The proton-driven dissociation of oestradiol-receptor dimers as a preparative tool

Isolation of a 32 kDa fragment from porcine uteri and assignment of C-terminal origin by partial sequencing

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Homodimers of the porcine oestradiol receptor dissociate at pH 6.4. The monomers reassociate after neutralization. This property is retained in a 32 kDa receptor fragment generated by co-adsorbed endopeptidases from cytosolic receptor bound to heparin–Sephacrose. The fragment was purified by successive gel filtrations in the dimer and monomer states. Precipitations with ethanol and $(\text{NH}_4)_2\text{SO}_4$ respectively served as concentrating steps. In all, 10–15 nmol of the homogeneous fragment were recovered from 8 kg batches of porcine uteri with a $\sim 10^5$ -fold enrichment and in $\sim 20\%$ yields. Its oestradiol-binding capacity was identical with that of the intact receptor. The N-terminus was blocked. Two decapeptides from a tryptic digest were sequenced. One of them corresponded to amino acids 353–362 of the human receptor, a sequence fully conserved in all species investigated. The second peptide differed in positions 553, 554 and 557 from the 549–558 sequence of the human protein.

INTRODUCTION

Porcine uteri are an abundant source for oestradiol receptor. Their receptor content, like that of calf uteri, varies with the season [1]. The porcine receptor, however, appears to be less stable in extracts. This is due to the presence of endogenous endopeptidases, which degrade the receptor to steroid-binding fragments with molecular masses ranging from 52 to 17 kDa. Attempts to avoid degradation by addition of proteinase-inhibitor cocktails have not been successful. Even the rapid adsorption of the receptor to resins like heparin–Sephacrose presents no safeguard, since proteolytic enzymes are co-adsorbed. The porcine receptor, unlike the calf protein [2], cannot effectively be eluted by salt or polyanions as an intact molecule from heparin–Sephacrose [3]. The co-adsorbed enzymes release defined fragments of ~ 32 kDa in the presence of dithiothreitol (DTT) at pH 7.5 [4].

The amino acid sequences of oestradiol receptor from various sources, delineated by the recombinant DNA technique [5–9], show considerable similarity. Features of secondary and tertiary structure have been derived from the sequences and ascertained by genetic manipulations [10]. The structures responsible for dimerization only recently focused the attention of several investigators [11,12]. The receptor dimer can be reversibly dissociated by subtle pH changes, as first demonstrated by Little *et al.* [13] in extracts of porcine uterus microsomes. These extracts contain receptor fragments roughly comprising the C-terminal half of the intact receptor (F. Jakob, H. H. Thole & P. W. Jungblut, unpublished work). The fragments, like those released from heparin–Sephacrose, harbour the oestradiol-binding domain and the regions responsible for dimerization. They are devoid of the cumbersome tendency of the intact receptor to form heteroaggregates, which is detrimental to both conventional

and affinity isolation techniques. We purified one of these fragments to provide sufficient natural product for epitope characterization.

EXPERIMENTAL

Materials

Grade A chemicals were used throughout. $[6,7\text{-}^3\text{H}]\text{Oestradiol}$, specific radioactivity 2.22 TBq/mmol, was purchased from New England Nuclear and purified to $> 99\%$ before use. The radioactive compound was diluted with unlabelled steroid to 148–222 GBq/mmol.

Purification of oestradiol-receptor fragments

All operations were carried out at 0–4 °C unless otherwise stated. A constant-tolerance shearing device was used for homogenization [14]. All column operations were performed with self-designed top and bottom adaptors and upward flow. Preparation of receptor-containing extracts from porcine uteri, binding of receptor to heparin–Sephacrose, release of receptor fragments by endogenous enzymes and their enrichment by DEAE-chromatography were performed as described for small-scale preparations [4].

Ethanol precipitation. DEAE eluates were paddle-stirred in a cryostat. Ethanol, cooled to -20 °C, was added dropwise (within 1 h) while the temperature of the eluates was lowered from 0 to -5 °C. The suspensions were kept at -5 °C overnight. The precipitates were collected at 10000 g (r_{av} 9.6 cm) for 1 h in an 8750 rotor, Zeta 20, at -5 °C. They were dissolved in 10 ml of Smith & Smith (S&S) (10 mM-glycylglycine/10 mM-piperazine hydrochloride) buffer, pH 8.2, containing 2 M-urea, 0.3 M-KCl, 5 mM-DTT and an excess of $[^3\text{H}]\text{oestradiol}$.

Abbreviations used: DTT, dithiothreitol; S&S, Smith & Smith buffer (for composition, see the text); TFA, trifluoroacetic acid; i.d., internal diameter.

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Fig. 1. Reversible dimerization of intact oestradiol receptor and of 32 kDa receptor fragment

Density-gradient analyses of intact receptor (a) and 32 kDa fragment (b) were performed at pH 6.4 (●) and 7.5 (○) respectively. Intact receptor was extracted from porcine endometrial nuclei [37]; 32 kDa fragment was from the peak fraction in Fig. 2(a). Aliquots (200 μ l each) were layered on 5–25%-(w/v)-sucrose gradients containing 0.3 M-KCl and buffered to either pH 6.4 or 7.5. The samples were spun in a SW 60 rotor at 257 000 g (r_{av} , 9.17 cm) for 19 h at 2 °C (Beckman L2-65B centrifuge). Fractions (0.15 ml) were collected by constant-volume sampling from the top. Inset: SDS/PAGE-Western blot of 50 μ l aliquots (of a and b) stained with anti-receptor antibody-Fab'-peroxidase. (The band at 52 kDa in lane b represents a minor degradation product.) Abbreviation: *M*, molecular mass.

Gel filtration at pH 8.2. The redissolved ethanol precipitate was applied to a Sephadex G-200 column (50 mm diameter; 770 mm height), equilibrated with the same buffer, but without DTT. The flow rate was set at 30 ml/h (Watson-Marlow pump) and the eluate monitored at 280 nm by a Uvicord S. Fractions (10 ml) were collected in LKB Ultrac. Aliquots (25 μ l each) were counted for radioactivity in an LKB Rac Beta instrument.

To the eluate, which contained the peak of receptor-bound radioactivity, solid $(\text{NH}_4)_2\text{SO}_4$ was added up to 40% saturation at 4 °C. The suspension was adjusted to pH 6.8, stirred for 2 h and the precipitate collected by centrifugation. The pellet was dissolved in 3–5 ml of 0.01 M-S&S buffer, pH 6.4, containing 2 M-urea, 0.3 M-KCl and an excess of [^3H]oestradiol. The clear solution was left for 24 h at 4 °C for complete dissociation of receptor dimers.

Gel filtration at pH 6.4. A column of Sephadex G-200 (50 mm $\times$ 780 mm), equilibrated with the pH 6.4 buffer, was used. All other conditions were as described above. The pool of receptor-oestradiol complex was saturated to 50% with solid $(\text{NH}_4)_2\text{SO}_4$ at pH 6.4, the suspension left overnight at 4 °C, the precipitate collected by centrifugation, dissolved in a small volume (0.25–0.5 ml) of neutral buffer and analysed for purity.

Sequence analysis of tryptic peptides

Pure, SDS-denatured, 32 kDa receptor fragments were carboxymethylated [15], dialysed against 100 mM-Tris/1 mM-EDTA, pH 8.2, and cleaved with trypsin (protein/enzyme ratio of 20:1) at ambient temperature for 15 h (overnight).

The cleaved sample was made 0.1% in trifluoroacetic acid (TFA) and applied to a narrow-bore RP column [MN Nucleosil

300; C_4 ; 5 μ m particle size; 2.1 mm internal diameter (i.d.)]. A linear gradient was run from A (water/0.06% TFA) to B (80% acetonitrile/20% water + 0.054% TFA) within 60 min at a flow rate of 0.2 ml/min at room temperature. The eluate was monitored at 215 nm (Kontron 735LC) and the peaks were manually collected. They were rechromatographed on a RP 18 column (MN Nucleosil 300; C_{18} ; 5 μ m particle size; 2.1 mm i.d.) using the same buffer system with a gradient running from 0 to 100% B in 150 min.

The peak fractions were applied to Biobrene-coated glass-fibre filters (maximum 90 μ l) and sequenced by Edman degradation on an A477 Applied Biosystems sequencer with on-line h.p.l.c. analysis (120A Applied Biosystems) of phenylthiohydantoin amino acid derivatives formed.

SDS/PAGE and Western blotting

SDS/PAGE was performed in slab gels of 3 mm thickness, as described by Margolis & Kenrich [16], with a linear gradient from 4% acrylamide (T)/5% bisacrylamide (C) to 20% T/5% C in 0.02 M-Tris/0.01 M-sodium acetate/0.001 M-NaEDTA/0.1 mM-thioglycolic acid/0.1% (w/v) SDS, adjusted to pH 7.4 with acetic acid.

The samples were denatured in 5% (w/v) SDS/0.02 M-NaEDTA/0.1 M-thioglycolic acid/10% (w/v) glycerol (5 min at 95 °C) before application to the gels. The gels were run at 50 V for 15 h at 8 °C.

Western blotting on nitrocellulose membranes (0.2 μ m thickness; Schleicher und Schüll) was carried out in a Tank Blot (Bio-Rad) apparatus for 5 h at 0–4 °C with 0.025 M-Tris/0.192 M-glycine, pH 8.5 [17], with adjustment to constant-current (0.7 A) conditions.

The nitrocellulose membranes were soaked for 30 min in 0.05 % (w/v) Tween 20 in phosphate-buffered saline (PBS; 0.01 M-phosphate/0.15 M-NaCl M-NaCl, pH 7.5) and incubated with 1–3 μ g of immunoconjugates in 25 ml of PBS/Tween 20 (0.05 % w/v) for 15 h at 2–4 °C. The Fab'-horseradish peroxidase conjugates [11], cross-linked with maleimidohexanoyl-N-hydroxysuccinimide ester), were prepared using IgG₁ from goat polyclonal antiserum (# 402) against C-terminal 32 kDa oestradiol-receptor fragment [19]. After three washes with PBS/Tween 20, peroxidase staining was performed at room temperature with 25 ml of a solution containing 0.1 M-imidazole buffered to pH 7.4, 0.08 mg of Co(NO₃)₂/ml, 0.2 mg of diaminobenzidine/ml and 25 μ l of 30 % H₂O₂ for 0.5–2 min. Counter-staining with Indian ink [20] was used.

Auxiliary techniques

Protein concentrations were assayed with Coomassie Blue [21] using BSA standards. ³H radioactivity was counted in an LKB Rac Beta with a Beckman Ready Organic scintillator at efficiencies of 50–60 %. Sucrose density gradients were centrifuged as specified in the legend to Fig. 1.

RESULTS

The 32 kDa porcine oestradiol-receptor fragment (lane b, inset to Fig. 1) with unimpaired ligand-binding capacity released from heparin-Sepharose [4] dimerizes like the intact 65 kDa receptor extracted from endometrial nuclei (lane a, inset to Fig. 1) and both dimers can be dissociated by proton addition. The fragment sediments as a 4.4 S dimer at pH 7.5 and as a 3.4 S monomer at pH 6.4 (Fig. 1b), as compared with the 5.3 S and 3.9 S entities present in nuclear extracts (Fig. 1a).

The fragment was purified for further characterization. We processed batches of 8 kg of uteri and recovered about half of the receptor with a 143-fold enrichment (Table 1) after the heparin-Sepharose/DEAE steps [4]. Concentration of the eluted receptor (150 ml of eluate) was achieved by precipitation with ethanol. [Both receptor dimer (pH 7.4 or 8.2) and receptor monomer (pH 6.4) are precipitated and their steroid-binding site remains intact up to 30 % (v/v) of ethanol. Precipitation at 0–25 % and at pH 8.2 gives a 2.5-fold enrichment of 32 kDa ER compared with only 1.2-fold at pH 6.4.] The recovery of receptor in the precipitate amounted to 97.5 % with 27.5 % ethanol at pH 8.2.

The pH-dependency of the dimer/monomer states of the receptor was used for purification by successive gel filtrations at pH 8.2 and pH 6.4. At pH 8.2 (Fig. 2a), the major peak of receptor-bound radioactivity corresponded to a molecular mass of ~ 70 kDa (Fig. 2a). The enrichment of receptor at this stage amounted to 1950-fold (Table 1). The peak was pooled asymmetrically to remove residual endopeptidases with low molecular masses; the pool was precipitated with (NH₄)₂SO₄ and dissolved in pH 6.4 buffer. An aliquot of the precipitate was subjected to SDS/PAGE followed by immunoblotting. A prominent immunoreactive band at 32 kDa was seen (Fig. 3, lane 2), well separated from a host of proteins with molecular masses of > 40–100 kDa (Indian-ink staining; Fig. 3, lane 1). [No immunoreaction was detectable with the # 402 preimmune serum (Fig. 3, lane 3) or with immunoconjugates preadsorbed with pure receptor (Fig. 3, lane 4).] The inclusion of 2 M-urea in the gel-filtration buffers does not affect the stability of the dimer at pH 8.2, but facilitates the complete dissociation to the monomer at pH 6.4 in the concentrated solution. The pH 6.4 gel filtration (Fig. 2b) resulted in a further 50-fold enrichment. A single peak of bound radioactivity was eluted at the trailing end of the absorbance profile at ~ 32 kDa molecular mass. The ~ 0.04 A₂₈₀ void-volume peak

represented aggregated non-receptor proteins formed during the (NH₄)₂SO₄ precipitation. The material in the peak centre was collected by precipitation with (NH₄)₂SO₄ and part of the dissolved sediment was analysed by SDS/PAGE. The immunostained Western blot showed one band at 32 kDa (Fig. 3, lane 6). (Additional faint immunoreactive bands at 30 and 28 kDa were rarely observed.) Other proteins were not discernible by either Indian-ink (Fig. 3, lane 5) or silver staining (result not shown).

The purification achieved by the schedule is listed in Table 1. In the experiment described, an approx. 105000-fold enrichment

Table 1. Purification of oestradiol receptor 32 kDa fragment

Receptor concentrations were calculated from [³H]oestradiol binding, assuming a 1:1 ratio.

Material/step	Receptor/fragment			Enrichment (-fold)
	Total (nmol)	Recovery (%)	(nmol/g of protein)	
Extract	62		0.3	
Heparin-Sepharose eluate/DEAE peak	28	46	43	143
Ethanol precipitation/gel filtration pH 8.2	17	27.5	586	1950
Gel filtration pH 6.4/(NH ₄) ₂ SO ₄ precipitation	12	19.5	31 500	105000

Fig. 2. Gel filtration of 32 kDa oestradiol-receptor fragment in the dimer (a) and monomer (b) state

(a) Sephadex G-200 gel filtration (pH 8.2) of ethanol-precipitated receptor. Pooled fractions are marked (—). (b) Sephadex G-200 gel filtration (pH 6.4) of (NH₄)₂SO₄-precipitated receptor. IgG, BSA and β -lactoglobulin were used for column calibration.

Fig. 3. SDS/PAGE/Western blot of purified 32 kDa porcine oestradiol-receptor fragment

Lanes 1–4, 40%-satd.-(NH₄)₂SO₄-precipitated receptor (~0.15 µg) after Sephadex G-200 gel filtration, pH 8.2; lanes 5 and 6, 50%-satd.-(NH₄)₂SO₄-precipitated receptor (~0.3 µg) after Sephadex G-200 gel filtration, pH 6.4; lanes 2 and 6 were immunostained with anti-receptor antibody-Fab'-peroxidase conjugates; lanes 1 and 5 were stained with Indian ink [20]. Lane 3 was incubated with goat (# 402) preimmune serum, followed by rabbit anti-goat antibody-peroxidase (Sigma) diluted 1:1000; lane 4 was treated with immunoconjugates (same concentration as in lane 2) pre-adsorbed with 1.5 µg of pure 32 kDa receptor (15 h; 4 °C). Molecular masses (*M*) of prestained standards (Sigma) are indicated.

over the starting material was obtained with a recovery of ~20%. Assuming a 32 kDa mass for the isolated fragment and a staining intensity in the Bradford assay comparable with that

of the protein standard used, the specific activity (31 500 nmol of oestradiol receptor/g of protein) indicates that the product is pure.

We investigated the reversibility of receptor dissociation by neutralizing a solution of the pure protein and warming it for 30 min at 20 °C. After cooling at 4 °C, an aliquot was re-adjusted to pH 6.4. The original pH 6.4 (Fig. 4a, ①), the neutralized and warmed (Fig. 4b, ②) and the re-acidified solutions (Fig. 4b, ③) were analysed by sucrose-density-gradient centrifugations. The observed shuttle:

supports the previous assumption, namely of homodimerization, derived from kinetic analyses [31].

The 32 kDa fragment was not susceptible to direct sequencing, probably due to a blocking of the *N*-terminus during purification. We subjected the 32 kDa fragment to tryptic cleavage. The digest was chromatographed on a C₄ column from which a complex pattern of partially resolved peaks was eluted with high concentrations of organic modifier (>45%) in the mobile phase (results not shown). Two fractions were chosen for rechromatography (Fig. 5). One peak each of the two chromatograms was sequenced. The decapeptide (P1) from the chromatogram in Fig. 5(a) was identical with the human 353–362 sequence and corresponded also to all known decapeptides of other species in this region. The peptide (P2) from the chromatogram in Fig. 5(b) contained seven amino acids of the cDNA-derived human 549–558 sequence [5] positioned downstream from the putative steroid-binding region. The deviation from known sequences of other species was even more pronounced, with a difference of eight out of ten in the *Xenopus* sequence. The sequenced peptides are approx. 27.4 kDa apart (calculated on the basis of the human cDNA sequence), spanning nearly the entire region E (Fig. 5).

DISCUSSION

Numerous cytosolic oestradiol-receptor-degrading activities have been described, ranging from trypsin-like enzymes in human [22] and rat uterus [23] to neutral proteinases in calf uterus [24], plasminogen activators in rat uterus [25] and chymotrypsin-like

Fig. 4. Reversible dimerization of pure 32 kDa oestradiol-receptor fragment

Density gradient analyses are shown (conditions were as in Fig. 1). (a) ①, Material of peak fraction in Fig. 2 (b) centrifuged at pH 6.4; (b) ②, same material adjusted to pH 7.4, kept at 20 °C for 30 min, then centrifuged at pH 7.4; (b) ③, (b) ② incubation mixture cooled to 0 °C, then re-adjusted to pH 6.4, left in ice/water for 30 min, then centrifuged at pH 6.4.

Fig. 5. Separation of tryptic peptides of carboxymethylated porcine 32 kDa oestradiol receptor by narrow-bore reverse-phase h.p.l.c.

The Figure shows a chromatogram (C_{18} reverse-phase column) of two fractions (a and b) obtained from C_4 reverse-phase chromatography of the tryptic digest. Fractions (100–200 μ l) were collected manually in Eppendorf tubes and stored at -20°C before sequence analysis. The insets show sequence alignment of porcine oestradiol receptor (pER) tryptic peptides (P1 + P2) with corresponding regions from human (hER [5]), bovine (bER [30]), mouse (mER [9]), rat (rER [7]), chicken (cER [6]) and frog (fER [8]) receptor. The numbers indicate the positions of amino acids in the corresponding sequence. The functional domains of human oestradiol receptor according to Kumar *et al.* [10] are shown in (c). The tryptic peptides P1 and P2 have been aligned by sequence comparison with domains E/F.

endopeptidases in $C_3\text{H}$ -mouse mammary tumour [26]. We took advantage of the limited receptor digestion by heparin-Sepharose-bound endogenous proteinase(s) from porcine uteri [4] and arrived at relatively stable, well-defined, fragments. The origin and the nature of the proteinase(s) responsible for cleavage to the major 32 kDa product are unknown; the fragmentation proceeds apparently from the *N*- to the *C*-terminus.

The product isolated resembles the 'basic' microsomal receptor [13] considering charge, electrophoretic mobility in agar electrophoresis [4], molecular mass (same Stokes radii) and pH-dependent reversible dissociation of fragment homodimers. We exploited the proton-driven dissociation as an essential step in the purification schedule, amended by the concentrating steps of precipitation with ethanol and $(\text{NH}_4)_2\text{SO}_4$. Proton-dependent dissociation is superior to the use of chaotropic salts for purification [28], since the steroid-binding capacity is not impaired. Ethanol precipitation could be used for receptor enrichment without affecting the ligand-binding ability. This contrasts with a previous report by Puca *et al.* [29], who noticed irreversibly destroyed oestradiol-binding capacity. Yields of some 10–15 nmol of pure receptor fragment were obtained. Reversible dissociation by protonation as an easy-to-use purification tool should permit the isolation of other receptor fragments and receptor mutants with intact dimerization regions.

The partial amino acid sequence analyses showed a high similarity of porcine oestradiol-receptor peptides to already known cDNA sequences. Decapeptide P1 and the first four

amino acids of P2, located in the steroid-binding region E, are fully conserved in all seven receptor sequences [5–9,30] (with the exception of the *Xenopus* sequence); the rest of peptide P2, corresponding to domain F, shows considerable diversity. Interestingly, Ratajczak *et al.* [30], who used h.p.l.c.-separated tryptic fragments from calf uterus receptor, sequenced the same two peptides from region E. The sequences of the two tryptic decapeptides analysed here are ~ 27.4 kDa apart, leaving an uncovered stretch of ~ 5 kDa that could extend to either the *C*-terminus or the *N*-terminal start of region E.

The (reversible) dimerization of steroid-hormone receptors, first reported by Little *et al.* [13] for microsomal oestrogen receptor, generates receptor homodimers, as previously shown by kinetic analysis [31]. Although the role of the ligand in receptor dimerization is considerable, ligand-free receptor monomers do form dimers as well [32]. Receptor dimerization has later been interpreted as an '*in vitro*' artefact [33]. The more recent detection by Kumar & Chambon [35] of palindromic oestrogen-responsive element ('ERE') [34] and the confirmation that oestradiol receptor binds as a ligand-induced dimer to its responsive element settled this dispute. These authors suggested that a dimerization activity might be contained within the ligand-binding domain E.

A recent study by Sabbah *et al.* [11] emphasized the importance of hydrophobic interactions for the formation of receptor dimers. They used ~ 30 kDa tryptic fragments of bovine receptor that still bind tamoxifen-aziridin and are devoid of DNA-binding

properties. The group used the chaotropic salt NaSCN at a neutral pH for dimer dissociation. Hydrophobic amino acids, claimed to be responsible for dimerization, have been analysed in more detail by Fawell *et al.* [12]. Point mutations at Arg-507, Leu-511 and Ile-518 of the mouse oestradiol-receptor cDNA sequence, a region partially overlapping the steroid binding domain, prevented receptor dimerization.

The proton-driven reversible dissociation is difficult to explain if only hydrophobic amino acids are involved (keeping in mind also the stability of dimers in the presence of 2 M-urea), but suggests that the receptor dimer is locked by ionic or pH-dependent hydrogen bonds as well. In 1986, Mueller & Traish [36] postulated that the oestrogen-receptor monomer contains a dimerization domain unrelated to the domains for DNA and oestrogen binding. They claimed a participation of ϵ -NH₂ and -SH groups in 4 S-5 S dimerization of intact oestradiol receptor. The dissociation of dimers observed after lowering the pH from 7.0 to 6.5 [13] rather points to an involvement of imidazole in the process.

We assume that, in addition to hydrophobic amino acids within the steroid-binding domain, other regions with polar side chains substantially contribute to the dimerization process. They are retained in the purified 32 kDa fragment, supporting the assumption by Fawell *et al.* [12] that a region C-terminal from the hydrophobic steroid-binding domain may be involved.

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