THE SUBUNIT-STRUCTURE OF THE UTERINE "OESTRADIOL-RECEPTOR"

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SUMMARY. Following the observation that the uterine "estradiol-receptor" denatured in 6 M guanidine-HCl + 0,1 M 2-mercaptoethanol is partially (up to 40 °/₀) renatured by dilution, several receptor preparations were filtered on a Sepharose 6/b column equilibrated in the above denaturing solvent. The fractions collected were renatured individually to establish the elution profile of the denatured receptor. Receptor preparations, which have a native molecular weight of about 120,000 dissociate into subunits of 55,000 ± 5,000 daltons in 6 M guanidine. The receptor obtained after mild proteolysis - molecular weight about 60,000 - dissociates into subunits of 28,000 ± 1.000 daltons. These results suggest that the uterine estradiol receptor is a dimer, and that one or both of the protomers is (are) composed of two identical fragments, the existence of which is revealed only by the mild proteolytic treatment.

INTRODUCTION

It is widely accepted today that estradiol interacts with uterine cells by a two-step mechanism in which the hormone first binds to a cytoplasmic receptor protein, this protein undergoing an alteration, called "receptor-transformation". The hormone-receptor complex is then translocated to the nucleus, where it associates with the chromatin. Neither the mechanism of these steps, nor the structure of the receptor is known at present (for a recent review-see (1)). As the receptor has not yet been isolated in amounts sufficient for the study of its structure[1], methods which allow for the utilisation of impure systems have to be employed. Particularly promising for this purpose is the method of "gel filtration is denaturing solvents", first described by Davison (2) and further developed in Tanford's Laboratory (3). This method is particularly advantegeous in that "molecular weights can be assigned to proteins without the uncertainties that arise when native molecules of unknown shape are studied" (2). Taking advantage of this method we analysed different receptor preparations by gel filtration in 6 M guanidine-HCl + 0,1 M 2-mercaptoéthanol. We wish to demonstrate in the present paper that the uterine estradiol receptor is composed of subunits.

Abbreviations : R uterine oestradiol-receptor ; RTF, Receptor Transforming Factor ; 6GM, 6M guanidine-HCl + 0.1 M 2-mercaptoethanol in 0.1 M Tris buffer pH 7.2 + 0.006 M EDTA.

[1] "Native" R has not been purified yet, only the purification of a modified, stabilised" R has been reported recently (4,5,6).

MATERIALS AND METHODS

Receptor preparations : 1) A crude extract of the uterine "cytoplasmic estradiol receptor" (R) was prepared from uteri of immature calves, as described previously (7), and was purified 5 times by filtration on a Sephadex G200 column with 95 °/₀ yield. 2) "Transformed cytoplasmic R" was prepared by ammonium sulfate fractionation, and 3) "Nuclear R" was extracted by 0,4 M KCl from chromatin previously incubated with labelled cytosol according to methods described by Jensens Laboratory (Ref (8) and (9) respectively). 4) R transformed by the "Receptor transforming factor" (RTF) was prepared according to Puca et al., (10). 5) "Trypsin-treated R" was prepared from preparation (1), according to methods described previously (11, 12, 13). 6) The transformation of the R by an endogenous protease was observed accidentally in this laboratory (14), during the preparation of "transformed R" (8) from a crude extract stored for 6 days at 0°C. To estimate M.W. of the R preparations, they were submitted to sucrose density gradient ultracentrifugation and Sephadex G200 chromatography in a 0.04 M Tris buffer, pH 7.4, 0.0015 M EDTA, 0.014 M 2-mercaptoethanol, 0.4 M KCl. In preparations 1-3, the peaks observed, especially those eluted from the Sephadex G200 column, showed certain heterogenity. Tentatively, molecular weights of approximately 120,000 were attributed to preps. 1-3. Preps 4-6 exhibit homogenous peaks, the estimated molecular weight being 60,000.

The assay of the R. The R was assayed according to a method described previously (15) : R preparations are incubated overnight at 0°C with saturating concentrations (1-3 x 10^{-8}M) of tritiated estradiol (50-100 curies/mmol C.E.A. or Amersham), and aliquots are adsorbed on small (0,5 ml) hydroxylapatite columns. Rinsing the columns with 50 ml 0,04 M Tris buffer pH 7,4 at 0°C, eliminates unbound estradiol ; estradiol bound to R remains adsorbed on the hydroxylapatite, which is then quantitatively transferred into counting vials. Bray's scintillation mixture (16) is added, and radioactivity measured in a Packard 3310 or Intertechnique scintillation spectrometer with a yield of 12-23 °/₀ respectively. "R concentration" is expressed as moles of estradiol bound in the R-estradiol complex stable at 0°C (17).

"Denaturation" of the R. The R-preparations were dialysed overnight at 4°C against a 0,1 M Tris buffer, pH 7,4, containing 6M guanidine-HCl (Carlo Erba) + 0,1 M 2-mercaptoethanol + 0,006 M EDTA (6 GM). The R does not bind estradiol in this solvent.

"Renaturation" of the R. The denatured R preparations were diluted 20 fold with a 0,04 M Tris buffer, pH 7,4, containing 0,1 M 2-mercaptoethanol +

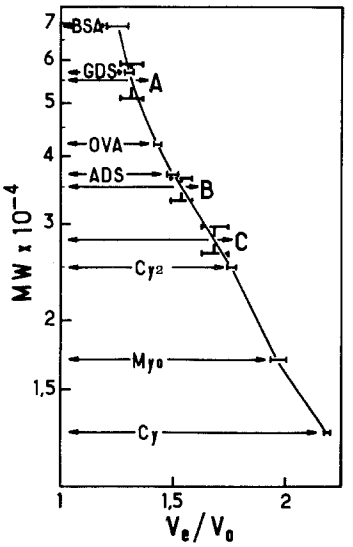

Fig.1. Calibration curve. Semilogarithmic plot of the molecular weight versus V_e/V_o. Horizontal bars represent V_e/V_o values of proteins of known subunit molecular weight, and indicate the extreme values of several (at least 3) experiments. To the proteins - denatured in 6GM - 1 mg Blue Dextran and 2 mg cytochrome c was added and volumes of 0.3-1 ml were filtered on the column. The proteins and Blue Dextran were monitored by adsorbance at 280 mμ and cytochrome c and the cytochrome c dimer (see later) at 410 mμ, with a Seive (France) recording spectrophotometer equipped by a monochromator allowing simultaneous recording at two desired wavelengths. Proteins used for calibration (abbreviations and subunit molecular weights in brackets) : Bovine serum albumine (BSA, 69,000) Serva ; glutamic dehydrogenase (GDH, 57,000) a gift of Dr. Pantaloni of this Institute Ovalbumine (OVA, 42,000) Serva ; Yeast alcohol dehydrogenase (ADS, 37,000) Worthington ; Whale myoglobine (MYO, 17,000) Sigma ; and horse-heart cytochrome c (Cy, 12,400) Boehringer. While recording adsorbance simultaneously at 280 mμ and 410 mμ to measure the elution volume of cytochrome c, we observed at both wavelengths a small peak which appeared ahead of the main cytochrome peak. As its position on the calibration curve corresponded to a M.W. of about 25,000 we concluded that it represents a cytochrome dimer, stable in 6GM, and used in all experiments as a very useful marker (2 Cy, 25,000). Vertical bars represent V_e/V_o values of experiments carried out with the different R preparations, and indicate the extreme values of 4-6 experiments. While only results of one typical experiment for each of the six different R preparations are shown in Fig.2, at 4-6 experiments were carried out with each preparation. A, B and C correspond to similar groupes in Fig.2. V_e/V_o values were calculated from data of elution volumes obtained under conditions described in text and in the legend of Fig.2.

0,0015 M EDTA + 10^{-8}M tritiated estradiol and kept at 4°C overnight. Measured by the hydroxylapatite technique, as described above, 40 °/₀ of the R was "renatured". Renatured R has the same apparent affinity constant as the native one, as measured according to methods described previously (12, 15). Renatured R in preparations 2, 4 and 5 has the same M.W. as "native" R when measured under the conditions described under "Receptor preparations" in this chapter. The other preparations were not tested.

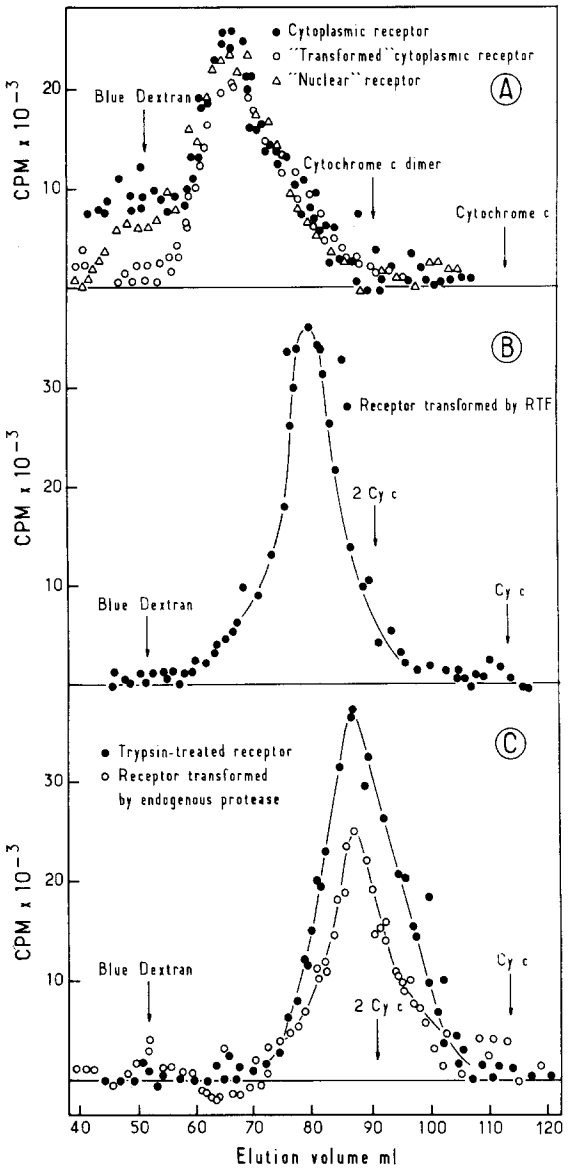

Fig.2. Elution profiles of different denatured R preparations. The R preparations were denatured in 6GM, 1 mg Blue Dextran and 2 mg cytochrome c were added to the samples and volumes of 0.3-1.5 ml, containing 2-5x10^{-11} moles of R and 6-16 mg protein, were filtered on the column. The R in the collected fractions was renatured and R concentration measured to establish the elution profile of the denatured R, as described under Methods. One typical experiment - carried out in duplicate - is shown for each of the six different R preparations. Elution volumes of Blue Dextran and cytochrome - added as markers in each run - show variations from experiment to experiment, but V_e-V_o values of cytochrome are practically constant. Therefore V_e of cytochrome c and V_o were measured in each individual run, and the position of the collected fractions normalised with respect to the average V_e-V_o value of the six experiments. Positions of Blue Dextran, cytochrome c (Cy c) and the stable cytochrome c dimer (2 Cy c) are marked with arrows.

Estimation of molecular weight in 6 M guanidine-HCl. The molecular weight of the denatured R was estimated according to the method described by Fisk, Mann and Tanford (3) : Sepharose 6/b was equilibrated in 6GM, and packed into a 20 mm diameter column to a height of 60 cm. The column was calibrated with proteins of known subunit molecular weight. To the different denatured R preparations Blue Dextran and cytochrome c were added as markers and 0.3-1.2 ml samples were filtered on the column at 22°C. Fractions of 0.5-1.5 ml were collected, renatured individually by dilution and their R concentrations assayed by the hydroxylapatite method in order to establish the elution profile of the denatured R.

RESULTS

Six different R-preparations were analysed on the Sepharose 6/b column equilibrated in 6GM, as described under Methods. Typical experiments are shown in Fig.2. The elution profiles of the denatured "cytoplasmic R", "transformed cytoplasmic R" and "nuclear R" (preps 1, 2 and 3, see Methods) seem to be superimposable. They show a considerable spread (Fig.2/A). Fig. 2/B shows the relatively sharp eluted peak of the denatured RTF-treated R (prep 4). Elution profiles of the denatured trypsin-treated R and the R transformed by an endogenous protease (preps 5 and 6, see Methods) also seem to be superimposable (Fig.2/C). Based upon the elution volumes (V_e) corresponding to the maxima of the peaks and on the elution volume of Blue Dextran (V_o) molecular weights were estimated with the aid of the calibration curve obtained with proteins of known subunit molecular weight (Fig.1). The results, together with the molecular weights of the corresponding "native" preparations are summarised in Table 1. Data presented in Table 1 show that the different R preparations denatured in 6GM exhibit three different molecular weights : 55,000 $\pm$ 5,000 (preps 1-3), 35,000 $\pm$ 100 (prep.4), and 28,000 $\pm$ 1,000 (prep. 5-6). As the "native" M.W. of cytoplasmic R (prep 1) is most probably 118,000 (10), the observed M.W. of 55,000 $\pm$ 5,000 in the denatured state suggests that the cytoplasmic R is a dimer. Due to the $\pm$ 10 °/₀ experimental error, it is not possible to state whether the two subunits are identical or slightly different. Preparations 1-3 show, whithin the limits of experimental error, similar molecular weights in the denatured state, suggesting that their subunit structures, and probably even their "native" molecular weights, may not be very dissimilar either. The"native" molecular weights of preps 5-6 (obtained by mild proteolytic treatment) is 60,000, they exhibit a molecular weight of 28,000 $\pm$ 1,000 in the denatured state, indicating dissociation into two similar - if not identical - subunits. Mild proteolysis transforms the R (M.W.120,000) into a form having a M.W. of 60,000 without affecting the estradiol binding site(s) (10, 11, 12, 13). It is not known whether the mole-

Table 1

N°	Receptor preparations References in brackets	App. molecular weights of: "native" R	denatured R
1	"Cytoplasmic" (7)	118,000 (10) 120,000	
2	"Transformed" (8)	120,000	55.000±5000
3	"Nuclear" (9)	120,000	
4	RTF-treated (10)	61,000	35.000±1000
5	Trypsin-treated (11)	60,000	28.000±1000
6	"Endogenous protease"treated(14)	60,000	

Table 1. Apparent molecular weights of the "native" and denatured uterine estradiol receptor. M.W. of the "native" R : There is controversy in the literature concerning the M.W. of preps. 1-3, since R undergoes various structural changes, including irreversible aggregation under even the mildest conditions of analysis or fractionation (1, 19, 20). Puca et al. (10), taking special measures to avoid aggregation, attribute a M.W. of 118,000 to cytoplasmic R (prep.1). DeSombre and Jensen (1, 8, 9) communicate identical (5 S) values for the sedimentation coefficient of "transformed" and "nuclear" R (preps. 2 and 3), without indicating the M.W. We find (see Methods) a M.W. of approximately 120,000 for preps. 1-3 measured in presence of 0.4 M KCl. R submitted to mild proteolysis does not reaggregate ; hence M.W. can be estimated with more confidence. The M.W. of preps. 4-6 is 60,000. M.W. of denatured R : M.W. was estimated on the basis of results shown in Fig.2 (elution volumes) with the help of the calibration curve shown in Fig.1.

cule is cut roughly into two "halves", or whether one "half" of the molecule undergoes more extensive proteolysis while the other remains intact. Whichever the case, we propose that the R (M.W. 118,000) is composed of two subunits (M.W. 55,000), mild proteolysis makes new sites accessible to cleavage in 6 M guanidine and the molecule dissociates further into two subunits (M.W. 28,000). Consequently, at least one of the R protomers (M.W. 55,000) is composed of two fragments, the existence of which is revealed only by the mild proteolysis treatment. The"native" M.W. of the R obtained by the action of RTF (prep.4) is 61,000 and in the denatured state 35,000 ± 1,000. As its elution peak is sharp and apparently symmetrical (Fig.2/B) and as the M.W. of the renatured R is identical to that of the native molecule, we conclude that the R dissociates into two subunits. Were this the case the reason for the contradiction between the values for the native M.W. and that of the sum of the subunits remains unsolved for the moment.

DISCUSSION

Gel filtration of impure R preparations (5-10 mg R/100 g protein) in

6M guanidine-HCl provided the first demonstration of the existence of R subunits. Though the only partial renaturation (20-40 °/₀) and relatively poor resolution[2] constitute a warning against generalised conclusions, we think that the model proposed concerning the quaternary structure of R is at least qualitatively correct. While estimation of "native" M.W. in preps. 1-3 is ambiguous, the estimation of M.W. in the denatured state is exempt of uncertainties introduced by the uncontrolled structural changes of the "native" molecule (see legend of Table 1). Therefore, the finding that the M.W.'s of denatured "cytoplasmic", "transformed", and "nuclear" R are very similar is in favour of the tentative suggestion (see "R preps" under Methods) that the "native" M.W.'s of these three R species are simular as well. At the same time these results offer the possibility of testing the validity of some biological models proposed : While all proposed models agree that, when estradiol binds to the cytoplasmic R, the R undergoes a "transformation" and that the resulting R-estradiol complex is translocated to the nucleus, there is controversy concerning the nature of this "transformation". Puca et al. (10) suggest that the R is transformed by RTL, a specific Ca^{++} activated endogenous protease, the resulting R molecule having a M.W. of 61.000. According to DeSombre and Jensen (1, 8) "transformation" involves a more subtle alteration of the molecule without a significant change of the M.W. Results presented in this report indicate that the subunit molecular weights of "nuclear R", and R transformed according to DeSombre and Jensen are very similar, if not identical ($55.000 \pm 5,000$), while that of "RTF-transformed R" is very different ($35,000 \pm 1,000$). While this finding does not prove that the theory proposed by DeSombre and Jensen is correct, it is certainly not in favour of the suggestion that RTF transformed-R is the precursor of the "nuclear R". RTF still might have some unknown biological role ; its action on the R molecule is different from that of trypsin or of the undefined endogenous protease(s), as revealed by the observed difference in the molecular weights of the corresponding subunits.

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[2] In fact the considerable spread of the eluted peaks in the experiments presented under Fig.2/A led us in some preliminary experiments (8) to an interpretation different from that of this report.

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