Radiation-inactivation size of transformed and non-transformed androgen receptors

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The nucleic acid sequence of the androgen receptor (AR) gene predicts that the protein structure possesses DNA- and steroid-binding domains that show high degrees of sequence similarity with those of other steroid receptors. Since the steroid-binding domain of the AR corresponds to a 30 kDa portion of the protein, and the AR structure may be monomeric or hetero-oligomeric depending on its transformation state, we have herein determined the AR radiation-inactivation size (RIS) in relation to the molecular structure whose binding activity toward methyltrienolone (R1881) is abolished by a radiation 'hit'. Soluble fractions from whole canine prostatic tissue were used as a source of non-transformed AR. The AR transformation was induced by the addition of 0.6 M-KCl, and these preparations were used together with high-salt nuclear extracts as a source of transformed AR. To maximize the binding activity, molybdate and dithiothreitol were included during AR extraction. Receptor transformation was verified by modifications of both the sedimentation coefficients (from 7.5 S to 4.1 S on sucrose gradients) and molecular masses (from 260 kDa to 115 kDa by gel filtration). The RIS values of the non-transformed and transformed ARs were not statistically different: 92 ± 19 kDa and 110 ± 25 kDa respectively. In addition, the inactivation of AR binding activity by radiation was attributed to a loss of binding sites, with no significant change in the K_d . When benzoic acid, a free-electron scavenger, was added together with dithiothreitol before and after irradiation, no change in the RIS value was observed. Thus, in the canine prostate, the RIS value of the AR represents the monomeric protein, independently of its association with other proteins, and this value corresponds to that predicted by cloning studies and photoaffinity-labelling of AR.

INTRODUCTION

Radiation inactivation is believed to occur through an all-or-none process, the molecules 'hit' by radiation being totally inactivated [1]. The method is versatile in determining the size of a molecule and, by relating it to the structure associated with activity, one defines the radiation-inactivation size (RIS) [2]. This method can be used directly on membranes or on crude extracts, and when applied to proteins it enables one to study some features of their structure without altering their subunit assembly and/or functional properties [1]. Proteins from variable sources have been inactivated and, for oligomers, the RIS value generally represents either the subunit or the whole oligomer [1]. In some instances the RIS corresponds to a fraction of the protein [3] which may represent a putative domain. However, this interpretation has rarely been challenged by irradiating proteins with known functional domains. In this respect, steroid receptors are of interest since their genes encode monomeric proteins presenting two well-defined and distinct domains for ligand and DNA binding [4]. This also holds for the androgen receptors (ARs) [5–8] selected for the present study.

On the other hand, hydrodynamic studies have indicated that steroid receptors such as the AR exist as either hetero-oligomers or monomers [9]. In the first instance, the AR found in high-speed supernatants from androgen target tissues is associated with heat-shock proteins and sediments as a 7–9 S entity; this represents the non-transformed AR. Secondly, the monomeric AR extracted from nuclear pellets in the presence of 0.6 M-KCl has a sedimentation coefficient of 4–5 S, and is referred to as the transformed receptor, which then has the capacity to bind DNA [9].

The aim of the present study was to submit the two forms of AR to radiation inactivation to establish their RIS after binding with [3 H]methyltrienolone and to monitor the effect of transformation on the RIS value of the AR. In order to ensure that transformation did occur, the prostatic fractions, soluble and nuclear extracts, were also submitted to gel filtration and centrifugation on sucrose gradients to determine the molecular masses and sedimentation coefficients of the ARs.

MATERIALS AND METHODS

Reagents

[3 H]Methyltrienolone ([17-methyl- 3 H]R1881) (3.18–3.22 TBq/mmol), unlabelled R1881, bovine serum [3 H]albumin (74–740 KBq/mg), Formula-989 scintillation fluid and vials were purchased from (NEN) DuPont Canada, Dorval, Québec, Canada. Bovine liver catalase, bovine intestinal mucosa alkaline phosphatase, bovine thyroglobulin, pig γ -globulin, horse myoglobin, molybdc acid, KCl, EDTA, dithiothreitol (DTT) and triamcinolone acetonide (TA) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Dextran T-70 and Sephacryl S-300 were purchased from Pharmacia Fine Chemicals, Montreal, Québec, Canada, and charcoal was from BDH Chemicals, Montreal, Québec, Canada. Radiolabelled R1881 was purified on a C_{18} minicolumn from Baker [10].

Extraction of prostatic tissue

Prostates (6–10 g) from normal mongrel dogs were excised surgically, dissected and minced on ice and processed as previously described [11]. Briefly, minces were suspended (1 g/2–

Abbreviations used: AR, androgen receptor; RIS, radiation-inactivation size; DTT, dithiothreitol; TA, triamcinolone acetonide; DCC, dextran-coated charcoal; D_{37} , radiation dose at which 37% of the initial activity remains; EGF, epidermal growth factor.

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3 vol.) in cold (4 °C) TEDG buffer (50 mM-Tris/HCl, 2 mM-EDTA, 1 mM-DTT, 10 % glycerol, pH 7.4) containing 10 mM-sodium molybdate. The preparations were homogenized by three pulses of 15 s at level 5 with a Polytron PCU-2-110 equipped with a PT-2 probe (Brinkmann Instruments, Rexdale, Ontario, Canada) and then centrifuged at 800 *g* for 30 min at 4 °C. The pellet was resuspended in TEDG/molybdate buffer containing 0.6 M-KCl and rehomogenized with five Polytron pulses of 5 s each. The 800 *g* supernatant and the KCl-treated pellets were then centrifuged at 105 000 *g* for 1 h at 4 °C. Freshly prepared high-speed supernatants were used for all further studies. In some instances, DTT and/or molybdate were omitted from buffer. To induce the AR transformation, the cytosolic fraction was treated with 0.6 M-KCl (30 min on ice) before further analysis. Protein concentrations (cytosol, 13–22 mg/ml; nuclear extracts, 6.5–11.0 mg/ml) were determined by the method of Bradford [12] using BSA as a standard.

Binding studies

Unless otherwise indicated, portions (0.2 ml) were incubated for 6 h at 4 °C in the presence of 1 nM-[³H]R1881 with or without a 100-fold excess of unlabelled ligand and in the presence of TA (0.5 μM) in all assays to prevent the binding of R1881 to progesterone and glucocorticoid receptors [13,14]. The preparations were then either used for gel filtration and sucrose gradients or treated with an equal volume of dextran-coated charcoal (DCC) (0.5 % Dextran and 0.5 % charcoal in TEDG/molybdate) for 20 min at 4 °C and centrifuged at 5000 *g* for 20 min to determine the specific binding after counting the radioactivity of the supernatants (LKB Rackbeta liquid scintillation spectrometer, model 1217) [11].

Centrifugation on sucrose gradients

Following incubation with the ligand as described above, prostatic fractions (0.2 ml) were layered on 5–15 % sucrose density gradients (4.5 ml) containing TEDG/molybdate buffer, in the absence and in the presence of 0.6 M-KCl, and centrifuged at 250 000 *g* for 20 h at 4 °C in a Beckman L8-M ultracentrifuge using a SW 60 Ti rotor. Gradients were fractionated (0.15 ml) and the radioactivity of each fraction was determined. As standards, catalase (11.3 S), alkaline phosphatase (6.3 S) and [¹⁴C]albumin (4.6 S) were run in parallel tubes, and [¹⁴C]albumin was also included with the AR preparations.

Catalase and alkaline phosphatase activities were measured spectrophotometrically by the hydrolysis of hydrogen peroxide and *p*-nitrophenyl phosphate respectively [15,16].

Gel-filtration chromatography

Prostatic fractions (0.2 ml) were incubated with [³H]R1881 as described above and were then submitted to gel filtration at 4 °C on Sephacryl S-300 columns (0.5 cm × 50 cm) equilibrated with either TEDG/molybdate (non-transformed AR) or TEDG/molybdate containing 0.6 M-KCl, pH 7.4 (transformed AR). Fractions of 0.5 ml were collected on a Gilson Micro-fractionator (model FC-80K), the absorbance at 280 nm was monitored on a Beckman DU-6 spectrophotometer and the radioactivity was determined. The molecular masses of ARs were calculated according to a standard curve determined from the relationship between K_{av} and the logarithm of the molecular mass of the following: thyroglobulin, 670 kDa; pig γ-globulin, 150 kDa; bovine serum [¹⁴C]albumin, 69 kDa; horse myoglobin, 17 kDa, which were eluted in parallel. The void volume was determined with Blue Dextran.

Radiation inactivation of the AR

Portions (0.5 ml) of soluble (treated or not with 0.6 M-KCl) and nuclear extracts in 1.5 ml Eppendorf microcentrifuge tubes were frozen in liquid N₂ and irradiated in crushed solid CO₂ (−78 °C) with gamma rays using a Gammacell model 220 (Atomic Energy of Canada, Ottawa, Ontario, Canada) delivering a dose of 1.3 Mrad/h at the time that the experiments were performed. The experimental set-up was as previously described by Noël *et al.* [17], but with a Gammacell of higher energy calibrated with enzymes of known radiation sensitivities both in the freeze-dried state [18] and frozen in solutions [19]. The tubes (in triplicate) to be exposed to various radiation doses, as well as controls (unirradiated tubes), were maintained in solid CO₂ throughout the experiment and were then kept at −78 °C until assayed for binding activity (in duplicate). Portions (0.1 ml) were incubated overnight at 4 °C with the ligand (1 nM-[³H]R1881 in the presence or absence of a 100-fold excess of R1881 and in the presence of 0.5 μM-TA) followed by the extraction of the free steroids with DCC and the determination of the radioactivity of the bound [³H]R1881 as described above. The data were analysed according to the classical target theory [20]. The D_{37} (in Mrad), corresponding to the radiation dose at which 37 % of the initial activity remains, was used to determine the RIS corresponding to the biologically inactivated unit, using the empirical equation:

$$\log \text{RIS} = 5.89 - \log D_{37} - 0.0028t$$

where t is the temperature; this equation is valid between $t = -200$ °C and $t = 200$ °C [2].

RESULTS

Effects of DTT and molybdate on AR stability during solubilization

The effects of DTT and molybdate on the yield of AR solubilization in soluble and nuclear preparations from whole prostatic tissue are summarized in Table 1. The absence of DTT during solubilization significantly decreased the binding activity ($P < 0.001$), whereas the presence of molybdate increased the binding activity by 3–4-fold in both the soluble and nuclear fractions. However, their addition in the assay did not result in any further binding (results not shown), indicating that DTT and molybdate affected the stability of the AR during solubilization rather than its binding activity *per se*.

Sedimentation coefficients of the transformed and non-transformed AR

When the soluble extract was incubated with [³H]R1881 and analysed on sucrose-density gradients at low ionic strength (Fig.

Table 1. Solubilization of AR in the presence of DTT and molybdate

Soluble and nuclear extracts were prepared with or without DTT and molybdate. Results are means ± S.D. and are expressed as percentages of specific binding activity of the controls (preparations extracted with TEDG buffer).

Conditions	Binding activity (%)	
	Cytosolic	Nuclear
Control	100 ± 7	100 ± 10
−DTT	60 ± 10	42 ± 8
+ Molybdate	472 ± 15	347 ± 11

Fig. 1. Centrifugation on sucrose gradients

Soluble (a) and soluble + KCl (b) extracts (0.2 ml) were centrifuged on 5–15% (w/v) sucrose gradients with [*methyl*- ^{14}C]albumin (4.6 S) as an internal standard. The preparations were incubated with 1 nM-R1881 with (○) or without (●) an excess of unlabelled ligand plus TA before centrifugation. Sedimentation coefficients were calculated from a linear relationship established with the three standards of known values centrifuged in parallel.

1a), one peak of radioactivity with a sedimentation coefficient of 7.5 S was detected. This binding was abolished when a 100-fold excess of unlabelled R1881 was included in the assay, indicating that binding was specific and therefore due to the non-transformed AR.

When a soluble preparation was incubated with 0.6 M-KCl to induce transformation during binding, and then submitted to centrifugation on sucrose-density gradients in the presence of 0.6 M-KCl, the specific binding of radiolabelled R1881 migrated as an entity with a sedimentation coefficient of 4.0 S (Fig. 1b).

The sedimentation coefficient of the nuclear preparation was similar (4.1 S) to that of the high-salt-treated soluble fraction (results not shown).

Gel-filtration chromatography of the transformed and non-transformed AR

Following their binding with $[^3\text{H}]\text{R1881}$, in the presence and in the absence of unlabelled ligand and TA, portions of soluble and nuclear prostatic fractions were chromatographed on Sephacryl S-300 columns in TEDG/molybdate without and with KCl. The results, illustrated in Fig. 2(a), showed that in the absence of KCl for the soluble extract, a single peak of specific binding, strongly diminished in the presence of an excess of unlabelled steroid, was eluted in a fraction corresponding to a 260 kDa protein. A second peak representing the free $[^3\text{H}]\text{R1881}$ was found at the total column volume.

When the nuclear extract, representing the high-salt-transformed AR, was analysed in TEDG/molybdate buffer containing KCl, the binding activity revealed, in addition to the presence of

Fig. 2. Gel filtration of the AR

Soluble (a) and nuclear (b) extracts (0.2 ml) were first incubated with $[^3\text{H}]\text{R1881}$ with (closed bar) or without (open bar) an excess of unlabelled ligand in the presence of $0.5 \mu\text{M}$ -TA before chromatography on Sephacryl S-300 columns. The absorbance at 280 nm (broken line) was monitored and the radioactivity of each fraction was determined. The apparent molecular masses of non-transformed and transformed AR were determined from the relation between K_{av} and $\log(\text{molecular mass})$ of protein standards analysed in parallel: 1, thyroglobulin, 670 kDa; 2, pig γ -globulin, 150 kDa; 3, BSA, 69 kDa; 4, myoglobin, 17 kDa.

the free ligand, another peak corresponding to a 115 kDa protein (Fig. 2b). This peak represented the AR, since the addition of an excess of unlabelled R1881 to the preparation before chromatography abolished it (Fig. 2b). Similar results were obtained with the soluble fraction treated in high-salt conditions (results not shown).

Radiation inactivation of the AR

Results of radiation inactivation of the soluble fractions extracted from canine prostatic tissue in TEDG/molybdate buffer are illustrated in Fig. 3(a): irradiation resulted in the inactivation of the AR. From the slope of the regression line ($r = -0.99$), a D_{37} of 13.99 Mrad, corresponding to an RIS of 92 ± 19 kDa, was calculated (Table 2). Similarly, D_{37} values of 12.92 and 11.68 Mrad, yielding RIS values of 99 ± 22 kDa and 110 ± 25 kDa respectively, were obtained for the transformed AR present in the high-salt-treated soluble fraction and the nuclear extracts respectively; the correlation coefficients were also -0.99 and the slopes calculated for the three types of preparations were not significantly different ($P > 0.5$).

To examine whether radiation inactivates the AR through a change in the number of binding sites or in the dissociation constant (K_d) [21], soluble preparations, irradiated with doses of 7.2 and 14.4 Mrad, were compared with an unirradiated control

Fig. 3. Radiation inactivation of the non-transformed AR

Radiation inactivation was performed on untreated soluble extracts. (a) Effect of dose (Mrad) on the binding activity. Data from different experiments were grouped in narrow dose ranges. Each value represents the average ($\pm$ S.D.) of five experiments, whereas each sample was irradiated in triplicate and assayed as duplicates. From the slope of the relation between the logarithm of residual binding activity and the radiation doses (Mrad), the D_{37} was calculated to determine the RIS as described in the Materials and methods section. (b) Effect of irradiation on the dissociation constant and the number of AR binding sites. Portions of soluble extracts exposed to 0 ($\square$), 7.2 ($\bullet$) or 14.4 ($\circ$) Mrad were analysed by saturation studies by incubation with increasing concentrations of [3 H]R1881 (1–10 nM), with and without a 100-fold excess of unlabelled ligand in the presence of 0.5 μ M-TA. The free ligand was eliminated by treatment with DCC and the radioactivity was determined. The data were analysed by Scatchard plots and the number of binding sites is expressed per mg of protein. (c) Effect of benzoic acid on the RIS value. Soluble extracts were treated with benzoic acid (10 mM) prior to (30 min on ice) and during irradiation ($\circ$) and compared with untreated samples ($\bullet$) for binding activity.

with respect to their specific binding activity with increasing concentrations of [3 H]R1881 up to 10 nM. The data, analysed by the method of Scatchard [22], are illustrated in Fig. 3(b). The

Table 2. RIS of the transformed and non-transformed AR

The different extracts were submitted to radiation exposure in triplicate as described in the Materials and methods section and assayed for binding activity in duplicate. The log of residual activity (%) as a function of the radiation exposure (Mrad) for all preparations was plotted to obtain the linear regression, which allowed the calculation of RIS value; means $\pm$ S.D. are given.

AR	Extracts	RIS (kDa)
Non-transformed	Cytosolic ($n = 5$)	92 ± 19
Transformed	Cytosolic (KCl, 0.6 M) ($n = 2$)	99 ± 22
Transformed	Nuclear ($n = 4$)	110 ± 25

slopes of Scatchard plots were not statistically different; the K_d values were 1.5 ± 0.4 , 1.2 ± 0.1 and 1.0 ± 0.4 nM, and the numbers of binding sites (zero-ordinate intercepts) diminished gradually from 216 ± 50 (0 Mrad) to 116 ± 40 (7.2 Mrad) and 93 ± 30 (14.4 Mrad) fmol/mg of protein.

It has been reported that the presence of reducing agents in samples submitted to radiation may, for certain proteins, affect the calculated RIS value [23]. To investigate this possibility, a free-electron scavenger (benzoic acid at 10 mM) was added to the soluble fraction already containing 1 mM-DTT prior to radiation inactivation. As can be seen in Fig. 3(c), the slopes in the presence of benzoic acid ($r = -0.96$) or in its absence ($r = -0.94$) were not different; D_{37} values of 16.10 and 14.89 Mrad, yielding RIS values of 80 ± 9 kDa and 86 ± 11 kDa respectively, were calculated; these values were not statistically different ($P > 0.5$).

DISCUSSION

The canine prostate, a known target for androgens *in vivo*, was used in this study because the dog develops benign prostatic hyperplasia and cancer with advancing age and is a model for similar diseases in man [24]. Upon homogenization and centrifugation, a fraction of AR, referred to as the non-transformed receptor, was recovered in the high-speed supernatant. The residual activity associated with the pellet, and referred to as the nuclear or transformed AR, was extracted in the presence of 0.6 M-KCl. In order to solubilize high levels of AR for RIS determinations, an optimization of the extraction procedure was undertaken (Table 1). For both extracts the binding activity was highly influenced by the presence of DTT and molybdate during AR extraction. These data are in agreement with other reports on steroid receptors and AR, and favour the idea that these agents prevent their inactivation and/or transformation [25–27].

In this study we wished to determine whether the presence of a hormone-binding domain or the structural changes occurring between the non-transformed and transformed AR had any effect on the RIS values calculated from the loss of steroid-binding activity after irradiation. To interpret the data from radiation inactivation, the characteristics of our AR preparations were assessed in parallel, as well as the effectiveness of the transformation process induced by increasing the ionic strength. Upon incubation at 0–4 °C with the hormone, the cytosolic AR formed a 'non-transformed' receptor complex with a 7.5 S sedimentation coefficient and an apparent molecular mass of 260 kDa; the transformed receptor, induced with KCl or present in nuclear extracts, was characterized by a 4.0 S sedimentation coefficient and an apparent size of 110 kDa. These data are in agreement with the concept that the non-transformed AR protein

is a complex composed of the receptor entity associated with other proteins devoid of hormone-binding capacity such as hsp 90 [28], and that transformation releases the AR from the complex, allowing DNA binding. Our sedimentation coefficient results (Figs. 1a and 1b) are also within the range of those reported for transformed and non-transformed ARs in the canine prostate [29,30] and clearly show that the transformation induced by high-salt treatment was effective. With respect to molecular masses, no values have been published for the canine prostate AR, but our results correspond to the molecular masses reported for the ARs of human and rat prostate extracts, with values ranging from 203 to 275 kDa for the non-transformed AR, and a value of 90 kDa for its transformed state [31–33]. Our preparations therefore represent the non-transformed and transformed AR of the canine prostate (Figs. 2a and 2b). Further information concerning the structure of the AR has become available from photoaffinity labelling with R1881 [34–36] and cloning of the gene [5–8]: the AR protein has a molecular mass ranging from 90 to 110 kDa, with an androgen-binding domain corresponding to a 30 kDa portion of the molecule [36].

Radiation inactivation followed by the measurement of AR binding shows that the RIS ranges from 90 to 110 kDa, even though the AR has multiple domains and may bind to other proteins. Clearly the RIS values obtained for the AR of the canine prostate represents the monomeric protein and not the steroid-binding domain (30 kDa). Thus the absorption of ionizing radiation energy anywhere in the AR protein would result in the cleavage of covalent bonds in the polypeptide chain [2] and would lead to its inactivation. On the other hand, the energy absorbed in associated polypeptides such as hsp 90 does not appear to be transferred to AR, since in the non-transformed state it still has a RIS value of 92–110 kDa (Table 2), which corresponds to that of the whole AR alone. The fact that the K_d does not change upon irradiation (Fig. 3b) also validates the 'all-or-none' hypothesis concerning radiation inactivation [1].

From the present data and RIS values obtained for other hetero-oligomers, it appears that no general rule can be used to interpret their structural significance with respect to a putative domain, a monomer or an oligomer. For instance, RIS values smaller than the α -subunit for various functions of the Na^+/K^+ -ATPase were obtained and attributed to putative domains [3,37]. Another example is the insulin receptor, a hetero-tetramer composed of two α -subunits for insulin binding and two β -subunits expressing a tyrosine protein kinase activity [38]. The RIS of the receptor function represents the monomer, i.e. the α -subunit (90 kDa) [38], whereas that of the tyrosine protein kinase is of the order of millions, as confirmed by chromatography [39], and corresponds to an aggregate. On the other hand, epidermal growth factor (EGF) receptor, a 170 kDa polypeptide, has an RIS value of 133 kDa as determined for two domains, the EGF-binding site and its tyrosine protein kinase activity. Interestingly, this RIS corresponds to the protein portion of the whole molecule [40]. The oncogene *v-erb B*, a 78 kDa glycoprotein which has a 90% sequence similarity with the tyrosine protein kinase domain of the EGF receptor [41], is also active *per se* [42,43]. Thus, as proposed by Beauregard *et al.* [19], the RIS value would represent the inactivated structure or the polypeptide chain along which the ionizing radiation energy migrated, rather than the structure required for activity [1]. In addition, the RIS would not necessarily represent domains when present such as in the AR and the EGF receptor [40].

In conclusion, the AR represented a good model with which to test certain hypotheses concerning radiation inactivation, due to the information available at the molecular level on its structure and monomeric and/or hetero-oligomeric states. For AR, RIS values do not correspond to the hormone-binding domain, nor

to the hetero-oligomer when present, and always reflect the whole AR polypeptide in all conditions tested.

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