FILTRATION OF CALF UTERINE CYTOSOL ON ULTROGEL ACA 34 PREVENTS AGGREGATION OF THE ESTRADIOL RECEPTOR

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1. Introduction

The Stokes radius of the estradiol receptor from calf uterus cytosol has been estimated to be 6.7 nm and 5.4 nm in low and high ionic strength medium, respectively [1,2]. However, the use of gel filtration (Sephadex G-200 or agarose) for the characterization of the 'native' estradiol receptor is questionable. In low salt medium, the receptor (8 S) is largely excluded from the gel, contrary to its behaviour in high salt medium (5 S–KCl) where it is not excluded. However in both cases, it is cluded in a broad peak, reflecting heterogeneous aggregation states of the receptor [3–5]. In presence of 0.1 M thiocyanate, it is possible to prevent the aggregation of the receptor, and its Stokes radius is 3.6 nm [6]. In the present paper it is reported that the filtration of the cytosol on ultrogel ACA 34, contrary to what is observed with the classically used gels, allowed the elution of the receptor as a symmetrical peak after the void volume of the column, in low as in high salt medium. The Stokes radius of the receptor was measured in low and high ionic strength buffers. A study of the filtration conditions for this type of gel was undertaken and led to a simple method of preventing aggregation of the estradiol receptor.

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2. Materials and methods

2.1. *Biological material*

Cytosol was prepared from calf uterus stored in liquid nitrogen, as previously described [7]. The tissue was homogenized in TE buffer (Tris–HCl) 50 mM, EDTA 1.5 mM, pH 7.4) at 0–4°C, the temperature at which all the subsequent operations were performed.

2.2. *Measurement of receptor concentration*

Cytosol was incubated immediately after preparation either with radioactive estradiol (^{3}H-E_2, 45–55 Ci/mmol, CEA, France) at a final concentration of 3.5–8 nM for at least 90 min at 0°C, or in presence of the same amount of radioactive estradiol diluted with nonradioactive estradiol (E_2 Roussel Uclaf) at a final concentration of 1 μM, in order to determine the contribution of the low affinity complexes.

The concentration of the hormone receptor complexes was measured by 'differential dissociation' techniques: charcoal method [8] or hydroxylapatite method [9].

2.3. *Gel-filtration*

The gel (ultrogel ACA 34) was washed several times with distilled water and with the experimental buffer and the fines were removed. The de gassed gel was packed in a column and equilibrated with the buffer. Two types of columns were used: column A (volume 200 ml, filtration flow rate 20–26 ml/h) and column B (volume 550 ml, filtration flow rate 100

ml/h). The volumes of the samples loaded on the columns were either 1% of the volume of the column (analytical purpose) or 5% of the volume of the column (preparative purpose). Calibration curves were established by filtration of standard proteins under the same conditions as the samples. V_o (void volume) and V_t (total volume accessible to the solvent) were determined by filtering dextran blue and potassium bichromate respectively. K_d was calculated from:

$$K_d = \frac{V_e - V_o}{V_t - V_o}$$

and results plotted according to Porath [10].

2.4. *Ultracentrifugation on sucrose gradient*

The samples were layered on a 5–20% linear sucrose gradient prepared in TE or TEK buffers (TEK = Tris–HCl 10 mM, EDTA 1.5 mM, KCl 0.4 M, pH 7.4), and centrifuged in SW 41 or SW 60 rotors (Beckman). Sedimentation coefficients were determined according to Martin and Ames [11], by using peroxydase (S = 3.6 [12]) as an internal standard.

2.5. *Radioactivity measurements*

The samples (0.1–0.9 ml) were counted in 10 ml Bray's solution with an efficiency of 26–35%.

2.6. *Proteins*

Protein concentration was measured according to Lowry [13].

3. Results

3.1. *Cytosol-filtration on ultrogel ACA 34*

3.1.1. Filtration of cytosol in low ionic strength medium

In fig.1 are presented the elution patterns obtained after filtration of cytosol preincubated with radioactive estradiol (see Materials and methods). It was observed that the first two radioactive peaks were suppressed by isotopic dilution of the steroid, indicating that the two peaks contained receptor. Peak 1 corresponds to the void volume of the column and contains aggregated receptor. Peak 2 is symmetrical and is unaggregated receptor. Peaks 3 and 4, which are not suppressed by isotopic dilution, correspond respectively to estradiol interacting non-specifically with small molecules

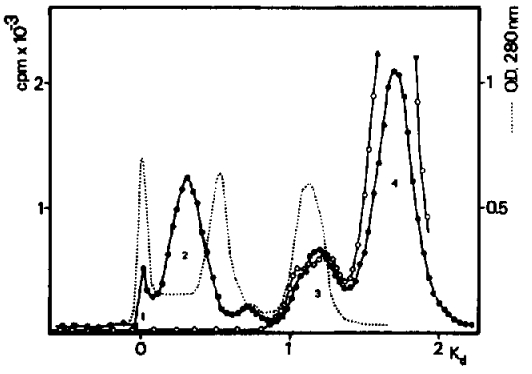

Fig.1. Filtration of cytosol through an ultrogel ACA 34 column in a low ionic strength medium. (•——•) 2 ml of cytosol preincubated with radioactive estradiol (see 2.2.) were filtered through a 200 ml column. 1.99 ml fractions were collected. (. . . . .) Proteins were detected by measurement of A_{280nm} and radioactivity measured in 0.5 ml of each fraction. (○——○) Simultaneously 2 ml of the same cytosol preincubated with estradiol isotopically diluted (see 2.2.) were filtered through a 200 ml column. 0.5 ml of the 1.75 ml collected fractions were used for radioactivity measurement. Peak numbers (1–4) are commented upon in the text.

eluted at the total volume and to free estradiol that is strongly retained by the gel. The receptor present in peak 2 was characterized by ultracentrifugation on sucrose gradient (fig.3A). In the TE buffer, it sediments at 6.3 S. This value is significantly lower than that obtained for the receptor ($7 < S < 8.5$) in the corresponding cytosol.

3.1.2. Filtration of cytosol in high ionic strength medium

In fig.2 are shown the elution patterns of cytosol treated as in fig.1. Peak 1 was suppressed by isotopic dilution and contained the receptor. As above, peaks 2 and 3 represent estradiol unbound to the receptor. The receptor eluted in peak1 was characterized by ultra-centrifugation on sucrose gradient. In high ionic strength medium (TEK buffer), its sedimentation coefficient is 4 S (fig.3B). This value is lower than that obtained for the receptor in the same cytosol ($4.6 < S < 6.2$). In TE buffer, it reassociates into a heavier form indicating that it is 'native' receptor and not a form partially proteolyzed during the preparation. For this last form the sedimentation coefficient would not be changed by the ionic strength.

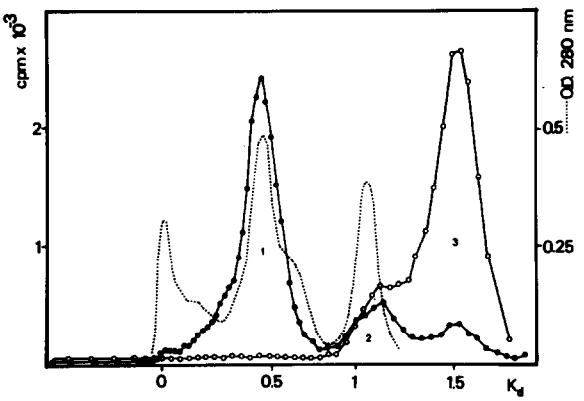

Fig.2. Filtration of cytosol through an ultrogel ACA 34 column in a high ionic strength medium. Experiment and conditions were the same as in fig.1, except that the column was equilibrated with TEK buffer. (•——•) Incubation with radioactive estradiol. Volume of the column 184 ml. Volume of the collected fractions 2.80 ml. (○——○) Incubation with isotopically diluted estradiol. Volume of the column 198 ml. Volume of the fractions 2.81 ml. In both cases radioactivity was measured in 0.3 ml of each fraction. Peak numbers (1–3) are commented upon in the text.

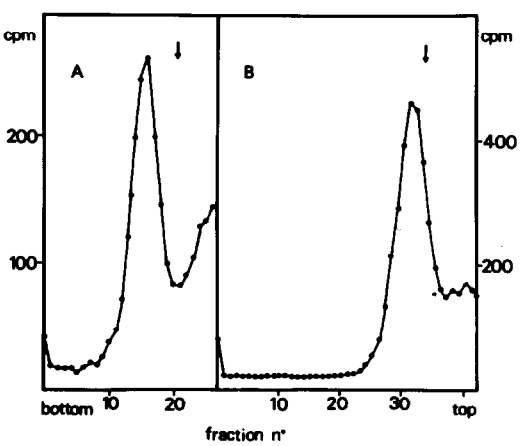

Fig.3. Ultracentrifugation on sucrose gradient of the receptor after gel-filtration. A. Low ionic strength medium ultracentrifugation. A 0.25 ml sample of the peak corresponding the non-aggregated receptor after gel-filtration in a low salt medium (binding activity 8885 cpm/ml) was run through a sucrose gradient for 14 h at 38 000 rev/min in the rotor SW 60. B. High ionic strength medium ultracentrifugation. A.1.2 ml sample of the receptor peak obtained after filtration of the cytosol in a high ionic strength medium (binding activity 6670 cpm/ml) was centrifuged for 16 h at 38 000 rev/min in the SW 41 rotor. The arrow indicates the position of the peroxydase used as an internal marker.

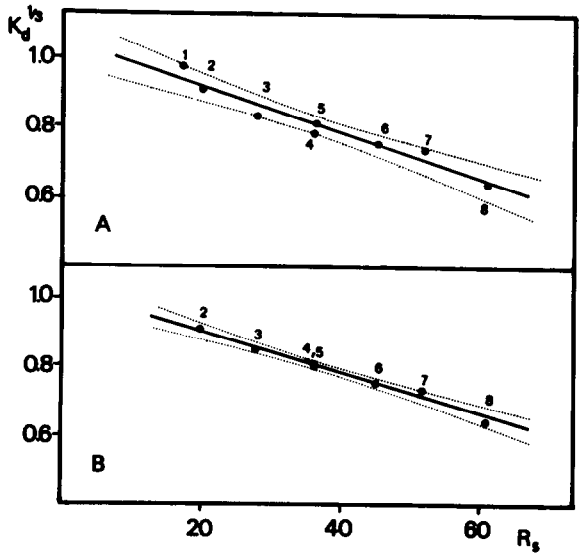

Fig.4. Stokes radius of the receptor in a low and high ionic strength medium. Calibration curves are plotted according to Porath [10]. Proteins used for the calibration are listed in table 1. Dotted lines represent the 95% confidence limit for the line. Error on the Stokes value was calculated from the confidence limit for the population [16]. A. Low ionic strength medium. Each point represents the mean of 2 independent determinations. For the receptor the K_d used for the determination of the Stokes radius was the mean of 12 independent determinations. B. High ionic strength medium. Each point represents the mean of 4 independent determinations. The K_d value used for the determination of the Stokes radius of the receptor was the mean of 16 independent determinations.

3.2. *Determination of the Stokes radius of the receptor*

Stokes radius of the receptor was determined in low and high ionic strength medium. Figure 4 presents calibration curves obtained in TE and TEK buffers. Proteins are listed in table 1. Results are plotted according to Porath [10]. The Stokes radii were 5.5. ± 1.3 nm and 3.7 ± 0.7 nm in TE and TEK buffers, respectively.

3.3. *Influence of extract protein concentration on receptor S-values and Stokes radius*

3.3.1. Influence on the sedimentation coefficient

The value of the sedimentation coefficient of the receptor systematically decreased after filtration through ultrogel ACA 34. Table 2 shows that the

Table 1
Standard proteins used for calibration of the columns

Protein		Source	R_S (nm)
1	Cytochrome *c*	Horse heart (Sigma)	1.74 [14]
2	Myoglobin	Equine skeletal muscle type I (Sigma)	2.02 [15]
3	Ovalbumin	(Nutritional biochemicals)	2.8 [15]
4	BSA	Bovine (Pentex)	3.63 [15]
5	Transferrin	Human pooled plasma (Calbiochem)	3.66 [15]
6	ADH	Yeast liver (Sigma)	4.55 [14]
7	Catalase	Bovine liver (Sigma)	5.22 [14]
8	Ferritin	Equine spleen (Boehringer)	6.14 [15]

See fig.4

Table 2
Variation of the sedimentation coefficient of the receptor in low ionic strength medium as a function of the protein concentration

Extract	Protein concentration (mg/ml)	Sedimentation coefficient (S)
Cytosol	15	8.50
Diluted cytosol	0.5	6.70
Peak column B[a]	0.7	6.70
Peak column B[a] diluted	0.14	6.20
Peak column A[b]	0.125	6.25

[a] Analytical procedure
[b] Preparative procedure (see 2.3.)
A. Volume of the column approx. 200 ml. B. Volume of the column 550 ml.

sedimentation coefficient of the receptor, measured in a low ionic strength medium, decreased when the proteins decreased, as was previously described for the cytosol estradiol receptor from rat uterus [17]. The same effect is obtained by dilution of the cytosol or by a preliminary partial purification of the receptor by filtration of the cytosol on ultrogel column in low salt medium. In TE buffer the sedimentation coefficient reaches its minimum measurable value at 6.2 S. In TEK buffer similar results were obtained: dilution of the cytosol (5–15-fold) or preliminary purification as described above led to a decrease of the sedimentation coefficient, reaching 4 S. Frequently a shoulder, also noted by other authors for rat cytosol estradiol receptor [17], appeared at the 5 S region.

3.3.2. Influence on the Stokes radius

The Stokes radius values measured above for the receptor were obtained by filtration of undiluted cytosol. Consequently it is important to know if the Stokes radius is a function of the protein concentration as the sedimentation coefficient is. The change in the protein concentration of the sample containing the receptor was obtained as described above for the sedimentation coefficient studies. In table 3 are compared the values of the Stokes radii obtained for the receptor in a low salt medium at different protein concentrations. It was observed that the decrease in the protein concentration led to a decrease in the Stokes radii values (0.5–0.9 nm less than the mean value calculated above from the cytosol) except when

Table 3
Variation of the Stokes radius of the receptor in low ionic strength medium as a function of the protein concentration

Extract	Protein concentration	R_S (nm)
Diluted cytosol	1.0	4.7
Peak column A^b	0.126	4.9
Peak column B^b	0.115	5.1
Peak column B^a	0.7	5.6

^{a,b} see table 2 for A, $B^{a\ b}$

the sample analysed on the column was partially purified under 'preparative' conditions (see Materials and methods).

For preventing aggregation of the receptor it is important that the volume of the loaded sample represents only 1% of the volume of the column, and that the collected fractions represent only the top of the receptor peak, thus preventing contamination of the receptor fraction by some aggregating proteins. In a high ionic strength medium, there was no variation of the Stokes radius obtained by varying the same parameters (results not presented here). Contrary to what was observed in a low salt medium, a preliminary partial purification of the receptor by filtration in a high salt medium did not prevent its aggregation.

4. Conclusion

Filtration of calf uterine cytosol on ultrogel ACA 34 alowed the characterization of the estrogen receptor. Its Stokes radius was estimated at 5.5 ± 1.3 nm and 3.7 ± 0.7 nm at low and high ionic strength, respectively. In a low ionic strength medium, it is necessary to note a slight variation of the Stokes radius with the protein concentration. The value obtained in high salt medium is comparable to the value obtained for the receptor in presence of thiocyanate [6] and higher than that obtained for the partially proteolysed form of the receptor obtained by trypsin treatment [18,19]. The estimation of the molecular weight of the receptor is difficult from these data. Indeed the sedimentation coefficient value varies considerably as a function of the protein concentration as indicated by the values presented in 3.1.1. and 3.1.2. Meanwhile, if one assumes that the lowest sedimentation coefficient and Stokes radius are closer to reality, it is possible to estimate the molecular weight of the receptor at approximately 120 000–140 000 and 60 000–70 000 in low and high ionic strength mediums respectively. The results obtained here do not allow us to show the 200 000 molecular weight form of the receptor described by other authors [1]. It is possible that a tetramer dissociates to a dimer during the filtration. Meanwhile the ultracentrifugation and the gel filtration results at low ionic strength suggest more non-specific aggregation of proteins to the receptor than dissociation of a tetramer to a dimer. This idea is strengthened by the observation that the partial purification of the receptor by filtration in a low salt medium of the cytosol prevents the aggregation of the receptor. This is not the case if the filtration is performed in a high salt medium. The low salt filtration allows the separation of the receptor from the major protein peak, contrary to the high salt filtration. However for obtaining such a result, the experimental conditions of the low salt filtration should be strictly controlled: if the resolution is insufficient (overloading of the column or pool of the edges of the receptor peak) the receptor reaggregates.

These observations are particularly important because they allow a simple method leading to unaggregated receptor in a low ionic strength medium. It is then possible to undertake the physicochemical characterization and the purification of the 'native' form of the estradiol receptor from calf uterus cytosol.

Acknowledgements

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