mono-Q HR 5/5 column were from Pharmacia (Montreal, Canada). The TSK-3000 SW SEC-column (0.75 × 30 cm) was provided by Varian Canada (Toronto, Canada), while the Protesil 300, diphenylsilylsilica-column (25 × 0.4 cm I.D.) was from Whatman (Clifton, NJ, U.S.A.). Hydroxylapatite and the dye reagent for protein determinations were from Bio-Rad (Toronto, Canada). Fresh human placentae obtained at birth were from the University clinic.

Microsomes. Crude placental microsomes were prepared (and if not used immediately, stored at −80°C in a preservation buffer) as described previously[8].

Solubilization and ammonium sulfate fractionation. These manipulations were conducted at 0–4°C in an ice-bath. Microsomes were suspended in 50 m*M* potassium phosphate buffer (pH 7.5) (KPB), containing 20% (v/v) glycerol, 1 m*M* DTT, 0.2

m*M* EDTA, and 10 μM 4-androstene-3,17-dione to give a concentration of 10–15 mg protein/ml. A 10% (w/v) solution of sodium cholate in the same buffer was added to give a final concentration of 1% detergent. The mixture was stirred for 30 min, then centrifuged at 105 000 *g* for 1 h. The supernatant was removed, and brought to 35% saturation with ammonium sulfate with continuous stirring. The precipitated proteins were pelleted (12 000 *g*; 15 min). To the supernatant was added ammonium sulfate to 60% saturation. This 35–60% ammonium sulfate cut was dialyzed overnight in 20 m*M* KPB containing 20% glycerol, 1 m*M* DTT, 0.2 m*M* EDTA, and 0.5% sodium cholate, and then used for the purification of the aromatase complex.

Affinity chromatography. This was carried out in the cold-room using a 10 × 1.5 cm I.D. column, packed with 2',5'-ADP Sepharose 4B, which was equilibrated with the dialyzing buffer described above. Reductase was eluted at a flow-rate of 10 ml/h with 20 m*M* KPB (pH 7.5), containing 20% glycerol, 1 m*M* DTT, 0.2 m*M* EDTA, 0.1% (w/v) Emulgen 913, and 2 m*M* 2'-AMP. The active fractions were dialyzed overnight against 20 m*M* Tris-acetate buffer (pH 7.5), containing 20% glycerol, 0.2 m*M* EDTA, and 0.05% Emulgen 913, and concentrated with Centricon-30 tubes (Amicon).

High-performance size-exclusion chromatography (HPSEC). The system used consisted of a LDC duplex minipump (Riviera Beach, FL, U.S.A.), a TSK 3000 SW column, cooled to 10°C by an external mantle, a Rheodyne injection valve, an Altex UV detector (Berkeley, CA, U.S.A.) set at 280 nm, a potentiometric recorder, and a LKB fraction collector (Bromma, Sweden).

High-performance ion-exchange chromatography. HPIEC was carried out at room temperature with a Varian-5020 instrument, equipped with a Valco injection valve containing a 2 ml loop, a prefilter, a Mono-Q (5 × 0.5 cm I.D.) column, an Altex-Hitachi variable-wavelength detector connected in tandem to a UV detector, a Kipp & Zonen recorder (Delft, The Netherlands), and a Pharmacia Model 100 fraction collector (Uppsala, Sweden).

Reversed-phase HPLC. This was also performed with a Varian-5020 chromatograph. The wide-pore Protesil column was protected with a precolumn, and a guard column, both packed with 40–50 μm silica. The parameters used for the above chromatographic purifications are detailed in the corresponding figure legends.

Enzyme assays. NADPH-cytochrome P-450 reductase activity was measured with cytochrome *c* as substrate[9]. The total protein content was determined by the Bio-Rad dye-binding assay[10]. Cytochrome P-450 concentrations were calculated from the spectral data of the carbon monoxide/cytochrome P-450 complex, after reduction with dithionite and using the value of 91 $mM^{-1}\ cm^{-1}$ as an extinction coefficient[11]. Aromatase activity assay was based upon stereospecific tritium loss of the [1β,2β-^{3}H]androstenedione substrate to tritiated water, according to Thompson and Siiteri[12], as simplified by us[8].

RESULTS AND DISCUSSION

NADPH-cytochrome P-450 reductase purification by HPIEC

Human placental reductase (HPR), purified 210-fold by 2',5'-ADP-Sepharose affinity chromatography, showed two major protein bands with some contaminants on SDS-PAGE. When this fraction was subjected to HPIEC, two reductase-active

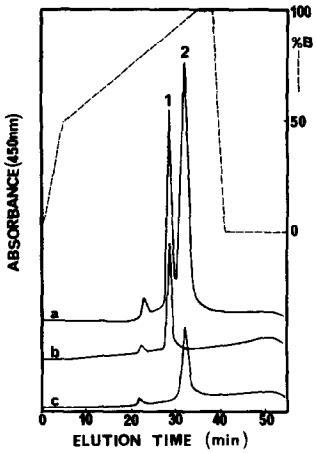


Fig. 1. Resolution of human placental reductase 1 (HPR-1) and HPR-2 by HPIEC. The reductase-active effluent collected from 2',5'-ADP Sepharose affinity chromatography was loaded onto a Pharmacia Mono-Q HR 5/5 column. Solvent A consisted of 20 m*M* Tris-acetate (pH 7.5), 20% glycerol, 0.2 m*M* EDTA, and 0.5% CHAPS. Solvent B was a solution of 0.6 *M* sodium chloride in Solvent A. A gradient from 0–100% buffer B was applied, as indicated by the program tracing (broken lines). Chromatography at room temperature; flow-rate, 0.3 ml/min; pressure, 500–560 p.s.i. (a) Crude human placental reductase (2.2 mg of protein); (b) purified HPR-1 (46 μg of protein); (c) purified HPR-2 (60 μg of protein).

peaks were obtained (Fig. 1a). Reductase 1 was eluted at 28.6 min (89% Solvent B) and Reductase 2 at 32.1 min (95% Solvent B) with a total recovery of *ca.* 80%. Each peak was collected, and subjected to a second HPIEC purification under identical conditions. Reductases 1 and 2 were again eluted at the same retention times and %B values as in the first HPIEC run (Fig. 1b and c, respectively). Estimation of their molecular weight by SDS-PAGE gave a value of 79 000 for Reductase 2, and of 70 000 for Reductase 1. The same analysis also revealed that, even after the first HPIEC, both Reductase 1 and 2 were obtained in an apparently homogeneous state, as judged by the appearance of a single protein band on SDS-PAGE (see Fig. 4B).

TABLE I

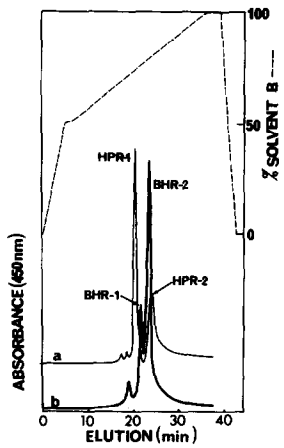
FINAL PURIFICATION OF NADPH-CYTOCHROME P-450 REDUCTASE BY HPIEC ON PHARMACIA MONO-Q COLUMN

Enzyme	*Specific activity (μmol/min/mg protein)*	*Purification-fold**	*Recovery (%)*
Human placental reductase (after affinity chromatography)	5.7	1.0 (210)	–
HPR-1 (first HPIEC)	22.6	3.9 (837)	79
HPR-2 (first HPIEC)	22.8	4.0 (844)	
HPR-1 (second HPIEC)	25.2	4.4 (933)	88
HPR-2 (second HPIEC)	25.5	4.5 (944)	73
Bovine hepatic reductase	4.2	1.0 (213)	–
Bovine hepatic reductase 1	12.0	2.8 (606)	81
Bovine hepatic reductase 2	10.0	2.4 (501)	

* Purification-fold from microsomes is indicated in parentheses.

Also, there was no further significant increasse of their specific activity when the reductases were subjected to a second HPIEC purification (see Table I).

Next, we purified the corresponding reductases from bovine liver in order to compare their properties with those from the human placenta. On the Pharmacia Mono-Q column, slight differences were noticeable (Fig. 2). Bovine hepatic reductase 1 (BHR-1) was eluted at a higher salt concentration than human placental reductase 1 (HPR-1), but BHR-2 was eluted at a slightly lower salt concentration than HPR-2. As shown in Fig. 3, their action, when added to a partially purified aromatase cytochrome P-450 preparation, was also nearly the same. Only their higher-molecular-weight forms, *i.e.* HPR-2 and BHR-2, had aromatase-reconstituting activity. Earlier, the presence of two molecular weight forms of NADPH-cytochrome P-450 reductase had been reported in the microsomes of rat liver, and in rabbit liver[13–16]. We point out in this regard that we have never obtained the two placental reductases in a constant ratio, but that this varied with each batch of microsomes, suggesting that one may be formed from the other through proteolysis during the purification procedure. To find out whether this may be the case, we have treated purified HPR-2 with trypsin. At different time intervals, samples were taken and the product formed was analyzed by SDS-PAGE as well as by enzyme essay. The results are depicted in Figs. 4A and 4B. Whereas reductase activity towards cytochrome *c* remained essentially unaffected, in contrast, aromatase-reconstituting activity decreased the longer HPR-2 was exposed to trypsin (Fig. 4A). After 1 h of tryptic digest, a protein band corresponding to a molecular weight of 70 000 began to appear. After 2 h, the origi-



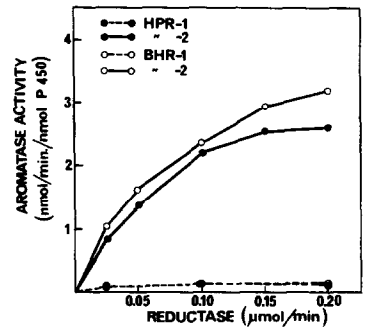


Fig. 2. Comparison of purified, human placental and bovine hepatic reductases. The lower, and higher-molecular-weight reductase forms from the human placenta (HPR-1 and HPR-2, respectively), and their counterparts from bovine liver (BHR-1 and BHR-2, respectively) were subjected to HPIEC on the Pharmacia Mono-Q column. The flow-rate was 0.5 ml/min. Otherwise, the chromatographic parameters are the same as described in Fig. 1. These chromatograms are reproducible. Broken-line tracing indicates gradient program.

Fig. 3. Reconstitution of the aromatase complex from purified reductases. To increasing amounts of homogeneous reductase were added 25 μl (0.0233 nmol) of partially purified human placental cytochrome P-450 (spec. content, 1.02 nmol/mg protein). Aromatase activities were assayed by the tritium-exchange method of Thompson and Siiteri[12].

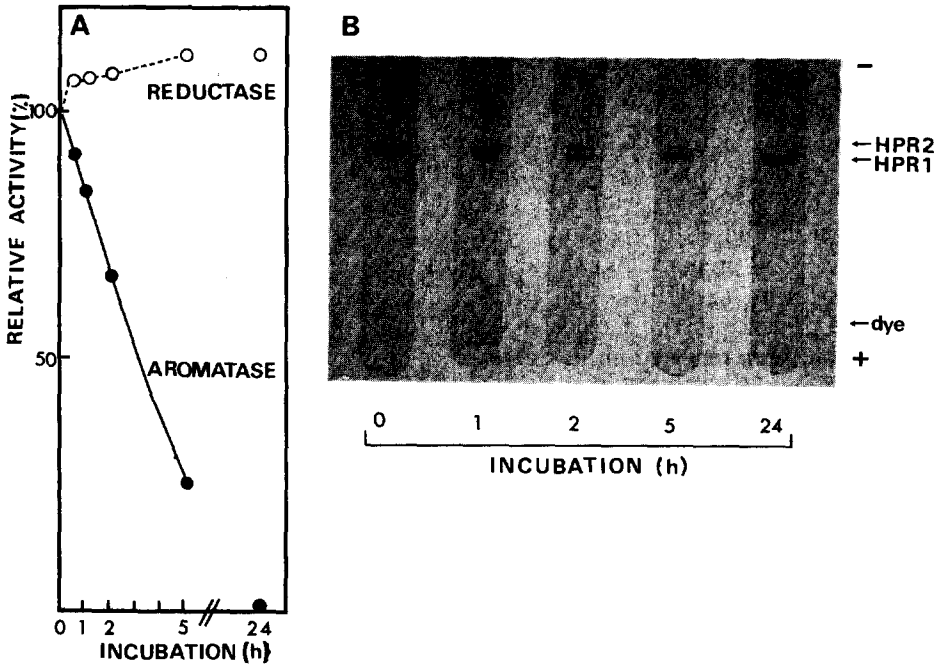
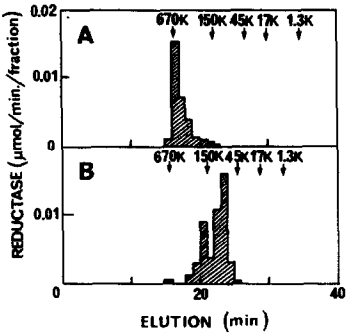


Fig. 4. Conversion of human placental Reductase 2 to Reductase 1 by trypsinolysis. HPR-2 (spec. activity, 13.2 μmol/min/mg; 162.2 μg/ml) was dialyzed against 10 m*M* KPB (pH 7.5) containing 10% glycerol and 0.1 m*M* EDTA. To the dialyzate was added trypsin (Type III-S, Sigma) at a substrate:enzyme ratio of 30:1. The digest was performed at 4°C. At the indicated time intervals, aliquots were withdrawn and transferred to Eppendorf tubes containing a solution of trypsin inhibitor (final conc. 0.02 mg/ml). (A) For measurement of reductase activity, 5 μl of the treated reaction medium were used, and for the aromatase assay 12 μl were mixed with 0.014 nmol of placental cytochrome P-450 (spec. content, 0.90 nmol/mg). (B) Results of SDS-PAGE analysis. At times 0, 1, 2, 5, and 24 h, 8-μl samples were withdrawn and applied to a 7.5% slab gel. The gels were stained with Coomassie Brilliant Blue R250.

nal 79 000 HPR-2 band and the newly formed HPR-1 band were of equal intensity. After 5 h, the HPR-2 band was barely visible, and after 24 h, only HPR-1 was present (Fig. 4B). We can deduce from this *in vitro* experiment that HPR-1 is very likely a proteolysis-derived product of the higher-molecular-weight HPR-2. As shown in Fig. 5, partially purified reductase undergoes aggregation when subjected to HPSEC. When no detergent was added, the bulk of the reductase-active fractions was eluted between 17 and 20 min, compatible with a protein of *ca.* 670 000 daltons molecular weight. Even in the presence of 0.5% cholate, the monomer that eluted after 22 min was still accompanied by a dimeric fraction of *ca.* 158 000 daltons. We therefore believe that HPSEC has only very limited usefulness for the purification of NADPH-cytochrome P-450 reductase. To confirm the homogeneity of the placental and the hepatic reductases, we subjected the purified enzymes to RP-HPLC analysis (see Fig. 6A and B). On a moderately hydrophobic, diphenylsilica column, and with a gradient of acetonitrile–2-propanol, the four reductases were eluted as single peaks at pH 2.2. However, we would like to stress the fact that after undergoing RP-HPLC, none of the reductases thus collected was found to retain enzyme activity. Therefore, RP-HPLC cannot be used for their purification with retention of bioactivity.



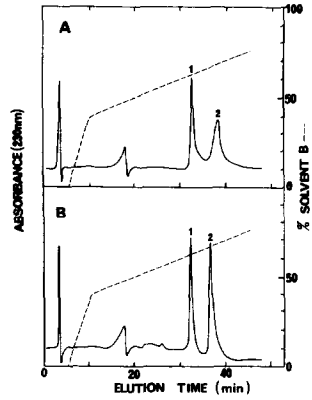
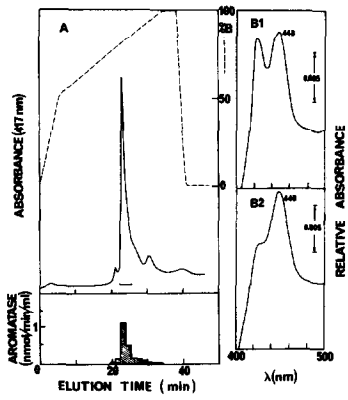


Fig. 5. Chromatographic behaviour of NADPH-cytochrome P-450 reductase on a size-exclusion column. (A) A TSK-3000 SW column was equilibrated with 0.1 *M* Na_2SO_4–0.02 *M* NaH_2PO_4 buffer solution (pH 6.8). Partially purified human placental reductase (200 μl = 0.10 μmol/min; spec. activity, 3.70 μmol/min/mg) was applied and eluted at a flow-rate of 0.44 ml/min and 10°C. The pressure was 400–500 p.s.i. Fractions (1 min) were collected and assayed for reductase activity. (B) The elution was carried out with addition of 0.5% sodium cholate to the mobile phase, and at a slightly higher flow-rate (0.46 ml/min).

Fig. 6. Reversed-phase HPLC of the purified reductase. A Whatman Protesil 300 diphenylsilylsilica column was equilibrated with Solvent A (15% acetonitrile–0.1% TFA). A gradient as indicated was applied with Solvent B (a 2:1 mixture of 95% acetonitrile–2-propanol containing 0.1% trifluoroacetic acid). Chromatographic conditions: flow-rate, 1.0 ml/min; temperature, 27°C; initial column back-pressure, 764 p.s.i.; detector, 0.1 a.u.f.s. at 230 nm. Prior to injection of the mixture, the retention time of each reductase was first established. (A) Injection of a mixture of 20 μg of HPR-1 and 18 μg of HPR-2 resulting in Peaks 1 and 2, respectively. (B) A mixture of 27 μg of BHR-1 and 35 μg of BHR-2 was injected and was eluted as Peaks 1 and 2, respectively.

Partial purification of aromatase cytochrome P-450

The hemoprotein component of the placental aromatase enzyme complex turned out to be much more difficult to purify. Because no laboratory has yet succeeded in purifying this enzyme to homogeneity, we have attempted the following strategy. The non-adsorbed, but aromatase-active fractions that eluted in the void volume during affinity-chromatography on 2',5'-ADP Sepharose, were directly introduced into a hydroxylapatite column (10 × 1.5 cm I.D.). This column was previously equilibrated with 20 m*M* KPB (pH 7.5), containing 20% glycerol, 1 m*M* DTT, 0.2 m*M* EDTA, 10 μ*M* androstenedione, and 0.5% sodium cholate. Elution was performed with a linear gradient from 20 to 300 m*M* phosphate. The active fractions were concentrated at 4°C with polyethylene glycol 20 000 in a dialysis bag. The concentrate was dialyzed overnight against 20 m*M* Tris buffer (pH 7.5), containing 20% glycerol, 1 m*M* DTT, 10 μ*M* androstenedione, 0.1% CHAPS, and 0.05% Emulgen 913. This sample had a specific aromatase activity of 0.352 nmol/min/mg protein. It was then subjected to HPIEC on a Mono-Q column in the presence of 0.1% CHAPS–0.05% Emulgen 913 as detergents. As shown in Fig. 7A, a major aromatase-active peak was eluted by 75–85% Solvent B. The reduced carbon monoxide difference spectra of the aromatase cytochrome P-450, obtained before and after HPIEC, are depicted in Fig. 7B1 and B2, respectively. Clearly, HPIEC was effective in removing much of the inactive P-420 form of the aromatase. At this stage of the



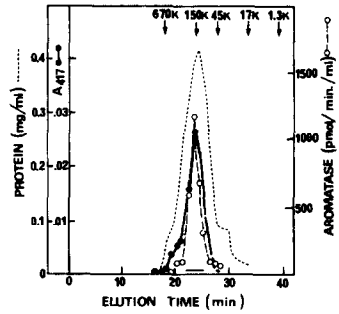


Fig. 7. HPIEC of crude aromatase cytochrome P-450 on Mono-Q column. (A) The aromatase-active eluate obtained by hydroxylapatite chromatography was, after dialysis, introduced into the column and eluted by applying a linear gradient of sodium chloride, as indicated, at a flow-rate of 0.3 ml/min, and a pressure of 580–650 p..s.i. The hemoprotein was detected at its characteristic absorption wavelength of 417 nm. Detector sensitivity, 0.2 a.u.f.s. (B1 and B2) Reduced carbon monoxide difference spectra of the aromatase fractions before and after passage, respectively, through the Mono-Q column.

Fig. 8. High-performance size-exclusion chromatography of crude aromatase cytochrome P-450. The active fractions obtained by HPIEC on the Mono-Q column were concentrated, then loaded onto a TSK-3000 SW column. Chromatographic conditions are as described in Table II. Marker proteins (left to right arrows): thyroglobulin, γ-globulin, ovalbumin, myoglobin, and cyanocobalamin.

procedure, we obtained four-fold purification of the aromatase with good retention of activity and 91% recovery (Table II). In the third step, the active fractions were further purified by HPSEC with the same detergent mixture and concentration. The results are shown in Fig. 8. The major aromatase-active peak (fractions 23–26) was collected. This pooled material had a specific aromatase activity of 3.995 nmol/-

TABLE II

AROMATASE-RECONSTITUTION ACTIVITY OF PLACENTAL CYTOCHROME P-450 AFTER PURIFICATION BY HPIEC ON MONO-Q COLUMN, FOLLOWED BY HPSEC

The aromatase-active fractions from HPIEC were concentrated at 4°C in Amicon Centricon 30 tubes and further purified by HPSEC on a TSK-G3000 SW column, which was previously equilibrated with 20 m*M* Tris buffer (pH 7.5) containing 20% glycerol, 1 m*M* DTT, 0.1% CHAPS, 0.05% Emulgen 913, 10 μ*M* androstenedione, and 0.1 *M* sodium chloride. Column temperature, 10°C; flow-rate, 0.4 ml/min; pressure, 500–600 p.s.i. Aromatase-reconstitution activity of each 1-min fraction was determined in the presence of purified HPR-2 (0.039 μmol/min).

	Protein (mg)	*Aromatase activity*			*Cytochrome P-450*		
		Spec. act. (nmol/min/mg)	*Total act. (nmol/min)*	*Recovery (%)*	*Spec. content (nmol/min)*	*Total (nmol)*	*Recovery (%)*
Impure sample	27.7	0.352	9.74	–	0.184	5.10	–
HPIEC fraction	5.64	1.565	8.83	91	0.819	4.62	91
HPSEC fraction	1.31	3.995	5.25	59	1.376	1:81	39

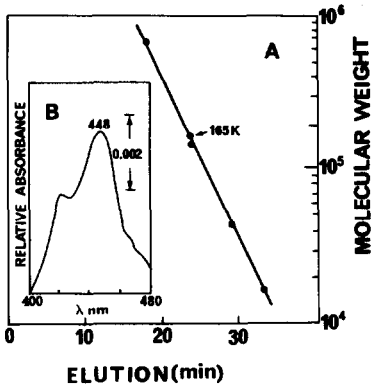


Fig. 9. Estimation of molecular weight of aromatase cytochrome P-450 by HPSEC. To estimate its molecular weight, the aromatase-active fraction, together with thyroglobulin, γ-globulin, ovalbumin, and myoglobin, was chromatographed on the TSK-3000 SW column under the same conditions as in Fig. 8; it eluted as a trimer of 165 000 daltons, despite the presence of 0.5% cholate detergent. This fraction had a specific activity of 4 nmol/min/mg protein and showed several bands in SDS-PAGE. (Inset) Difference spectrum of the reduced carbon monoxide complex.

min/mg protein and a specific cytochrome P-450 content of 1.376 nmol/mg protein (Table II). These values amount, respectively, to a 60-fold and a 23-fold purification of the aromatase from the original, microsomal state of the enzyme. The reduced carbon monoxide difference spectrum of the partially purified aromatase had a maximum at 448 nm (inset, Fig. 9B). Estimation of its molecular weight by HPSEC in the detergent-containing buffer gave a value of 165 000 daltons (Fig. 9A). We remark again that the aromatase cytochrome P-450 enzyme has not been obtained in a homogeneous state by any laboratory; neither from the human placenta, nor from any other source.

In conclusion, we have purified by a short, two-step chromatographic procedure, the reductase component of human placental aromatase in high yield and with full retention of activity. Its homogeneity was judged by two criteria: (1) the appearance of a single band in SDS-PAGE, and (2) the elution of a single peak in RP-HPLC. HPIEC on a Pharmacia Mono-Q column was found to be particularly efficient and reproducible for the purification of this membrane-bound enzyme. On the other hand, by applying an analogous approach, we have been able to achieve only partial purification of the second, heme-containing cytochrome P-450 component, which therefore must have physicochemical properties very different from those of the reductase.

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