J. steroid Biochem. Vol. 23, No. 5A, pp. 547–551, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

CHARACTERIZATION OF THE 4-HYDROXYTAMOXIFEN (4-OHTAM) BOUND ESTROGEN RECEPTOR OF MCF-7 CELLS SOLUBILIZED BY MICROCOCCAL NUCLEASE

AVRAHAM GEIER, MICHAL HAIMSOHN, RACHEL BEERY and BRUNO LUNENFELD
Institute of Endocrinology, Chaim Sheba Medical Center, Ramat Gan 52621, Israel

(*Received* 12 *March* 1985)

Summary—In order to get an insight into the molecular mechanism of antiestrogen action at the chromatin level, we characterized the physical–chemical properties of the chromatin fragments released by micrococcal nuclease digestion of nuclei isolated from MCF-7 cells previously exposed to [^{3}H]4-OHTAM. The [^{3}H]4-OHTAM bound solubilized fragments were characterized in a low ionic strength buffer and in a high ionic strength buffer without and with urea. The following parameters were determined: sedimentation coefficients (S) on a sucrose gradient, Stokes radii (Rs) by gel filtration on a Sephadex G-200 column and the binding ability to a DNA-cellulose column. The molecular weights (Mr) and frictional ratios (f/fo) were calculated from the S and Rs values. Following mild nuclease digestion, the solubilized [^{3}H]4-OHTAM bound receptor sedimented as an abundant 6–7 S form and a less abundant ~12 S species. Increasing the extensiveness of digestion resulted in one receptor form sedimenting at 5.2 S, Rs = 7.25 nm and Mr = 155,000. About 45% of the applied receptor bound to a DNA-cellulose column could be eluted by a high salt concentrated buffer. Dissociation of the micrococcal nuclease solubilized receptor in 0.4 M KCl resulted in a smaller receptor form with a 4.9 S, Rs = 5.87 nm and Mr = 119,000. Further dissociation in the presence of 3 M urea resulted in a receptor with a 3.5 S, Rs = 5.78 nm and Mr = 83,000. These results suggested that the antiestrogen bound estrogen receptor in chromatin, is associated with a tightly bound protein component and with an additional less tightly bound protein, complexed with DNA.

INTRODUCTION

Antiestrogens are a class of compounds which prevent estrogens from expressing their full effect on estrogen target tissues and thus antagonize a variety of estrogen dependent progresses [1]. Antiestrogens are thought to act on the target cell preferentially through binding to the estrogen receptor [2, 3]. According to the recently published model for the mechanism of action of steroid hormones, the estrogen receptor resides in the target cell nucleus, presumably chromatin bound [4, 5]. Thus the antiestrogen would affect the target cell through its chromatin bound estrogen receptor. The retention time of the receptor in the nucleus was found to be much longer when it was bound to antiestrogen compared to when it was bound to an estrogen [6]. This may indicate a different mode of interaction of the antiestrogen bound receptor with some of the chromatin components compared to the estrogen bound receptor.

In the present study we characterized the antiestrogen bound chromatin fragments released by micrococcal nuclease digestion of isolated nuclei from MCF-7 cells previously exposed to [^{3}H]4-OHTAM (an antiestrogen with high affinity to the estrogen receptor) and discussed these results with the results previously published for the estradiol bound receptor [7, 8].

EXPERIMENTAL

MCF-7 cells were generously provided by Dr Charles M. McGrath (Michigan Cancer Foundation, Detroit, MI, U.S.A.) and grown as previously described [9]. Confluent cells in Falcon flasks were exposed for 1 h to 10 nM-Z-4-Hydroxy [*N*-methyl-^{3}H] tamoxifen (sp. rad. 70–90 Ci/mmol; Amersham, U.K.). The flasks were washed in cold phosphate buffered saline [10] and were stored 1–7 days at −80°C until assayed.

Nuclei were purified by the methods described previously [10, 11]. Isolated nuclei were resuspended (800 μg/ml DNA) in digestion buffer (10 mM-Tris–HCl, pH 7.9, 0.1 mM $CaCl_2$, 1.5% sucrose) and incubated with 5–30 U/ml of micrococcal nuclease (Sigma Chemical Co., St Louis, U.S.A.) at 0°C for 5–120 min. Digestion was terminated by addition of EDTA to a final concentration of 5 mM. The hydrolysate was centrifuged at 110,000 *g* for 30 min at 2°C. In some experiments digestion was continued with DNase I (DPFF, Worthington Biochemical Corp., New Jersey U.S.A.) as described previously [8].

Portions (250 μl) of the nuclease hydrolysate were fractionated by centrifugation in a linear 5–20% sucrose gradient prepared in TED buffer (10 mM-Tris–HCl, 1.5 mM EDTA and 0.5 mM Dithiothreitol, pH 7.4) or in TEDK buffer (TED buffer plus 0.4 M KCl) or in TEDK buffer containing 3 M

urea. The tubes were centrifuged in a Beckman SW 60 rotor at 2°C. In some experiments (see Fig. 1) the hydrolysate was fractionated by centrifugation in a sucrose gradient prepared in TED buffer in a Beckman SW 41 rotor as previously described [8]. Myoglobin (2 S) and γ-Globulin (6.6 S) were used as external standards, while [^{14}C] bovine serum albumin (4.4 S) and catalase (11.3 S) were included in the gradients as internal standards.

Gel chromatography was performed on a Sephadex G-200 column as previously described [8]. Briefly, nuclei were digested with 30 U/ml of micrococcal nuclease at 0°C for 60 min. Digestion was terminated by adjusting the hydrolysate to 5 mM EDTA. The obtained supernatant was applied to the column and eluted with TED buffer or TEDK buffer without or with 3 M urea. The protein standards used to calibrate the column were: Ferritin, catalase, bovine serum albumin and myoglobin. The distribution coefficients (K_d) were measured according to the equation $K_d = (Ve - Vo)/(Vt - Vo)$, where *Vo*, the void column was determined with blue Dextran 2000; *Vt* the total volume of the gel bed was measured with [^{3}H] leucine; and Ve is the elution volume of the receptors or the standard proteins. The binding ability of the micrococcal nuclease hydrolysate to a DNA-cellulose column was performed as described previously [8].

RESULTS

Sedimentation behaviour of the solubilized [^{3}H]4-OHTAM bound chromatin fragments

Figure 1 shows the sedimentation profile of the chromatin fragments solubilized by micrococcal nuclease digestion of nuclei isolated from MCF-7 cells 1 h after exposure to 20 nM [^{3}H]4-OHTAM. Mild digestion (Fig. 1a) released a [^{3}H]4-OHTAM bound receptor entity sedimenting predominantly as a 6–7 S form and a less abundant ~12S species which sedimented slightly more rapidly than the mononucleosome fraction (11 S). Some of the radioactivity sedimented with the heavier molecular weight chromatin fragments. The u.v. absorbance shows the familiar pattern of DNA fragments separation into mono, di- and trinucleosomes, while heavier molecular weight forms sedimented toward the bottom of the gradient. Increasing the extensiveness of the micrococcal nuclease digestion released more of the slower sedimenting receptor form. Furthermore a stepwise decrease in the sedimentation coefficient of the 6–7 S receptor occurred reaching a 5–6 S form (Fig. 1b). In order to characterize further the micrococcal nuclease released receptor form, we tried to dissociate it into smaller [^{3}H]4-OHTAM receptor forms using 0.4 M KCl and 3 M urea. Sedimentation of the hydrolysate in the presence of 0.4 M KCl resulted in a 4.9 S receptor form (Fig. 2a), while a 3.5 S receptor from was obtained by sedimenting of the hydrolysate in the presence of 0.4 M KCl and 3 M urea (Fig. 2b).

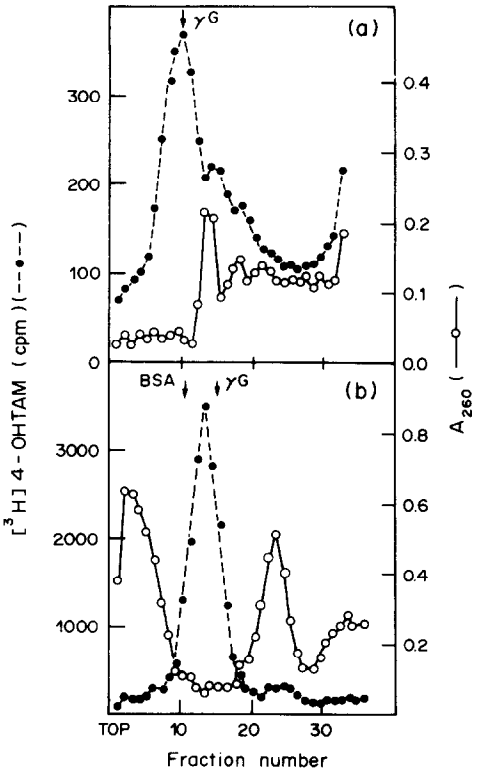

Fig. 1. Sedimentation of [^{3}H]4-OH-TAM bound receptor solubilized by micrococcal nuclease digestion. Nuclei were digested with micrococcal nuclease and the solubilized chromatin sedimented on a sucrose gradient in a SW 41 rotor for 18 h. (a). 5 U/ml enzyme incubated for 5 min and centrifuged at 77,000 *g*. (b). 30 units enzyme incubated for 60 min and centrifuged at 150,000 *g*. Arrows indicate the positions of [^{14}C]Bovine Serum Albumin (BSA) and γ-Globulin (γG).

Estimation of Stokes radii, molecular weights and axial ratios of the micrococcal nuclease solubilized receptor forms

Figure 3 illustrates the Sephadex G-200 elution pattern of the micrococcal nuclease solubilized receptor in low salt buffer or in high salt buffer without or with 3 M urea. The molecular Stokes radii of the different receptor forms were estimated by comparing their elution parameters on the Sephadex G-200 column with those of other standard proteins whose molecular Stokes radii are known. The elution profile of the receptors forms and the position of the protein standards are shown in Fig. 3 (top) and the column calibration line in Fig. 3 (bottom). In agreement with previous reports the elution pattern of the protein standards were identical under the different buffer conditions indicating no alteration in their molecular Stokes radii [8, 12, 13]. The receptor released by micrococcal nuclease digestion eluted from the column in a low salt concentrated buffer as one peak just after ferritin with an estimated Stokes radius of 7.25 nm. Elution in a 0.4 M KCl containing buffer resulted in a receptor form eluting just before catalase

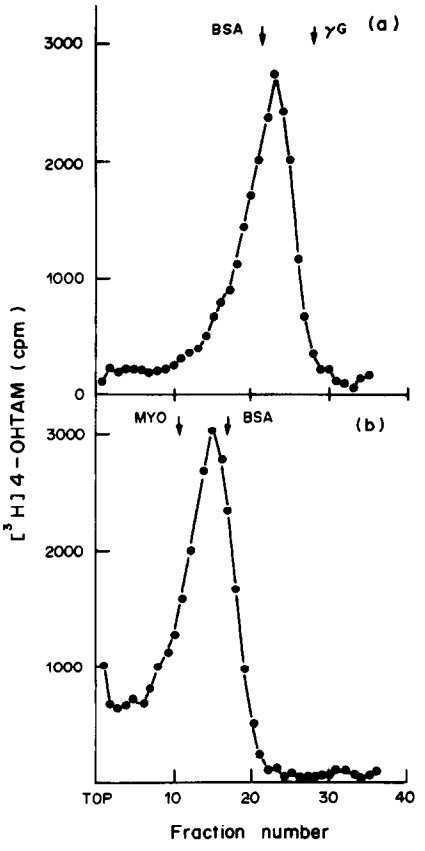

Fig. 2. Effect of 0.4 M KCl and 3 M urea on the sedimentation of the micrococcal nuclease solubilized receptor. Nuclei were digested with 30 U/ml micrococcal nuclease for 60 min. The hydrolysates were adjusted to 0.4 M KCl (a) or 0.4 M KCl containing 3 M urea (b) and sedimented on a sucrose gradient prepared with the appropriate buffers, in a SW 60 rotor at 210,000 *g* for 18 h. Arrows indicate the position of Myoglobin (MYO), [^{14}C]Bovine Serum Albumin (BSA) and γ-Globulin (γG).

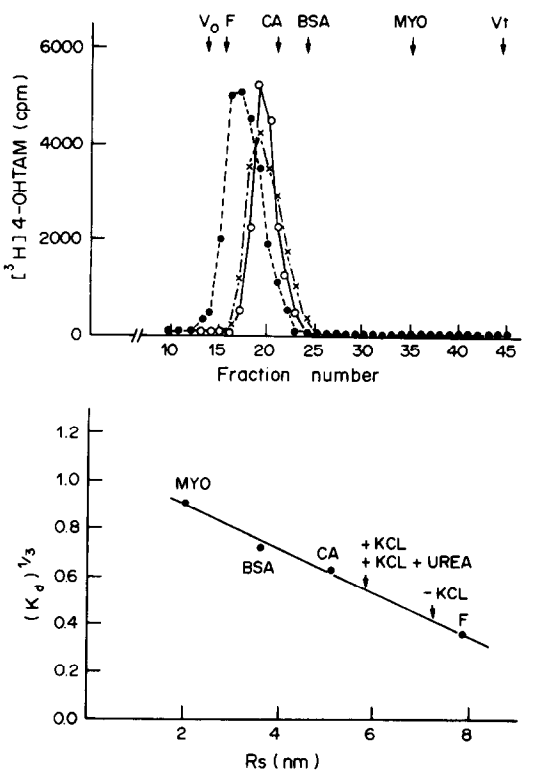

Fig. 3. Estimation of the Stokes radii of the micrococcal nuclease solubilized receptor by Sephadex G-200 column chromatography. Micrococcal nuclease hydrolysates were prepared as described under Experimental and eluted on a Sephadex G-200 column in the appropriate buffers. For each buffer condition the protein standards were: Ferritin (F), Rs = 7.9 nm; Catalase (CA), Rs = 5.13 nm; [^{14}C] Bovine Serum Albumin (BSA), Rs = 3.59 nm; Myoglobin (MYO), Rs = 2.01 nm. The elution patterns of each protein standard were identical under the different buffer conditions. *Vo* is the void volume and *Vt* is the total volume of the gel bed. Top, the elution profile of the solubilized receptor in TED buffer (--●--), in TEDK buffer (—○—) and in TEDK plus 3 M urea buffer (–·– × –·–). Bottom, the relationship between Rs and $(K_d)^{1/3}$ for the protein standards and the solubilized receptor forms.

with an estimated Stokes radius of 5.87 nm. A similar Stokes radius of 5.78 nm was estimated for the receptor in a 0.4 M KCl containing 3 M urea buffer. Utilizing the Stokes radii estimated from Sephadex G-200 column chromatography and the sedimentation coefficient determined from sucrose gradient analysis, the molecular weights and shapes were calculated by the equation of Siegel and Monty [14] and are summarized in Table 1. The micrococcal nuclease solubilized receptor in low salt buffer has a calculated mol. wt of 155,000 and a fractional ratio of 1.88. Following dissociation in a 0.4 M KCl containing buffer a mol. wt of 119,000 and a fractional ratio of 1.66 were calculated. Further dissociation in

Table 1. Molecular parameters of the [^{3}H]4-OHTAM bound estrogen-receptor determined from gel chromatography and sucrose gradient centrifugation*

Buffer	Sedimentation coefficient (S)	Stokes radius†	Molecular weight‡	Frictional ratio§ (f/fo)	Axial ratio¶
TED	5.2 ± 0.06 (12)	7.25 ± 0.28 (9)	155,000	1.88	17.1
TEDK	4.9 ± 0.04 (9)	5.87 ± 0.34 (5)	119,000	1.66	12.6
TEDK-3 M urea	3.5 ± 0.03 (6)	5.78 ± 0.32 (6)	83,000	1.85	16.5

*Experimental values given as mean ± standard error of the mean with number of determinations indicated in parentheses.

†Determined as described in Fig. 3.

‡§Calculated by the equation of Siegel and Monty [14] assuming a partial specific volume of 0.725 cm^3 per g and a solution factor of 0.2 g of solvent per g of protein.

¶Calculated for prolate ellipsoids by Schachman tables [15].

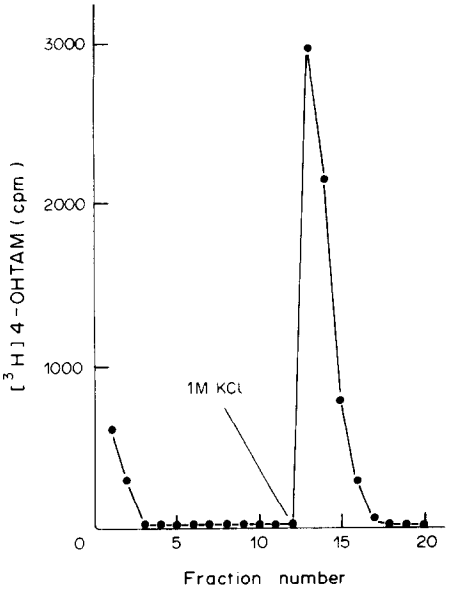

Fig. 4. Binding of the micrococcal nuclease solubilized receptor to a DNA-cellulose column. Nuclei were digested with 30 U/ml micrococcal nuclease for 60 min. The hydrolysate was chromatographed on a 1 ml DNA-cellulose column. The amount of the receptor bound to the column and eluted by 1 M KCl represents 43% of total receptors applied to the column.

the presence of 3 M urea resulted in a smaller receptor form with a 83,000 calculated mol. wt and 1.85 frictional ratio.

DNA binding of the solubilized receptor

The binding ability of the micrococcal nuclease solubilized receptor to DNA was studied by DNA-cellulose column chromatography. As shown in Fig. 4, 43% of the receptor present in the original sample was retained on the affinity resin and could be successfully eluted with high salt buffer.

DISCUSSION

Our approach in investigating the molecular mechanism of antiestrogen action at the chromatin level was to analyse the antiestrogen bound estrogen receptor chromatin fragments, released from chromatin by micrococcal nuclease digestion. We assumed that those fragments may contain the receptor in association with other molecules which comprise the acceptor i.e. receptor and molecules which bind it to DNA.

Mild micrococcal nuclease digestion excised the receptor from the bulk of chromatin as an abundant 6–7 S form and a less abundant ~12 S species. A similar sedimentation profile was found following mild micrococcal nuclease digestion of nuclei isolated from MCF-7 cells exposed to [^{3}H] estradiol [8]. Similar sedimentation forms were found for the micrococcal nuclease released estrogen receptor in rat uterus [17] and lamb endometrium [18, 19]. We proposed that the ~12 S species was the mononucleosome in association with a part of the linker DNA to which the receptor is associated and that the 6–7 S receptor was bound to the linker region between the nucleosomal particles [8]. Extensive digestion with DNase I did not affect its sedimentation behaviour, indicating that this form may not contain additional DNA residues (results not shown). We found similar results for micrococcal nuclease solubilized estradiol-bound receptor [8].

Using the Stokes radii value estimated by gel filtration and the sedimentation coefficient, we calculated a mol. wt of 155,000, which is slightly smaller than the molecular weight (Mr = 163,000) of the estradiol-bound receptor solubilized by micrococcal nuclease digestion [8]. Reexamination of the sedimentation coefficient of the estrogen-bound receptor under identical experimental conditions described in the present paper resulted in a more accurate sedimentation coefficient of 5.2 S and a calculated mol. wt of 151,000. On the other hand, in the lamb endometrium extensive digestion with micrococcal nuclease released the nuclear estrogen receptor as two molecular forms [18, 19]. Further characterization of the 155,000 mol. wt 4-OHTAM bound receptor form was accomplished by its dissociation into smaller receptor containing forms. Using 0.4 M KCl, a receptor form with a mol. wt of 119,000 was obtained. Under identical experimental conditions a smaller receptor form with a mol. wt of 77,000 was calculated for the estradiol-bound receptor [8]. This may indicate that the 4-OHTAM bound receptor form is still associated with an additional protein molecule(s). Using 3 M urea, which is known to dissociate protein molecules held together by weak interaction [12, 16], we dissociated this 119,000 mol. wt receptor form into a smaller 83,000 mol. wt receptor form. Similarly, a heavier 4-OHTAM bound estrogen receptor form with a mol. wt of 137,000 was extracted by high ionic strength buffer from the crude nuclear pellet of the MCF-7 cells by Eckert *et al.*[16]. This receptor form was further dissociated in the presence of 3 M urea into a 81,000 mol. wt receptor form. From these results we would suggest the following model for the functional organization of the estrogen receptor in chromatin: The receptor (Mr ~ 80,000) is tightly bound to a protein (Mr ~ 35,000) when it is ligated to an anti-estrogen and less tightly when it is ligated to an estrogen. An additional protein (Mr ~ 35,000) is bound to this (Mr ~ 120,000) complex. The whole complex (Mr ~ 155,000) is bound to DNA. Further investigation is required to determine whether this model could be applied to other estrogen target cells.

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