Pergamon

0960-0760(93)E0051-8

*J. Steroid Biochem. Molec. Biol.* Vol. 48, No. 5/6, pp. 463–466, 1994

0960-0760/94 $7.00 + 0.00

# The Side Chains Responsible for the Dimerization of the Estradiol Receptor by Ionic Bonds Are Lost in a 17 kDa Fragment Extending Downstream from aa 303

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**Fragments of 32, 26 and 17 kDa of the porcine estradiol receptor were prepared, all of which contain the ligand-binding site. While dimers of the 32 and 26 kDa fragments like those of intact receptor can be dissociated by protonation, the dimer of the 17 kDa fragment obtained by trypsination of the 26 kDa fragment is resistant to lowering the pH from 7.0 to 6.5 and below. Its dissociation can be achieved by 0.5 M $MgCl_2$ at pH 7.0. All fragments are recognized by the MAB 13H2 in Western blots. The antibody also reacts with native receptor and the three fragments, both in their monomer and dimer states. The combining ratios of antibody with receptor, or its fragments, in the monomer and dimer states and the weakening of the estradiol–receptor bond by antibody attachment support the back to back and head to toe model of receptor dimers.**

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## INTRODUCTION

The estradiol receptor is a member of the receptor family acting in the nucleus by attaching as homo- or heterodimers to responsive elements of the DNA [1]. Most of these receptors like the PR and the GR do not form stable dimers [2]. The estradiol receptor, in contrast, readily dimerizes at neutral pH even in the absence of ligands [3]. The speed of dimerization is accelerated at least 10-fold after hormone binding to the monomers. The dimers can be reversibly dissociated either by subtle change to pH 6.5 as first demonstrated by Little *et al.* [4], or by treatment with chaotropic salts [5]. The reversible proton-driven dissociation of receptor dimers is shown by the intact 65 kDa receptor and by a 32 kDa fragment [6]. This fragment contains all of domain E with short extensions into D and F. We recently reported on the isolation of a 26 kDa estradiol-binding receptor fragment, which can be cleaved by trypsin to a 17 kDa fragment, which still contains the steroid binding site [7]. The N-terminal sequence of the 17 kDa fragment starts at amino acid 303, the N-terminus of domain E (human receptor numbering). The dimerizations of the 32, 26 and 17 kDa receptor fragments were studied.

Received 1 June 1993; accepted 18 Nov. 1993.

## EXPERIMENTAL

### *Preparation of estradiol receptor fragments*

The 32 and the 26 kDa fragments were prepared as described previously [6, 7]. Papain was removed from the digestion mixture by $(NH_4)_2SO_4$ precipitation followed by anion-exchange chromatography on DEAE-EMD Fractogel (Merck). The protease-free solution was stored at 4°C after the addition of 1 $\mu$M E 64 (final).

The 17 kDa fragment was generated by tryptic digestion of the 26 kDa pool at a substrate–enzyme ratio of 20:1 for 24 h at 4°C in the presence of 1 mM dithiothreitol (DTT). The digestion was stopped by 10 $\mu$M 3, 4 DCI (final).

### *Monoclonal antibody*

The mouse MAB 13H2 of the $IgG_1$ subclass directed against the 32 kDa estradiol receptor fragment [6] was purified from hybridoma supernatants by protein A-Sepharose chromatography [8].

### *Sucrose density gradients*

Samples of 200 $\mu$l were layered on 3.7 ml of (a) linear 5–15% (w/v) sucrose gradients (containing 0.815 M KCl, 0.1 M sodiumphosphate pH 7.5 or 6.5, respectively, 0.5 M $MgCl_2$, 50 mM MOPS pH 7.0) or (b) linear 5–20% (w/v) sucrose gradients (containing 0.3 M

KCl, 0.1 M sodiumphosphate pH 7.5 or 6.5, respectively) in SW 60 polyallomer tubes. The tubes were centrifuged in SW 60 rotors at 50,000 rpm for 15 h at 2°C in a Beckman L2-65B. Fractions of 150 $\mu$l were collected by upward constant-volume displacement with 55% sucrose solution. Radioactivity was measured in LKB-Wallac $\beta$-counters with $^3$H efficiencies of 50–58%.

*SDS–PAGE and Western blotting*

SDS–PAGE was performed in 0.75 mm slab gels using the Tris/Tricine system of Schägger and Jagow [9] with 4 and 10% acrylamide in the stacking and 16% in the separating gel. Samples were denatured according to [9] and run for 2 h at 100 V, 80 mA at 8°C. The gels were equilibrated in 25 mM Tris, 190 mM glycine buffer pH 8.3 containing 10% methanol and blotted onto ProBlott membranes (Applied Biosystems) at 0.5 A for 30 min. Immunostaining with 1 $\mu$g of 13H2–peroxidase conjugates has been described elsewhere [8].

## RESULTS

Hormone-binding estradiol receptor fragments of different molecular weights (32–17 kDa) have been prepared. All cleavage steps were performed at 4°C for minimizing the loss of bound steroid. The 32 kDa fragment [6] [Fig. 1(A)] was obtained by plasmin cleavage [10] and the 26 kDa fragment [7] [Fig. 1(B)] by papain treatment of ligand-filled estradiol receptor attached to heparin–Sepharose. The 26 kDa fragment

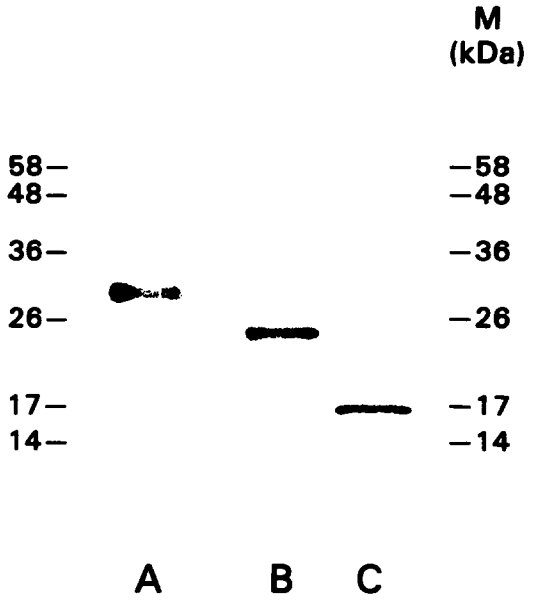


**Fig. 1. SDS-PAGE survey of hormone binding estradiol receptor fragments. Samples of 32 kDa receptor (plasmin cleaved), lane A; 26 kDa fragment (papain treated), lane B; and 17 kDa peptide (papain and trypsin incubate), lane C; were run on Tris/Tricine SDS-PAGE gels and blotted. The PVDF membranes were incubated with 1 $\mu$g 13H2-peroxidase conjugates and stained [8].**

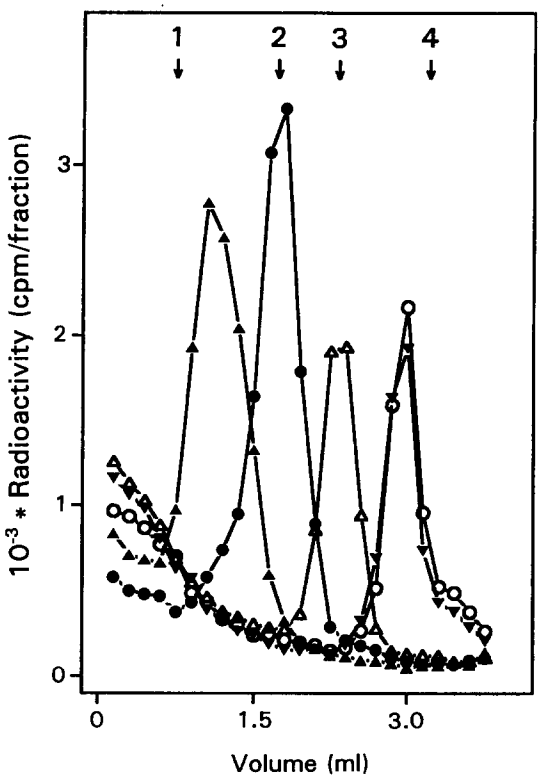


**Fig. 2. Sedimentation of 32 kDa estradiol receptor fragment with MAB 13H2 in the monomeric (pH 6.5) and dimeric (pH 7.5) state. Aliquots (200 $\mu$l) of receptor preparation, incubated with 10 nM [6, 7$^3$H]$E_2$ at 37°C for 30 min were charcoal-treated [4], buffered with sodium phosphate to pH 6.5 or 7.5 and mixed with 10 $\mu$l of MAB 13H2 solution (4 $\mu$g) or buffer. After 4 h at 4°C, the sample was layered on top of the 5–20% sucrose gradient buffered at pH 6.5 ▲, △ and pH 7.5 ●, ○, ▼, and spun for 15 h at 50,000 rpm. ▲: 32 kDa receptor alone, pH 6.5; ●: 32 kDa receptor alone, pH 7.5; △: 32 kDa receptor plus MAB 13H2, pH 6.5; ○: 32 kDa receptor plus MAB 13H2, pH 7.5; ▼: 32 kDa receptor plus MAB 13H2 incubated at pH 6.5 for 3.5 h. The sample was then buffered to pH 7.5 and allowed to stand for 30 min at RT. The positions of marker proteins (1) cytochrome C from horse heart 2.1 S, (2) bovine serum albumin 4.5 S, (3) pig IgG 7 S and (4) $\beta$-amylase from sweet potato 8.9 S are indicated.**

was digested with trypsin to the 17 kDa fragment [Fig. 1(C)]. The fragments were identified by MAB 13H2 [8] on Western blots.

MAB 13H2 also binds to native receptor and accelerates its sedimentation in sucrose gradients [8]. The stoichiometry of monomer/dimer interaction with MAB 13H2 was analysed. The antibody, when incubated with the 32 kDa receptor fragment at pH 6.5, sediments to 2.25–2.4 ml of the gradient (Fig. 2, △), corresponding to approx. 7 S. Incubation at pH 7.5 results in a faster sedimenting complex (3.0 ml, ○) of approx. 8.5 S speaking in favour of a 1:1 ratio in the monomeric state and the binding of 2 molecules of antibody to the receptor dimers. The dimerization process is not impaired by the antibody. This can be shown by incubation of the monomer receptor with the antibody at pH 6.5, followed by neutralization and warming which generates the same complex as obtained by the reaction of dimers with the antibody at pH 7.5 (Fig. 2, ▼).

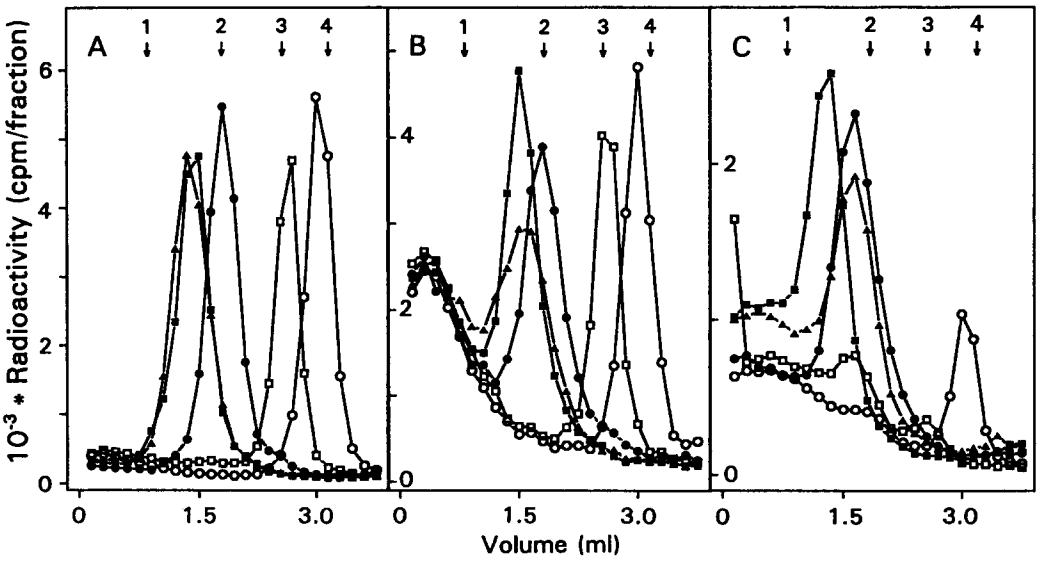


**Fig. 3. Dimerization of hormone binding estradiol receptor fragments of 32 kDa (A), 26 kDa (B) and 17 kDa (C). Density gradient analyses of 32 kDa estradiol receptor (A), of 26 kDa fragment (B) and of 17 kDa tryptic peptide were performed at pH 6.5 (▲), pH 7.5 (●) and in the presence of 0.5 M $MgCl_2$ (■), respectively. Incubates with 4 μg MAB 13H2 were centrifuged at pH 7.5 (○) and at pH 7.0 in the presence of 0.5 M $MgCl_2$ (□). Aliquots of 200 μl were layered on top of 5–15% sucrose density gradients containing 0.815 M KCl (▲, ● and ○) or 0.5 M $MgCl_2$ (■, □) and spun for 15 h at 50,000 rpm.**

The monomer/dimer states of the 32 kDa fragment generated by plasmin, of the 26 kDa fragment obtained by papain digestion and of the 17 kDa tryptic fragment were analysed before and after incubation with MAB 13H2 (Fig. 3) in sucrose density gradients. All three fragments sedimented as dimers in the density gradients buffered at pH 7.5 [Fig. 3(A–C), ●]. Their sedimentation velocities rose to 8.5–9 S after incubation with 13H2 [Fig. 3(A–C), ○]. Both the dimers of the 32 and 26 kDa fragments—like that of intact receptor—can be dissociated by lowering the pH to 6.5 [Fig. 3(A + B), ▲ and [6]]. The dimer of the 17 kDa fragment, however, remains stable at pH 6.5 and even at pH 6.2, when the loss of ligand becomes substantial [Fig. 3(C), ▲]. Its dissociation can be achieved by 0.5 M $MgCl_2$ at pH 7.0 [Fig. 3(C), ■]. This condition also leads to the dissociation of the dimers of the two larger fragments. MAB 13H2 binds to the fragment monomers in the presence of 0.5 M $MgCl_2$. The complexes sediment at ≈7 S [Fig. 3(A–C), □]. The antibody binding, however, results in a loosening of the steroid receptor bond, which is most pronounced for the 17 kDa fragment, less so for the 26 kDa fragment. At pH 6.5, the immunocomplexes of both fragments release all of the steroid (data not shown) while the loss from the 32 kDa receptor–antibody complex starts at pH 6.3 [11].

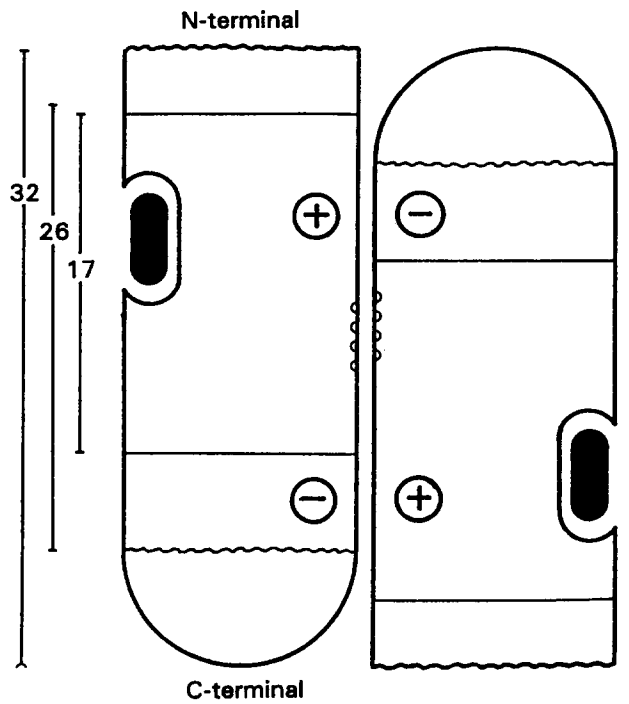


**Fig. 4. Model of the interaction of estradiol receptor (fragment) dimers. Black symbols represent estradiol. The symbol ∩∩∩ indicates hydrophobic amino acids. The "face" side is the one which contains the steroid-binding niche.**

## DISCUSSION

The dimerization of a steroid hormone receptor was first reported by Little *et al.* [4] for estradiol receptor extracted from microsomes of porcine uteri. The authors postulated the formation of homodimers by kinetic analysis and gave evidence for a linkage by ionic bonds [12]. Jungblut and coworkers suggested a back to back/head to toe model of the receptor dimers [4, 12]. Later on, Kumar and Chambon [13] analysed the dimerization of wild type and of mutated human estradiol receptor by gel shift experiments. They found a constitutive link for dimerization in the DNA binding domain C and a second ligand-dependent site in the

hormone binding domain E. Using the same technique, Fawell *et al.* [14] assigned the second linkage to the amino acid sequence 507–518 of the mouse estradiol receptor and emphasized the importance of R 507 and of the hydrophobic L 511 and I 518 which are highly conserved in steroid hormone receptors [15]. Hydrophobic interactions were also considered instrumental by Sabbah *et al.* [16] who prepared a tryptic fragment of ≈30 kDa, covalently labelled with tamoxifen-aziridine. The dimer of this fragment could be reversibly dissociated by 0.5 M sodiumthiocyanate as shown by density gradient centrifugation.

The precise localization of the polar amino acid side chains, held responsible for the ionic interactions, was as yet not possible. The intact 65 kDa porcine receptor and the proteolytic 32 kDa fragment, which contains all of the ligand binding domain E, can be reversibly dissociated by a simple pH shift [6]. The dissociation proceeds between pH 6.8–6.5 with similar kinetic parameters for both types of receptor [12]. The dimers are stable in the presence of 2–4 M urea at neutral pH as shown by density gradient centrifugation and size exclusion chromatography [6, 12, 17]. This could not be expected if the dimers would be stabilized by hydrophobic interactions only. The dimers of the 26 kDa fragment described here can still be dissociated by protonation. This property is lost in the tryptic fragment. Since the N-termini of the fragments are close [aa 298, 26 kDa (unpublished results); aa 303, 17 kDa [7]], the residue(s) responsible for the proton-driven mechanism must reside in the extended C-terminal portion of the 26 kDa fragment. The participation of an imidazol side chain has been suspected from its pK and the pH of dissociation. This is corroborated by the ethoxycarboxylation of histidines with diethylpyrocarbonate, which abolishes the proton-driven process in 32 kDa receptor [18]. Which of the nine histidines [474, 476, 488, 501, 513, 516, 524, 547 and 550; human numbering; all of which are conserved in the porcine sequence (unpublished results)] of the 26 kDa fragment is involved, remains to be shown. The absence of this/these histidine(s) in the 17 kDa fragment could then allow for a strengthening of hydrophobic interactions as suggested in the model.

The sketch (Fig. 4) locates a couple of ionic bonds adjacent to a central region of hydrophobic interactions. While the head to toe arrangement of the (homo)monomers appears logical for sterical reasons (homopolymers are not formed), the back to back arrangement of the model could be disputed. It is supported by the reactivity of 32 kDa receptor monomers and dimers to the MAB 13H2. Complexes of 32 kDa monomers with 13H2 can be reversibly dimerized. Also, receptor dimers bind 2 molecules of 13H2, which are dissociated by protonation to 2 receptor monomer–13H2 complexes. Most important, however, is the observation that the dimers of the 17 kDa monomer also bind two molecules of 13H2 and that in this process estradiol is released from the binding niche at pH 6.5. On these grounds, the rather simplistic model appears as reasonable.

*Acknowledgements*—The author would like to thank Professor P. W. Jungblut for his helpful advice and encouragement. The excellent technical assistance of Uwe Grunenberg, Karsten Mävers and Alexander Prokscha is gratefully acknowledged.

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