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Estrogen synthetase in the horse

Comparison of equine placental and rat liver NADPH-cytrochrome *c* (P-450) reductase activities

A. Vibet, T. Dintinger, J.C. Maboundou, J.L. Gaillard, D. Divoux and P. Silberzahn

Laboratoire de Biochimie, Université de Caen, Esplanade de la Paix, 14032 Caen cedex, France

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NADPH-cytochrome *c* (P-450) reductases from horse placenta and rat liver were purified and their biological activities compared using cytochrome *c* as substrate. Rat liver reductase was purified to electrophoretic homogeneity in one chromatographic step on 2′,5′-ADP agarose, and had a relative mass of 85 000 Da as estimated by SDS-PAGE. Equine placental reductase was separated from cytochrome P-450 on aminohexyl-Sepharose 4B and further purified on 2′,5-ADP agarose; this preparation exhibited two bands, one of 85 000 and one of 80 000 Da, on SDS-PAGE. The lower molecular weight form was assumed to be a proteolytic product of the higher molecular weight form. A high retention of activity was obtained in both preparations. Equine placenta and rat liver enzymes were found to exhibit very similar V_{max} and K_m, suggesting that they are not species specific.

Reductase; Aromatase; Cytochrome *c*; Equine placenta

1. INTRODUCTION

Estrogen biosynthesis is catalyzed by a microsomal mixed-function oxidase system termed estrogen synthetase (EC 1.14.14.1). This enzyme complex includes NADPH-cytochrome *c* (P-450) reductase and cytochrome P-450. In the human, C19 androgens (androstenedione, testosterone and 16α-hydroxytestosterone) are far more rapidly aromatized than are 19-norandrogens [1–3]. In contrast, we have shown that mare placental estrogen synthetase is able to aromatize 19-norandrogens and androgens in vitro with approximately the same rate [4]. These observations, together with our previous findings on equine testicular and ovarian aromatases [5,6], indicate that equine aromatase is species specific and differs from the human model.

There is general agreement that the substrate specificity of mixed-function oxidases is determined by the cytochrome P-450 moiety, and that NADPH-cytochrome *c* (P-450) reductase is common to all cytochromes P-450. However, immunological and/or chemical differences between NADPH-cytochrome *c* (P-450) reductases from different tissues or species have been reported, thus suggesting a tissular and/or species specificity of the enzyme [7,8].

Osawa et al. reported that antibody against human placental NADPH-cytochrome *c* (P-450) reductase inhibited androstenedione aromatization by human, baboon and horse placental microsomes with different efficiencies [9]. However, the biochemical properties of horse placental NADPH-cytochrome *c* (P-450) reductase have not previously been studied. To investigate whether this component of the equine placental aromatase complex exhibits or not such specific characteristics in comparison to the widely used rat liver enzyme, we decided to purify NADPH-cytochrome *c* (P-450) reductases from horse placenta and phenobarbital-treated rat liver, and to compare the reductase activities of these enzymes using horse heart cytochrome *c* as substrate.

Correspondence address: A. Vibet, Laboratoire de Biochimie, Université de Caen, Esplanade de la Paix, 14032 Caen Cedex, France

2. MATERIALS AND METHODS

2.1 *Chemicals*

$NADP^+$, NADPH, cytochrome *c* (equine heart), dilauroylphosphatidylcholine, FAD, FMN, Chaps 2′,5′-agarose, aminohexyl-Sepharose 4 B, sodium cholate, Lubrol and concanavalin A were purchased from Sigma Chemical Co. Pharmacia Fine Chemicals supplied the 2′,5′-ADP agarose resin. Biobeads and chemicals employed for SDS-PAGE were from Bio-Rad. [1β-^{3}H]Androstenedione was obtained from NEN. Other chemicals and solvents were from Sigma or Merck.

2.2. *Animals and preparation of microsomes*

Six Sprague–Dawley male rats (300 g) were injected intraperitoneally with a 4% (w/v) solution of phenobarbital in 0.9% NaCl at a dose of 40 mg/kg daily for 5 days. Full-term placenta from a thoroughbred mare was collected immediately after delivery and processed as described previously [4]. Microsomes from equine placenta and rat liver were prepared according to the same method [4]. Rat liver microsomes were resuspended in 0.01 M sodium

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phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA and 1 mM dithiothreitol (Buffer A) to a final protein concentration of 27.2 mg/ml. Equine placental microsomes were resuspended in 0.05 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mg/l butylated hydroxytoluene and 2 μM androstenedione (Buffer B), to a final protein concentration of 28.9 mg/ml.

2.3. *Protein determination*

Protein concentrations were assayed with the Biorad dye-binding assay, using serum bovine albumin as standard.

2.4. *Enzyme assays*

Reductase activity was determined by measuring the reduction of cytochrome *c* at 550 nm. Incubations were performed at 30°C in 2.5 ml of 0.2 M sodium phosphate buffer, pH 7.5, containing 0.4 mM KCN, 0.04 mM EDTA, 0.04 mM NADPH and 20 μM cytochrome *c*. A molecular extinction coefficient of 19 $mM^{-1} \cdot cm^{-1}$ was used. In the kinetic study versus cytochrome *c* concentration (2–40μM), incubations were at 25°C for 5 min in 2.5 ml of 0.2 M sodium phosphate buffer, pH 7.5, containing 0.04 mM EDTA, 0.04 mM NADPH and 20% glycerol.

Aromatase activity was determined by measuring the release of tritium from [1β-^{3}H]androstenedione. Incubations were in 0.5 ml of 0.05 M Tris-maleate buffer, pH 7.4, containing ethylene glycol (3%), dilauroylphosphatidylcholine (40 μg/ml), 0.25 units of purified rat liver NADPH-cytochrome *c* (P-450) reductase, and 200 nM substrate. Reaction was initiated by the addition of 0.3 mM NADPH and stopped with 2 ml chloroform. Samples were then processed as described previously [4]. In all assays, except for tests for aromatase activity in elution fractions, detergents were extracted using Biobeads (40 mg/ml) with stirring for 1 h at 4°C.

2.5. *Solubilization and purification of equine placental NADPH-cytochrome c (P-450) reductase and partial purification of cytochrome P-450*

Equine placental microsomes were diluted with Buffer B and a 10% Chaps solution was added dropwise to a final concentration of 2.3% Chaps and a final protein concentration of 13 mg/ml. Microsomes were stirred for 30 min at 4°C and centrifuged for 1 h at 120 000 × g. The supernatant (76 ml) was diluted with 20 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM dithiothreitol, 0.2 mM EDTA, 2 μM androstenedione and 0.3% Chaps (Buffer C), to a final volume of 750 ml. The mixture was pumped (35 ml/h) directly to an aminohexyl-Sepharose 4B column (2.6 × 14 cm) previously equilibrated with 200 ml of Buffer C according to the method of Tan and Muto [10]. The column was washed with 450 ml of Buffer C and then eluted with a linear gradient of NaCl (0 to 1 M) in Buffer C. The eluted fractions were collected and assayed for cytochrome *c* reduction and aromatase activities. The fractions with reductase activity were pooled (97.5 ml), dialyzed for 18 h against 0.1 M sodium phosphate buffer, pH 7.7, containing 0.4% sodium cholate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20 μM butylated hydroxytoluene, 5 μM FMN and 20% glycerol, and then applied directly to a 2′,5′-ADP agarose column (2.5 × 4 cm) at a flow rate of 1 ml/min according to the procedure of Ardies et al. [11]. The active fractions were pooled and dialyzed on Sephadex G25 (PD 10, Pharmacia) to remove $NADP^+$, and stored at -20°C. The fractions eluted from the aminohexyl-Sepharose 4B column and containing aromatase activity were pooled (75 ml) and applied to a ConA-Sepharose 4B column (1.6 × 12 cm) previously equilibrated with Buffer C. The column was washed with 300 ml of Buffer C containing 1 M NaCl and then eluted with Buffer C containing 1 M NaCl, 0.05% Emulgen 913 and 0.2 M methyl α-D-mannoside. The eluted fractions containing aromatase activity were pooled, dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1% Nonidet P 40, 0.1 mM EDTA, 0.1 mM dithiothreitol and 2 μM androstenedione, and stored at -20°C. These preparations remained stable for several weeks when stored at -20°C.

2.6. *Solubilization and purification of rat liver NADPH-cytochrome c (P-450) reductase*

Rat liver microsomes were diluted to about 18 mg protein/ml with Buffer A and solubilized by dropwise addition of a 10% (w/v) solution of Chaps to a final concentration of 2% and a final protein concentration of 9.5 mg/ml. After stirring for 30 min at 4°C, microsomes were centrifuged (120 000 × g, 1 h). Purification was carried out as described above for equine placental NADPH-cytochrome *c* (P-450) reductase using a 2′,5′-ADP agarose column (2.5 × 4 cm). This preparation was stable for several weeks when stored at -20°C.

2.7. *Gel electrophoresis*

SDS-PAGE was performed using 12% gels according to the method of Laemmli [12].

2.8. *Spectral study*

The flavin content of reductase preparations was measured with a dual beam Perkin-Elmer spectrophotometer (Lambda 15), using an extinction molecular coefficient of 10.7 $mM^{-1} \cdot cm^{-1}$.

3. RESULTS AND DISCUSSION

Use of the zwitterionic detergent, Chaps, resulted in an effective solubilization of equine placental cytochrome P-450 and of both equine placental and rat liver NADPH-cytochrome *c* (P-450) reductases with an increase in their specific activities. Sodium cholate anionic detergent was not used for solubilization since a deleterious effect on cytochrome P-450 activity was observed during storage (data not shown); this observation is consistent with the results of Nakajin et al. [13] who reported that purified human placental cytochrome $P\text{-}450_{arom}$ had a half-life of only 2.5 days when stored at -80°C in a buffer containing small amounts of Emulgen and sodium cholate.

The total recovery for purification of rat liver en-

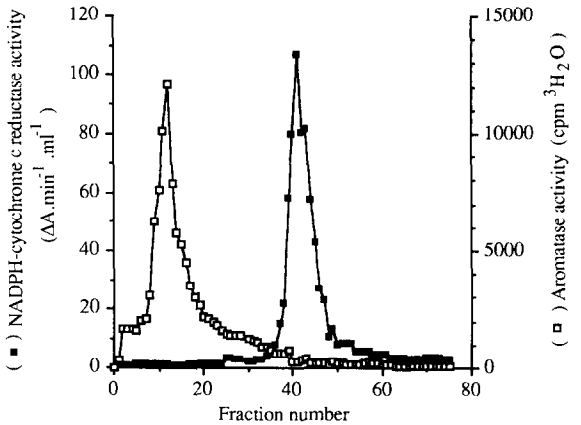

Fig.1. Aminohexyl-Sepharose-4B chromatography of the solubilized fraction of equine placental microsomes. The solubilized fraction, obtained by incubation of microsomes with 2.3% Chaps at 4°C for 30 min, was applied to an aminohexyl-Sepharose 4B column (2.6 × 14 cm), and eluted with a linear gradient of NaCl (0 to 1 M). 10 μl of each elution fraction (8 ml) was assayed for reductase and aromatase activities. Details of the chromatographic procedure and biological activity assays are given in section 2.

Table 1

Purification of equine placental NADPH-cytochrome *c* (P-450) reductase

	Proteins (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)	Purification fold
Microsomes	1300	325	0.25	100	-
Solubilized supernatant	758	349	0.46	107	1.8
Aminohexyl-Sepharose 4B	63	189	3	58	12
2′,5′-ADP agarose	4.35	174	40	54	160

Reductase activity was assayed at 30°C in 0.2 M sodium phosphate buffer, pH 7.5.

zyme (75%) was comparable to that reported by Ardies et al. [11]. The specific activity found in microsomes (0.36 units/mg protein) was brought to 25 units/mg protein in the purified preparation.

Fig.1 shows that a clean separation of equine NADPH-cytochrome *c* (P-450) reductase from cytochrome P-450 was obtained by chromatography on aminohexyl-Sepharose 4B, confirming the results of Tan and Muto [10]. Tables 1 and 2 summarize the results of the purification procedure of equine placental enzymes. The 2′,5′-ADP agarose chromatography step resulted in the greatest increase in purity and specific activity of the reductase. The yield for equine placental enzyme was lower (54%), due to the aminohexyl-Sepharose 4B step. A high purification factor (×160) was obtained for the equine placental enzyme; the smaller value (×70) obtained for the rat liver enzyme, although comparable to that reported by Ardies et al. [11], could be related to the high bulk of enzyme present in the liver of rats treated with phenobarbital.

The results of the analysis of equine placental and rat liver NADPH-cytochrome *c* (P-450) reductases by SDS-PAGE are shown in fig.2. Rat liver enzyme exhibited a single band corresponding to a molecular mass of 85 000, indicating the efficiency of the one step purification on 2′,5′ ADP-agarose affinity column. Equine placental enzyme was resolved into two bands corresponding to molecular masses of 80 000 and 85 000. Vermilion and Coon [14] have reported that the native enzyme can be converted by trypsin to a smaller protein by loss of a 5 000–6 000 hydrophobic membrane-binding part of the protein [15]; the lower band obtained for equine placental enzyme could

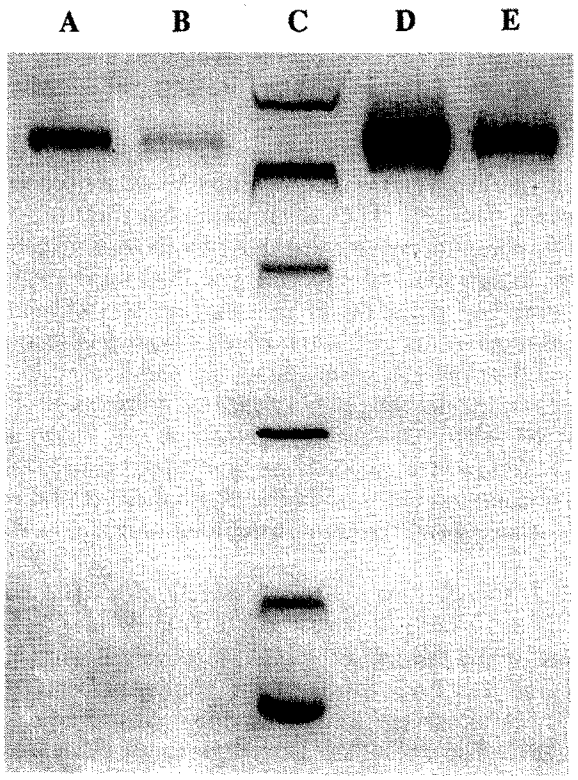

Fig.2. SDS-PAGE of equine placental and rat liver reductases. The proteins were treated with 2.4% SDS and 5% β-mercaptoethanol at 100°C for 5 min and subjected to electrophoresis on a 12% polyacrylamide gel. Gels were stained with Coomassie blue. (Lanes A and B) 20 μg (A) and 10 μg (B) of rat liver enzyme. (Lane D and E) 40 μg and 20 μg of equine placental enzyme. (Lane C) Molecular mass markers obtained from Bio-Rad consisted of rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31.1 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa).

Table 2

Partial purification of equine placental cytochrome P-450

	Proteins (mg)	Total activity (nmol/min)	Specific activity (pmol/min/mg)	Yield (%)	Purification fold
Microsomes	1300	110.1	84.7	100	-
Solubilized supernatant	757	105.1	138.7	95.5	1.6
Aminohexyl-Sepharose	64.2	15.6	242.5	14	2.8
Con A	6.1	5	828.1	4.6	9.8

Aromatase activity was assayed at 20°C in 0.05 M Tris-maleate buffer, pH 7.4, using 0.25 units of purified rat liver NADPH-cytochrome P-450 reductase.

therefore result from proteolysis of the native enzyme. If the upper band can be assumed to be the native NADPH-cytochrome *c* (P-450) reductase, the molecular weights of equine placental and rat liver enzymes found in the present study would be identical and comparable to those reported for human placental [9] or house flies [16] enzymes. However, it is to be noted that a range of values extending from 72 000 to 83 000 can be found in the literature [7,11,14,17–20]; the reasons for this variance are not known. Coon et al. [21], who compared the use of Renex 690 or deoxycholate for the solubilization of rat liver enzyme noted a variation in electrophoretic mobility corresponding to a difference of 2 000 in the molecular weights obtained by SDS-PAGE.

The flavin content of the rat liver enzyme (1.3 mol flavin/mol enzyme) was comparable to values reported elsewere [14–17]. The absorption spectrum of the equine placental enzyme was that of a flavoprotein with peaks at 380 nm and 454 nm. The flavin content was found to be 1.8 mol flavin/mol enzyme, and was in the same range as the values reported in most publications for rat liver [14,17], swine testis [18], and house fly [16] enzymes. Incubations of both enzyme preparations (1 h at 4°C) with 100-fold concentrations of FMN and FAD, followed by dialysis on Sephadex G25 (PD 10, Pharmacia) did not modify the original flavin content, indicating a good fixation of the flavin on the purified proteins.

High cytochrome *c* reductase activities have been reported for both the small and high molecular weight forms of the reductase [7,15]. Moreover, Bellino [22] reported that both forms of the human placental enzyme exhibited very similar K_m for cytochrome *c* and NADPH. These results prompted us to use cytochrome *c* as substrate to compare our two purified reductases. As shown by double reciprocal plots, V_{max} of horse placental NADPH-cytochrome *c* (P-450) reductase (1.4 units/mmol flavin) was similar to that of rat liver enzyme (1.36 units/mmol flavin). The K_m value of the horse placental enzyme (8.2 μM cytochrome *c*) and of the rat enzyme (13.5 μM cytochrome *c*) were of the same order of magnitude.

It would thus seem that these enzymes are not species specific. This is in apparent disagreement with the findings of Osawa et al. [9] who reported that antibody against NADPH-cytochrome *c* (P-450) reductase from human placenta suppressed cytochrome *c* reduction activity of human, baboon and horse purified reductases with different efficiencies, thus suggesting a species specificity. Nevertheless, immunological differences between enzymes may not necessarily be correlated to biological specificity, i.e. reductases from different sources (species, tissues) could contain different antigenic determinants corresponding to parts of the enzyme not involved in catalysis or binding, thus resulting in immunological but not biochemical differences with regard to the reduction of cytochrome *c*. It must be noted that the similarity in behaviour we observed between the rat liver and equine placental reductases with cytochrome *c* as a substrate does not exclude the possibility of differences between other properties of these two enzymes.

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