Solubilization of a tamoxifen-binding protein

Assessment of its molecular mass

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Recent findings point to a role of Antioestrogen-Binding Site (ABS) in some of the growth-modulatory effects of antioestrogens. In the present study, a method for the solubilization of ABS from rat uterus microsomal fractions by using 3-(3-cholamidopropyl)dimethylammonio-1-propanesulphonate (CHAPS; 20 mM) and KCl (0.4 M) is described. Decreasing the CHAPS concentration below the critical micelle concentration led to long-term stabilization of the protein. All of the membrane-bound ABS was recovered in the extract, and only one class of binding site, with a high affinity for [³H]tamoxifen ($K_A = 5 \times 10^8 \text{ M}^{-1}$) was detectable. This binding was time-dependent and reversible: at 4 °C, the association rate constant was $k_a = 7.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, and the reverse rate constant was $k_d = 1.0 \times 10^{-4} \text{ s}^{-1}$. Solubilized ABS exhibited an affinity and specificity similar to those of the membrane-bound sites. Under disaggregating conditions, solubilized ABS had an apparent sedimentation coefficient, $s_{20,w}$, of 5.2 S and a Stokes radius of 6.4 nm. From these two values, molecular masses of 160 000 Da for the detergent–ABS complex, and 110 000 for the protein moiety, were estimated. Assessment of the size of the membrane-bound ABS by a radiation inactivation technique is also described. The ‘radiation inactivation size’, corresponding to the mass of 1 mol of protein structure(s) whose associated tamoxifen-binding activity is abolished after a single hit by ionizing radiation, was estimated to be 80 000 Da.

INTRODUCTION

The growth of certain human breast tumours is oestrogen-dependent [1]. Treatment with competitive oestrogen antagonists could offer an attractive strategy for the control of such tumours. Non-steroidal antioestrogens derived from triarylalkanes or alkenes have such properties, and a compound of this type, tamoxifen, is currently used in the treatment of breast cancer [2]. Many studies have shown that its antiproliferative action involves the oestrogen-receptor system [3–5]. However, it now appears that the pharmacology of this drug is extremely complex, and that some of its effects cannot be solely explained by interactions with the oestrogen receptor [6–10]. Among the numerous proteins known to interact with the antioestrogens, one has attracted widespread attention because of its particularly high affinity (in the nanomolar range) for these molecules. This protein has been termed ‘Antioestrogen Binding Site’ (ABS) [11], and recent results suggest a role of ABS in inhibiting cell growth, since: (i) a clonal variant of the MCF-7 breast-cancer cell line (RTx-6), still containing the oestrogen receptor but lacking ABS, is resistant to tamoxifen [12]; (ii) the growth of numerous breast-cancer cell lines which lack the oestrogen receptor but contain ABS is inhibited by tamoxifen [6]; (iii) newly synthesized compounds which bind selectively to ABS, but not to oestrogen receptor,

inhibit growth of MCF-7 cells, but are ineffective on RTx-6 cells [13,14].

The existence of ABS has been reported in cytosol, microsomal and nuclear fractions of tissues from various species (see review in [15]). In rat uterus, rat liver and MCF-7 cells, ABS seems to be predominantly localized in microsomal membranes [16–18]. The membrane localization and the absence of specific photoaffinity ligands have probably hampered the physicochemical characterization of ABS. Partial characterization of the detergent-solubilized microsomal ABS from rat liver has been reported by Watts & Sutherland [19]. Their technique of gel filtration in the presence of cholate indicated a heterogeneous molecular mass of the protein–detergent complex ranging from about 70 000 to 700 000 Da, with a peak at 490 000. In the present study on rat uterus microsomal fractions a similar approach was adopted. With a different detergent, 100 % of the ABS, with full binding activity, could be solubilized. Binding affinity and specificity were compared before and after solubilization. Because of the presence of the detergent, a new method for separation of protein-bound and free tamoxifen had to be developed. The molecular mass of the solubilized protein–detergent complex was estimated to be about 160 000 Da. Its sedimentation velocity and its behaviour on h.p.l.c. gel filtration indicated that it was homogeneous. In addition, the size of the protein still imbedded in the membrane was

Abbreviations used: ABS, antioestrogen-binding site; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulphonate; DMF, dimethylformamide; CMC, critical micelle concentration; K_i , equilibrium dissociation constant of competing ligand.

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evaluated by the radiation inactivation technique, and was found to be close to that of the solubilized protein.

MATERIALS AND METHODS

Chemicals

[³H]Tamoxifen (sp. radioactivity 86 Ci/mmol) was from New England Nuclear. Unlabelled steroids were from Sigma. Unlabelled tamoxifen, hydroxytamoxifen, nitromifene and nafoxidine were gifts from Imperial Chemical Industries, Parke Davis and Upjohn Laboratories. Tris, KCl, NaN₃, glycerol, propanediol and dimethylformamide (DMF) were from Merck. Thioglycerol was from Sigma, and CHAPS from Serva.

Preparation of membrane fractions

Adult castrated Sprague-Dawley rats received daily subcutaneous injections of oestradiol-17 β (25 μ g, in propanediol) for 3 days, to increase the uterine ABS concentration [20]. Uteri were homogenized (1:4, w/v) at 0–4 °C in 20 mM-Tris/HCl buffer, containing 12 mM-thioglycerol and 0.01% NaN₃, pH 7.4 at 20 °C (TT buffer), in a glass/glass homogenizer. The microsomal membrane fraction was prepared as described by Watts & Sutherland [17], except that it was carried out in TT buffer.

Solubilization procedure

The microsomal membrane pellet (3 mg of protein/ml) was suspended in TT buffer adjusted to 0.4 M-KCl (TTK buffer) containing 20 mM-CHAPS (20 mM-CHAPS/TTK buffer). The suspension was sonicated twice (Biorblock, type 20-200SV) at 4 °C, on setting 1.25 W for 5 s each with a 30 s interval for cooling, and immediately centrifuged at 105 000 *g* for 1 h. Incubation of membranes in the presence of detergent for 15 min, 30 min, 1 h or 2 h before centrifugation did not affect the efficiency of solubilization. For binding and kinetic studies, the supernatant (containing solubilized ABS) was diluted in TTK buffer to decrease the CHAPS concentration (2.5 mM) below the critical micelle concentration (CMC). In this buffer system, the CMC of CHAPS as a function of ionic strength was determined spectroscopically [21]. 8-Anilino-naphthalene-1-sulphonate (ANS) was used as a CMC indicator. When ANS changes from an aqueous (hydrophilic) to a micellar (hydrophobic) microenvironment, the probe shows a dramatic enhancement of the quantum yield of fluorescence, and a blue-shift in the fluorescence peak [21]. Four different TTK buffers (KCl 0.0, 0.15, 0.4, 1.0 M) were prepared and adjusted to CHAPS concentrations ranging from 0 to 30 mM. Each sample, containing 3 μ M-ANS, was continuously irradiated at 360 nm. The quantum yield of the fluorescence was determined from 450 nm to 500 nm. The peaks of the emission curves were measured by comparing CHAPS-containing buffers with the reference samples (TTK buffers without CHAPS).

Binding assay

The binding assay was carried out in the presence of 1 μ M-oestradiol in order to eliminate interaction of [³H]tamoxifen with the oestrogen receptor. Membranes (suspended in TT buffer adjusted to 7% DMF) and diluted CHAPS extracts were incubated for 18 h at 4 °C

with increasing concentrations of [³H]tamoxifen (0.05–20 nM) in the absence or the presence (unspecific binding) of 1 μ M unlabelled tamoxifen. For the microsomal membranes, specific binding of [³H]tamoxifen was determined by adsorption of unbound ligand with dextran-coated charcoal [16,19]. For the solubilized preparations, samples (50 or 75 μ l) were chromatographed on Sephadex LH-20 columns (bed volume 2.5 ml) at 4 °C. Columns were pre-equilibrated, and eluted by gravity with 1.1 ml of TTK buffer. The void volume contained the bound [³H]tamoxifen. The separation of bound ligand from free ligand is particularly efficient with this type of Sephadex, suggesting that it displays some affinity for tamoxifen. The elution pattern of solubilized ABS was the same whether or not the columns were equilibrated with buffer containing 2.5 mM-CHAPS. This suggested that enough molecules of CHAPS remained associated to ABS during the short time of the experiment, preserving ABS in a soluble state, and enabling elution in the void volume of the column. On the other hand, CHAPS concentrations above the CMC in the incubation medium decreased the effectiveness of the gel-filtration procedure (increase in non-specific binding and variability between triplicates). In some experiments, a single saturation dose of [³H]-tamoxifen (20 nM) was used to assess specific binding capacity.

Kinetic studies

A diluted CHAPS extract was used for time-course studies of [³H]tamoxifen (2 nM) in association with, and in dissociation from, solubilized ABS at 4 °C. Stability of binding throughout the experiments was checked. For the association analysis, [³H]tamoxifen (2 nM) and oestradiol (2 μ M) were incubated with the soluble fraction in the absence or the presence of unlabelled tamoxifen (2 μ M). At different times, total and non-specific binding were determined as described above. The dissociation rate was studied by the isotopic-dilution technique. The diluted extract was equilibrated for 18 h with [³H]tamoxifen (2 nM) and oestradiol (2 μ M). After incubation, a 1000-fold excess of unlabelled tamoxifen was added, and specifically bound labelled tamoxifen was determined at different times. The association and dissociation rate constants were calculated as described by Weiland & Molinoff [22].

Competition studies

To determine the ability of various ligands to compete for tamoxifen binding to ABS, a constant concentration of [³H]tamoxifen (1 nM or 5 nM, for the membrane or the solubilized preparation respectively) was incubated in the presence of increasing concentrations of the competing ligand over the range 0.5–1000 nM. The concentration which inhibited tamoxifen specific binding by 50% at equilibrium (IC₅₀) was determined for each compound, and used to determine values of K_i [22]:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}} \quad (1)$$

where [L] represents the concentration of free labelled tamoxifen, and K_D is the equilibrium dissociation constant of tamoxifen, determined by Scatchard analysis.

H.p.l.c.

Gel-filtration h.p.l.c. on TSK 3000 SW (7.5 mm × 300 mm; Beckman) was performed as previously described, with a Waters model 6000 A chromatography system equipped with a model 440 detector and a 280 nm filter [23,24]. The column was equilibrated and eluted with 20 mM-CHAPS/TTK buffer adjusted to 20 % glycerol. The non-diluted extracts were preincubated in the presence of 5 nM-[³H]tamoxifen, 1 μM-17β-oestradiol ± 1 μM-tamoxifen, then applied to the column. Total and unspecific binding were assayed on each collected fraction by Sephadex LH-20 chromatography. In other experiments, h.p.l.c. after labelling was carried out on each collected fraction. The calibration curve was obtained by using the same buffer, with 14 water-soluble standard proteins of known Stokes radius [23,24]. The void volume and total volume were measured with Blue Dextran and NaNO₂ respectively. The results are presented in accordance with the procedure of le Maire *et al.* [24]. The Stokes radius of ABS was obtained from the calibration curve.

Molecular-mass determination

The Stokes radius (R_s) of ABS was used with the sedimentation coefficient ($s_{20,w}$) to calculate the molecular mass of the protein-detergent complex (M), by using the following formula [25]:

$$M = \frac{6\pi\eta\rho NR_s s_{20,w}}{1 - \bar{v}_i\rho} \quad (2)$$

where N is Avogadro's number, η is the viscosity and ρ is the density of the solution, and $\bar{v}_i$ is the partial specific volume of the detergent-protein complexes.

Thus:

$$\bar{v}_i = \frac{\bar{v}_p + \delta_D \bar{v}_D}{1 + \delta_D} \quad (3)$$

where $\bar{v}_p$ and $\bar{v}_D$ are respectively the partial specific volume of the protein and the detergent, and δ_D is the detergent binding ratio (g/g of protein). The value $\bar{v}_D$ of CHAPS is 0.81 cm³/g [26]; $\bar{v}_p$ and δ_D are taken as 0.74 and 0.5 cm³/g respectively [23,27]. The molecular mass of the protein part of the complex (M_p) is related to M by the following formula [25]:

$$M = M_p (1 + \delta_D) \quad (4)$$

In this section, we assumed that, at the high detergent concentrations employed, almost all the lipids were removed from the protein ABS. In most cases, the contribution of the bound lipids to the determination of M_p is negligible, since the partial specific volume is close to 1 [23].

Radiation inactivation procedure

Irradiation was carried out in a Gammacell model 220 apparatus (Atomic of Canada, Ottawa) equipped with a 17000 Ci ⁶⁰Co source at the time the experiments were performed. The source was calibrated with standard enzymes as described elsewhere [28].

Microsomal membranes were prepared as described above, except that thioglycerol was omitted from the buffer (T buffer). They were suspended (2 mg of protein/ml of T buffer) and dispersed in 1.5 ml Eppendorf microcentrifuge tubes. Samples were immediately plunged into liquid nitrogen, and stored at -80 °C before and after

irradiation. Tubes were put in a 15 cm crystallizing dish and exposed to isodoses [29] at -78 °C in crushed solid CO₂. At least three tubes were exposed for each radiation dose. Appropriate controls of non-irradiated preparations were run concurrently.

Irradiated and control samples were quickly thawed, diluted (1:4) with T buffer, adjusted to 7 % DMF and 12 mM-thioglycerol, and then assayed for [³H]tamoxifen-binding activity. The natural logarithm of the ratio of specifically bound labelled tamoxifen at doses D and O Mrad ($\ln A_D/A_O$) was plotted against radiation dose (Mrad). According to the terminology used by Beauregard *et al.* [30], the parameter determined from the slope of the inactivation curve is termed 'Radiation Inactivation Size' (RIS) and defined as the mass of 1 mol of protein structure(s) whose associated biological activity (tamoxifen-binding activity in the present case) is abolished after a single hit by an ionizing radiation. RIS may be obtained from the equation [30]:

$$\text{RIS} = \mu_{(t)} \times 10^{-8} Q_{(t)} \quad (5)$$

where $\mu_{(t)}$ represents the slope of the semi-logarithmic plot of remaining biological activity versus dose at temperature t (°C), and $Q_{(t)}$ is the amount of radiation energy required to inactivate 1 mol of protein at temperature t . At -78 °C, it can be calculated that $Q_{(-78^\circ\text{C})} = 12.836$ MJ/mol [30]. It follows that:

$$\text{RIS} = \mu_{(-78^\circ\text{C})} \times 1.2836 \times 10^6 \quad (6)$$

Protein determination

This was done by the method of Lowry *et al.* [31]. Standard curves for bovine serum albumin were always run in the same medium in which the protein concentration of the samples was measured.

RESULTS

Optimization of the solubilization procedure

Treatment of microsomal membranes with KCl (0.4 M) and CHAPs released ABS in a soluble form. The amount liberated depended on the detergent concentration (Fig. 1). Maximal binding activity was recovered at a CHAPS concentration of 15 mM, above which progressive inactivation occurred, probably masking the real efficiency of solubilization. To prevent gradual inactivation after solubilization, the solubilized preparation was diluted. ABS could be kept in a soluble and active state in the range of CHAPS concentrations between 2.5 and 5 mM (see below). The experiment was therefore repeated with each extract adjusted to a final CHAPS concentration of 2.5 mM immediately after the 105000 g-centrifugation step (Fig. 1). Under these conditions, all of the original microsomal binding activity was solubilized at a CHAPS concentration of 20 mM. Further increases in detergent concentration did not improve the efficiency of solubilization. The efficiency of extraction was also found to be dependent on the ionic strength of the detergent solution (Table 1). At constant protein and detergent concentrations (3 mg of protein/ml in 20 mM-CHAPS/TT buffer), maximal recovery of ABS was obtained at 0.4 M-KCl. Higher ionic strengths (0.6 or 0.8 M) did not improve the efficiency (results not shown). The influence of ionic strength on the solubilization process can be partly attributed to the effect of the detergent on the CMC. We measured the fall

Fig. 1. Solubilization of [^3H]tamoxifen-binding activity plotted as a function of CHAPS concentration

Microsomal membranes (3 mg of protein/ml of solubilizing buffer) were solubilized with increasing concentrations of CHAPS. After centrifugation at 105 000 g for 1 h, either undiluted extracts ($\blacklozenge$, $\blacklozenge$) or diluted extracts (CHAPS concn. lowered by dilution to 2.5 mM; $\blacksquare$, $\blacksquare$) were assayed for [^3H]tamoxifen binding: $\blacklozenge$, $\blacklozenge$, total binding of [^3H]tamoxifen (20 nM); $\blacksquare$, $\blacksquare$, non-specific binding determined in the presence of 2 μM unlabelled ligand. The dilution was taken into account for the expression of [^3H]tamoxifen binding activity per 50 μl of extract. The data are means of three measurements from one experiment which was reproduced twice.

in CMC with increasing salt concentration. At 25 $^{\circ}\text{C}$, the CMC of CHAPS fell from 5.2 ± 0.1 to 3.8 ± 0.1 mM (mean $\pm$ S.D.) when the KCl concentration was raised from 0 to 0.15 M, and then to 3.2 ± 0.1 mM at 1 M-KCl. Under our experimental conditions (0.4 M-KCl), the CMC was observed at 3.5 ± 0.1 mM.

The ABS-binding activity of the 20 mM-CHAPS extract decreased, with a half-life of 24 h, and could not be restored by subsequent dilution. However, it was

Fig. 2. Scatchard analysis of [^3H]tamoxifen binding to membrane-bound ABS ($\circ$) and solubilized ABS ($\bullet$) of rat uterus microsomal fractions

The membranes and corresponding diluted extract were incubated at 4 $^{\circ}\text{C}$ with increasing concentrations of [^3H]tamoxifen (0.07–6 nM). In this particular experiment, K_D values were 1.3 and 1.9 nM respectively for membrane-bound ABS and solubilized ABS. Correlation coefficient, $r = 0.95$ ($\bullet$) and 0.93 ($\circ$).

much more stable in the diluted extract (2.5 mM-CHAPS) either in the presence ($> 90\%$ after 7 days) or, to a lesser extent, in the absence of ligands (60% after 7 days). Inactivation of solubilized ABS was also prevented by addition of 20% glycerol to the 20 mM-CHAPS extract immediately after the 105 000 g -centrifugation step. In these conditions, the binding activity of the extracts was preserved for at least 1 week at 4 $^{\circ}\text{C}$. Finally, 90% of the solubilized activity was destroyed by treatment with 300 μg of trypsin/ml for 15 min at 30 $^{\circ}\text{C}$ (results not shown).

Retention of binding characteristics of ABS after solubilization

Both binding affinity and specificity of ligands to membrane and solubilized ABS were compared. Typical results are shown in Figs. 2, 3 and 4 and Table 2. The same membranes were used for determination of the equilibrium dissociation constant of membrane-bound

Table 1. Effect of KCl concentration on the solubilization of protein and [^3H]tamoxifen-binding activity

Portions of microsomal membranes were solubilized with 20 mM-CHAPS in TT buffer adjusted to various KCl concentrations. After centrifugation, the extracts were diluted (1:8) to decrease the detergent concentration to 2.5 mM, while the original concentration of KCl was maintained. Binding activity (B_{max}) was calculated from Scatchard analysis as described in the Materials and methods section. Extracted ABS and protein are expressed as percentages of the original values in the microsomal fraction.

	Membranes	KCl extract		
		0.0 M	0.2 M	0.4 M
Protein (mg/ml)	3	1.1	1.7	2.1
(% extracted)	100	36.7	56.7	70
ABS (pmol/mg of protein) ($n = 3$)	1.00 ± 0.08	0.54 ± 0.04	1.35 ± 0.05	1.38 ± 0.05
(% extracted)	100	18.7 ± 1.4	76.2 ± 2.8	98.7 ± 4.0

Fig. 3. Time course of [^3H]tamoxifen binding to solubilized ABS

A diluted extract was incubated at 4 °C with 2 nM-[^3H]-tamoxifen and 2 μM -oestradiol in the absence (■, total binding) or in the presence of 2 μM -tamoxifen (□, non-specific binding). Data shown are averages of triplicate determinations. Inset: association is plotted according to the pseudo-first-order equation (data ranging from 0 to 30 min). The use of this simplified equation is suitable [22], since under our conditions less than 10% of the total radioligand was bound.

$$A = k_a \times t = \frac{[\text{LR}]_e}{[\text{L}]_T[\text{R}]_T} \times \ln \frac{[\text{LR}]_e}{[\text{LR}]_e - [\text{LR}]}$$

where $[\text{LR}]_e$ = concentration of ligand-ABS complex at equilibrium, $(\text{L})_T$ = total concentration of [^3H]-tamoxifen, $(\text{R})_T$ = total concentration of binding sites and (LR) = concentration of ligand-ABS complexes at time t . The slope of the line gives an estimate of the association rate constant, k_a (correlation coefficient, $r = 0.99$).

and solubilized ABS after incubation for 18 h with ligands at 4 °C (Fig. 2). Care was taken to keep the protein concentration in the same range in both preparations. Whereas tamoxifen was completely solubilized by 2.5 mM-CHAPS, it was only partially (60–70%) solubilized in the membrane fraction, despite the addition of DMF to the buffer [16]. For the calculation of K_D , the real [^3H]tamoxifen concentration available had therefore to be determined in each sample. The mean value of K_D in the soluble preparations was 2.1 ± 0.9 nM, quite comparable with the K_D value of 2.1 ± 0.8 nM determined in the membrane preparations (means $\pm$ S.D., $n = 7$).

With [^3H]tamoxifen at 2 nM, the calculated association rate constant, k_a (measured between 0 and 30 min), was $7.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 3a). Fig. 4(b) shows the time course of [^3H]tamoxifen dissociation from solubilized ABS, with a $t_{1/2}$ of 90 min and a dissociation rate constant k_d (measured between 0 and 5 h) of $1.0 \times 10^{-4} \text{ s}^{-1}$. The equilibration dissociation constant $K_D = 1.4$ nM, estimated from the kinetic studies (k_d/k_a), is in agreement with the K_D value obtained from equilibrium studies. The relative potencies of four non-steroidal antioestrogens to inhibit binding of [^3H]tamoxifen were similar in both membrane and soluble fractions (Table 2), although the K_i values decreased apparently by a factor 2 in the soluble fraction, presumably because ligands were better solubilized in this system. Steroids (oestradiol, cortisol, progesterone, dihydrotestosterone) did not compete for bound [^3H]tamoxifen in either preparation.

Fig. 4. Determination of dissociation rate constant for [^3H]-tamoxifen-ABS complexes at 4 °C

A diluted extract was equilibrated with 2 nM-[^3H]-tamoxifen and 2 μM -oestradiol for 16 h. At zero time, 2 μM -tamoxifen was added to the incubation mixture. At the indicated times, samples were removed to determine bound [^3H]tamoxifen. Inset: specific binding refers to the amount of [^3H]tamoxifen specifically bound at equilibrium before the addition of unlabelled tamoxifen (% of control). The value of specific binding (% of control) is plotted on a log scale as a function of time (correlation coefficient, $r > 0.99$).

Table 2. Competition of three non-steroidal antioestrogens for [^3H]tamoxifen binding

The K_i value for each compound was determined in membrane and solubilized preparations as described in the Materials and methods section.

Drug	K_i (nM)	
	Membrane-bound	Solubilized
Tamoxifen	6	3
Nitromifene	12	4
Nafoxidine	18	8
4-Hydroxytamoxifen	24	12

Sedimentation-velocity analysis

Undiluted CHAPS extracts, containing 2.5 mg of protein/ml, were first analysed on 5–20% (w/v) sucrose gradients prepared in TTK buffer adjusted to either 2.5 mM- or 20 mM-CHAPS. Binding activity was measured in each collected fraction. Runs in 2.5 mM-CHAPS showed a profile suggesting protein aggregation with a predominant peak at 9 S. No binding activity was detectable in gradients adjusted to 20 mM-CHAPS. Runs in 20 mM-CHAPS were repeated with an extract with a higher protein concentration (5 mg of protein/ml): the measurable activity (15% of the total activity loaded) covered a range of sedimentation coefficient between 5 and 7 S, with a predominant peak at 5 S, suggesting again protein aggregation. When undiluted CHAPS extracts containing 2.5 mg of protein/ml were analysed on 10–35% (v/v) glycerol gradients at a CHAPS concentration of 20 mM, no binding activity could be detected in the collected fractions (results not shown). Activity

Fig. 5. Glycerol-gradient analysis of prelabelled solubilized ABS

For this 20 mM-CHAPS extracts (2.5 mg of protein/ml) were adjusted to 5% glycerol and preincubated (18 h) with 5 nM-[³H]tamoxifen + 1 μ M-oestradiol, in the presence and the absence of 1 μ M-tamoxifen. Samples corresponding to total and non-specific binding were directly layered on two separate gradients containing 20 mM-CHAPS and adjusted to the same ligand concentrations of the sample layered. Centrifugation was carried out for 14 h at 260 000 *g*. Samples of each fraction were chromatographed on Sephadex LH-20 to determine total (●) and non-specific (○) binding of [³H]tamoxifen (recovery = 30–40% of specific binding layered on the gradients). The data are means of three measurements from one experiment which was performed twice. The arrows indicate γ -globulin (γ G; 7.1 S) and ovalbumin (OV; 3.55 S).

could only be recovered by analysis of prelabelled extracts (incubated for 18 h in the presence of 5 nM-[³H]tamoxifen $\pm$ 1 μ M-oestradiol $\pm$ 1 μ M-tamoxifen) in gradients containing the same concentrations of ligands. Under these conditions, the specific binding activity of each collected fraction measured by Sephadex LH-20 chromatography presented a well-defined peak with an apparent sedimentation coefficient, $s_{20,w}$, of 5.2 S (Fig. 5).

Gel-filtration analysis

The high-performance gel-chromatographic behaviour of 14 water-soluble standard proteins, in the presence of 20 mM-CHAPS/TTK buffer containing 20% glycerol, is illustrated in Fig. 6(a). As proposed by le Maire *et al.* [24], the Stokes radius is plotted against the partition coefficient. The experimental points were fitted to a polynomial of degree 5. The calibration curve obtained was similar to that of le Maire *et al.* [24], indicating the validity of the method at high concentrations of CHAPS and glycerol.

To investigate the homogeneity of the solubilized ABS, and to determine a Stokes radius, glycerol was added to freshly prepared CHAPS extracts (undiluted) to a final glycerol concentration of 20%. The samples were then filtered through the h.p.l.c. column. As shown in Fig. 6(b), the binding activity of ABS, determined on

Fig. 6. H.p.l.c. size-exclusion chromatography

(a) Calibration curve for water-soluble proteins on a TSK 3000 SW column. The elution and equilibration medium was 20 mM-CHAPS/TTK buffer adjusted to 20% glycerol. The standard proteins were: 1, myoglobin (R_s 1.9 nm); 2, lysozyme (2.0 nm); 3, trypsin inhibitor (2.2 nm); 4, haemoglobin dimer (2.4 nm); 5, β -lactoglobulin (2.75 nm); 6, ovalbumin (2.8 nm); 7, bovine serum albumin (3.5 nm); 8, transferrin (3.6 nm); 9, aldolase (4.6 nm); 10, catalase (5.2 nm); 11, aspartate transcarbamoylase (6.0 nm); 12, ferritin (6.3 nm); 13, β -galactosidase (6.9 nm); 14, thyroglobulin (8.6 nm). The partition coefficient, defined by the equation $V_e - V_0 / (V_t - V_0)$, where V_e = elution volume of the protein and V_t and V_0 are total and exclusion volumes of the column, is plotted against the R_s of the standard protein (mean of three independent determinations). The continuous line is the fit obtained with a polynomial of degree 5 (correlation coefficient = 0.99). (b) Freshly prepared undiluted extracts were adjusted to 20% glycerol and chromatographed (200 μ l sample) on a TSK 3000 SW column. Portions of each fraction were assayed for total (■) and non-specific (□) binding of [³H]-tamoxifen. In a separate experiment, the h.p.l.c. system was calibrated by use of Blue Dextran (B), NaNO₂, and four pure proteins: aspartate transcarbamoylase (ACT), albumin (Alb), haemoglobin (Hb) and myoglobin (Myo).

each fraction, presents a narrow symmetrical peak with 20% recovery. The width of this peak is comparable with that of the standard proteins. When the extracts were labelled before gel filtration, similar profiles were obtained, with an approx. 80% recovery of ABS activity (results not shown). Identical patterns were obtained 0

Fig. 7. Radiation inactivation analysis of microsomal ABS

Microsomal membranes prepared from 35 rat uteri were frozen and irradiated as described in the Materials and methods section. The natural logarithm of fractional surviving activity [$\ln(A_D/A_0)$] against radiation dose (Mrad) was assessed by measuring [^3H]tamoxifen specific binding over the concentration range 1.6–27 nM ($\blacklozenge$, 1.5 nM; $\diamond$, 2.5 nM; $\square$, 16.0 nM; $\blacksquare$, 27.0 nM). The regression line ($r = 0.84$, $n = 34$) shown has the equation $\ln(A_D/A_0) = -\mu_{(-78^\circ\text{C})} \times \text{Dose}$. The slope of the line was used to calculate RIS as described in the Materials and methods section.

and 30 h after extraction, indicating an absence of aggregation. By using the equation of the calibration curve, a R_s value of 7.5 nm was calculated. It has been shown that the membrane proteins solubilized in detergent have an apparent R_s which is overestimated by 10–20 % if the calibration curve is obtained with water-soluble proteins [23,32]. The true R_s was thus taken as 6.4 nm, giving with the 5.2 S sedimentation coefficient a protein–detergent molecular mass of 160 000 from eqn. (2) (see the Materials and methods section).

Radiation inactivation analysis

ABS activity in microsomal membranes disappeared after freeze-drying (results not shown), and samples were thus kept in the frozen state (-78°C). At this temperature, no changes in binding affinity and capacity were observed for at least 2 months. The decay in ABS-binding activity was assessed at concentrations of labelled tamoxifen both below and above the K_D of the ligand–ABS complexes. We also checked that the affinity was not affected by irradiation (results not shown). Fig. 7 shows the irradiation data. ABS appeared to be inactivated as a single exponential function of the radiation dose. The mean RIS of ABS was 80 000 Da.

DISCUSSION

Although the biological role of ABS is the subject of intense research [33–38], there are few data on the molecular characteristics of this binding site. In the present study, microsomal ABS from rat uterus were characterized both in membranes and in the solubilized state.

Watts & Sutherland [19] have reported that microsomal ABS from rat liver could be solubilized by using 1 % cholate in conditions of high ionic strength (1 M-NaCl), but not with CHAPS. It was not clear, however, whether the original conformation of the cholate-solubilized ABS was preserved, since binding characteristics were not reported. Moreover, their results on gel filtration indicated that the detergent–ABS

complexes were not in a homogeneously dispersed state [19]. In the present study, we demonstrated that ABS from microsomal membranes of rat uterus could be solubilized in an active state, and that aggregation could be prevented. Among the detergents tested (Triton X-100, Nonidet P-40, CHAPS, sodium deoxycholate), CHAPS was found to be the most effective in solubilizing and maintaining ABS in a functional state. Efficient solubilization of microsomal membrane proteins (3 mg/ml) could only be achieved in a medium containing high concentrations of CHAPS (20 mM) and KCl (0.4 M). The additive effect of ionic strength has also been reported for rat liver microsomal ABS [19], and is well recognized for the bile-salt detergents [39]. We found that the CMC of CHAPS depended somewhat on ionic strength; CMC decreased from 5.2 to 3.2 mM as KCl concentration was raised from 0 to 1 M. It is possible that high salt concentrations disrupt membranes, via an alteration of the micellar properties of CHAPS.

Unfortunately the high concentration of CHAPS required for the solubilization (20 mM) was not compatible with the binding assay of [^3H]tamoxifen, when Sephadex LH-20 chromatography is used for the separation of bound and free ligands. This assay could only be performed after dilution of the extracts to a final CHAPS concentration of 2.5–5 mM. Under these conditions, the assay was reproducible, the variability between triplicates was less than 5 %, and non-specific binding was under 30 % of the total binding. Even at this low detergent concentration, the classical dextran-coated-charcoal technique could not be used (results not shown). Moreover, it was observed that dilution immediately after extraction strongly stabilized the binding activity of the solubilized ABS. Denaturation was also prevented by addition of glycerol to the undiluted extract. The two-step procedure described above, consisting of solubilization with a high CHAPS concentration followed by dilution for assay of binding activity, enabled complete recovery of active ABS. Comparison of the binding characteristics with those of the membrane-bound ABS indicated that the solubilized ABS had conserved their native-like conformation. The denaturing properties of high CHAPS concentrations and the incompatibility of this detergent with the dextran-coated-charcoal technique may account for previous unsuccessful attempts to solubilize ABS with this detergent [17,19].

In the second part of the study, the molecular size of ABS was estimated. Sedimentation-velocity analysis demonstrated the value of a high CHAPS concentration in the gradient in order to prevent progressive aggregation. However, the 20 mM-CHAPS concentration required to prevent aggregation decreased the binding activity, probably by disruption of important protein–protein interactions, and/or harsh delipidation [40,41]. A well-defined peak of binding activity could only be detected when the non-diluted extract was labelled before loading on the glycerol gradients in the presence of 20 mM-CHAPS. Owing to the rapid dissociation rate of [^3H]tamoxifen–ABS complexes, the ligand had to be included in the gradient buffer to favour re-association during the run, and thus to protect the binding site from denaturation. This sedimentation coefficient was combined with the estimated Stokes radius to calculate an approximate molecular mass of the complexes [25,42], although the results should be

interpreted with caution for membrane proteins [25]. An approximate molecular mass of 160 000 Da was obtained. Assuming that 0.5 g of CHAPS was bound per g of ABS [23,27,43], the molecular mass of the protein part of ABS (M_p) was estimated to be 110 000. A 40% difference in detergent-binding ratio (δ_D) would lead to only a 12% difference in M_p . The molecular mass of the membrane-bound ABS was also measured by the radiation inactivation technique. The observations showed that a single hit by ionizing radiation led to the total destruction of the binding activity, and did not generate partially inactivated structures. The Radiation Inactivation Size of ABS (RIS) could thus be determined. It should be borne in mind that the RIS does not take account of potential lipid or carbohydrate residues associated with ABS [30,44]. The calculated RIS of 80 000 Da was in good agreement with a molecular mass of the solubilized protein part of the ABS-CHAPS complexes of 110 000 Da, estimated from the sedimentation-velocity and gel-filtration analyses. In summary, the non-denaturing and high-yield solubilization procedure described here should facilitate characterization of the biological function of ABS and further understanding of its role in the therapeutic action of tamoxifen.

We thank Dr. J. Teissie for helpful discussions and suggestions, R. Baritaud for technical assistance, and Mrs. M. Larribe and Miss S. Chaieb for typing the manuscript.

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