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COVALENT CHROMATOGRAPHY BY THIOL-DISULFIDE INTERCHANGE OF THE HIGHLY-PURIFIED NON-TRANSFORMED RAT LIVER GLUCOCORTICOID-RECEPTOR

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Summary—Highly-purified non-transformed rat liver [^{3}H]triamcinolone acetonide–receptor complex was shown to be covalently adsorbed on activated thiol sepharose 4B, a reactive sulfhydryl matrice. Elution by mercaptoethanol in excess and inhibition of binding by previous treatment of the complex with *N*-ethylmaleimide clearly demonstrated the specificity of the binding by thiol disulfide interchange. The transformed [^{3}H]triamcinolone acetonide–receptor complex, partially purified by DNA-cellulose chromatography, was also retained on activated thiol sepharose 4B. The physicochemical characteristics of both the transformed and non-transformed glucocorticoid receptor complexes eluted from the covalent chromatography column were studied by HP size exclusion chromatography on a TSK G 3000 SW column and were found to be identical to those of the starting complexes. These results provide direct evidence for accessible sulfhydryl groups on the glucocorticoid receptor complex surface, probably distinct from the steroid binding essential sulfhydryl group.

INTRODUCTION

An early and critical event in the interaction of glucocorticoids with their target cells is the binding of steroids to specific receptor proteins which play an essential role in the mediation of glucocorticoid effects [1]. Glucocorticoid–receptor complexes, once formed, undergo a "transformation" step by which they acquire affinity for nuclei and DNA [2]. Glucocorticoid–receptors in rat thymus and liver cytosol are sensitive to sulfhydryl modifying reagents. The marked inactivation of the unbound receptors obtained by treatment with such reagents is well documented [3, 4]. The presence of bound steroid protects the receptor against this inactivation [3, 5, 6]. However sulfhydryl modifying reagents are able to impede glucocorticoid–receptor complex transformation and DNA binding [4, 5, 7]. Thus the existence of at least two distinct sulfhydryl groups on the receptor protein is generally postulated: one essential to the steroid binding and the other to the transformation of the preformed hormone receptor. Nevertheless no direct evidence of the presence of accessible sulfhydryl groups on the steroid–receptor complex was available until very recently [8]. Thus we used covalent chromatography on a reactive sulfhydryl matrice to study these accessible sulfhydryl groups on highly-purified rat liver glucocorticoid receptor.

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EXPERIMENTAL

[1,2(*n*)-^{3}H]Triamcinolone acetonide, 25 Ci mmol^{-1}, was from Amersham International (Amersham, U.K.) and unlabeled triamcinolone acetonide was from Serva (Heidelberg, F.R.G.). Sepharose CL 4B and activated thiol sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). DNA cellulose was from Sigma Chemicals (St Louis, MO, U.S.A.), Ultrogel ACA 202 from Industrie Biologique Française (Villeneuve-la-Garenne, France) and the TSK G 3000 SW column was from LKB produkter (Bromma, Sweden).

Cytosol preparation

Adrenalectomized male Wistar rats (250 g body weight) were killed by cervical dislocation. The livers were removed and perfused with ice-cold TMG buffer [pH 7.4] (20 mM Tris–HCl–20 mM mercaptoethanol–10 mM Na_2MoO_4 and 10% glycerol). The tissue was blotted free of excess buffer, weighed and homogenized (1.5 ml TMG/g of tissue) using a Teflon-glass Potter homogenizer. The homogenate was centrifuged at 105,000 *g* for 2 h. The supernatant was recovered by aspiration and pH was adjusted to 7.4 with 1 M Tris. In the case of transformed receptor preparation TMG buffer was replaced by TEM buffer [pH 7.4] (20 mM Tris–HCl–20 mM mercaptoethanol–3 mM $MgCl_2$–50 mM KCl–4 mM EDTA–1 mM phenylmethylsulfonylfluoride–10% glycerol).

Protamine sulfate precipitation

The precipitation of the unbound receptor was performed as previously described [9] and the precipitate was extracted with a phosphate buffer (pH 7.4) comprising 160 mM potassium phosphate–20 mM mercaptoethanol–10 mM Na_2MoO_4–10% glycerol.

Affinity chromatography

A glucocorticoid receptor-specific matrix was prepared using cyanogen bromide activated sepharose CL 4B coupled to oxidized dexamethasone through a diaminononyl spacer arm as previously described [9]. The protamine sulfate extract was adsorbed on this matrix and, after careful washing, elution was obtained by incubating with tritiated triamcinolone acetonide.

Size exclusion chromatography

The various molecular forms of the glucocorticoid receptor complex were analyzed by chromatography on a LKB TSK G 3000 SW column (7.5 × 600 mm) equilibrated with ice-cold phosphate buffer (pH 7.4) (160 mM potassium phosphate–20 mM mercaptoethanol–10 mM Na_2MoO_4). Flow rate was 0.5 ml/min and pressure in the range 400–500 psi. Solvent delivery and sample injection devices were supplied by Waters (Milford, MA, U.S.A.). Stokes radius calibration was obtained using the following standards: thyroglobulin (8.5 nm), ferritin (6.1 nm), catalase (5.2 nm), bovine serum albumin (3.5 nm), ovalbumin (3.0 nm) and chymotrypsinogen (2.0 nm).

Determination of binding sites

The samples were incubated in duplicate at 0°C with 30 nM [^{3}H]triamcinolone acetonide. After 19 h, the bound radioactivity was determined in duplicate by the charcoal adsorption assay [10]. The non-specific binding was measured by incubating parallel samples in the presence of a 1000-fold excess of non-radioactive triamcinolone acetonide.

Miscellaneous

Thiol free bovine serum albumin was obtained by incubating overnight 100 mg protein in presence of 5 mM *N*-ethylmaleimide. Excess reagent was removed by gel filtration. The absence of retention of the maleimide treated protein on the activated thiol sepharose 4B was checked.

The method of Bensadoun[11] was used for protein determination. Radioactivity was measured in an Intertechnique SL 4000 spectrometer, using Aqualyte (Baker Chemicals, Deventer, The Netherlands) as scintillation cocktail (35% tritium efficiency).

RESULTS

Retention on thiol sepharose 4B of the highly purified molybdate stabilized glucocorticoid receptor complex

Rat liver glucocorticoid receptor purification was performed in presence of molybdate in two steps as previously described [9]. A 2000–7500-fold purification is easily obtained by this method yielding a 15–40% homogeneous receptor preparation (from experimental specific activity and a putative mol. wt of 90,000). Table 1 summarizes the purification experiment used to prepare the eluate submitted to thiol sepharose chromatography. The purified receptor was obtained in a non-transformed, molybdate stabilized form. A preliminary report of the main characteristics of this purified receptor has already been published [12]. It is a high molecular weight form sedimenting at 8.3S in sucrose gradient ultracentrifugation and yielding a sharp elution peak with an apparent 7.0 nm Stokes radius when analyzed by size exclusion chromatography on LKB TSK G 3000 SW column (*vide infra*). Activated thiol sepharose 4B is a mixed disulfide formed between 2,2-dipyridyl disulphide and gluthatione coupled to CNBr-activated sepharose 4B [13]. The hydrophilic gluthatione residue acts as a spacer group thereby decreasing steric interference to disulphide exchange reactions with the terminal 2-pyridyl sulphide group. This reactive sulfhydryl column is able to bind covalently any thiol bearing compound which can then be eluated by adding an excess of disulfide reducing reagent like β-mercaptoethanol.

The highly-purified non-transformed glucocorticoid receptor complex was submitted to covalent chromatography on this gel. The affinity chromatography eluate containing the purified complex was first submitted to gel filtration on an Ultrogel AcA 202 column equilibrated in thiol free phosphate EDTA buffer (pH 7.6) in order to remove the β-mercaptoethanol present in the elution buffer. *N*-Ethylmaleimide treated bovine serum albumin was added to the eluate sample in order to stabilize the receptor during the chromatographic steps without introducing any interfering proteinaceous sulfhydryl group into the medium. AcA 202 excluded peak containing the receptor and the maleimide-treated serum albumin was then assayed for binding to thiol sepharose 4B. As shown in Fig. 1, two radioactive peaks were obtained and submitted to subsequent

Table 1. Purification of the non-transformed rat liver glucocorticoid receptor

	Volume (ml)	Protein (mg)	Total [^{3}H] steroid bound.cpm × 10^{-6}	Specific activity cpm mg^{-1} × 10^{-6}	Purification fold	Yield (%)
Cytosol	96	3400	31.6	0.0093	1	100
Protamine	24	324	19.1	0.059	6.32	60
Affinity column eluate	3.8	0.24	6.5	27	2914	21

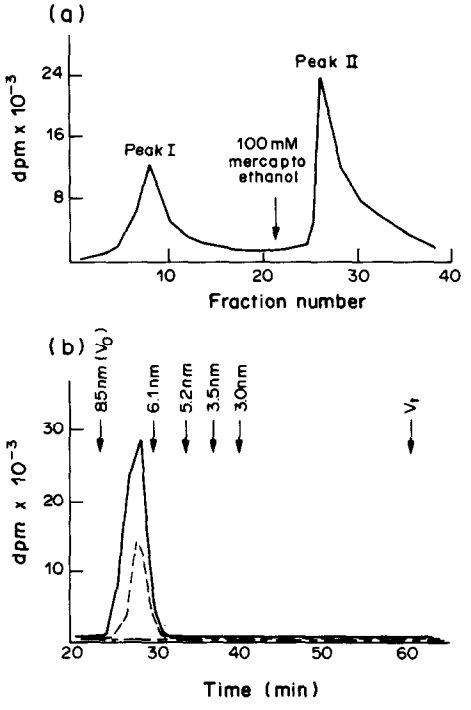

Fig. 1. Covalent chromatography of highly-purified non-transformed glucocorticoid receptor. A. 0.5 ml of affinity column eluate containing the purified [^{3}H]triamcinolone acetonide receptor complex was supplemented with 0.5 mg *N*-ethylmaleimide treated bovine serum albumin and submitted to gel filtration on an Ultrogel AcA 202 column equilibrated in thiol-free buffer [pH 7.6] (100 mM potassium phosphate–200 mM NaCl–10 mM Na_2MoO_4–1 mM EDTA). The excluded peak was loaded on a 1 ml thiol sepharose 4B column equilibrated in the same buffer. After a 90 min incubation period at 0°C, washing was performed (0.1 ml mn^{-1} flow rate) and followed by elution with 100 mM β-mercaptoethanol. Four min fractions were collected and assayed for radioactivity. B. Radioactive peaks I (–––––) and II (-----) obtained in A were analyzed by size exclusion chromatography on a TSK G 3000 SW column and compared to native affinity column eluate (——).

HPLC analysis by size exclusion chromatography on a LKB TSK G 3000 SW column. The excluded peak obtained with sulfhydryl affinity column represented 22% of the radioactivity and contained only free [^{3}H]steroid. The peak eluted by β-mercaptoethanol represented 61% of the radioactivity and contained the receptor complex in an unchanged 7.0 nm form (Fig. 1b). This Stokes radius value is in good agreement with results reported for both crude [14] and highly purified molybdate-stabilized rat liver glucocorticoid receptor [15]. The remaining 14% radioactivity was found on the gel and corresponded probably to non-eluted steroid hormone receptor complex. The yield of receptor complex obtained in the mercaptoethanol eluted peak was only 35%. The 65% loss could easily be explained by the highly purified receptor complex unstability, particularly in the absence of sulphydryl-reducing compounds. However our results clearly demonstrate the presence of at least one accessible sulphydryl group on the purified non-transformed rat liver glucocorticoid receptor complex. The specificity of the retention of the receptor complex through sulphydryl group was strongly suggested by the elution obtained with sulphydryl-bearing compounds like β-mercaptoethanol. Additional evidence of this specificity was afforded by pretreatment of the glucocorticoid receptor complex with *N*-ethylmaleimide 5 mM which resulted in complete exclusion of this complex when applied to the reactive sulphydryl column (not shown). This last result was obtained using glucocorticoid receptor complex partially purified by protamine sulfate precipitation. These receptor preparations give very similar results to those observed with the purified complex. Here again no glucocorticoid receptor was found in the thiol sepharose excluded peak and all the steroid binding activity was encountered in the β-mercaptoethanol eluted peak. Owing to the better stability of the protamine sulfate extract, 58% receptor yield was obtained. Protein content was determined in all of the fractions of the covalent chromatography step and the purification factor appeared to be very low (2- to 3-fold). However this was not surprising since the presence of accessible cysteine residues on the surface of proteins is nothing less than rare.

Retention on thiol sepharose 4B of the partly purified transformed glucocorticoid receptor complex

The cytosolic glucocorticoid receptor complex prepared in the absence of molybdate was submitted to partial purification using a two step DNA cellulose procedure [16]. The partly purified transformed glucocorticoid receptor obtained in the eluate of the second DNA-cellulose column was submitted to gel-filtration to remove any low molecular weight sulfhydryl-bearing compound and then applied to the thiol sepharose 4B column. Here again two radioactive peaks were obtained (Fig. 2a), one excluded and one β-mercaptoethanol eluted peak. These peaks were submitted to high performance size exclusion chromatography analysis and results were compared to those obtained with the native cytosolic steroid–receptor complex and heat-transformed complex (Fig. 2b). As expected the native non-transformed receptor complex appeared to be a 7.0 nm species whereas heat activation caused a shift towards the 5.0 nm transformed form and another smaller species (2.0 nm) obtained in inconstant yield. These last two species were the only constituents of the DNA-cellulose eluate. The 5.0 nm transformed complex appeared to be covalently adsorbed on the thiol sepharose column. No clear conclusion could be drawn in the case of the smaller species which was obtained in poor yield. However the appearance of this species was strikingly reduced by the addition of protease inhibitors like phenylmethylsulfonylfluoride during cytosol preparation (not shown). Here again

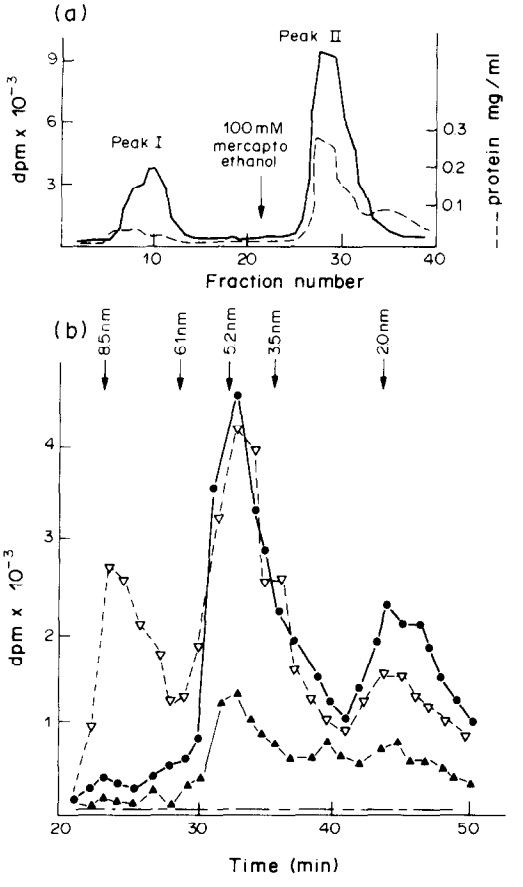

Fig. 2. Covalent chromatography of the partly purified transformed glucocorticoid receptor complex. A. Rat liver cytosol (12 ml) prepared in TEM buffer (pH 7.4) was incubated 4 h at 0°C with 30 nM [^{3}H]triamcinolone acetonide and applied to a DNA-cellulose column (2.5 × 2.0 cm) equilibrated in TEM buffer. The eluate was warmed to 26°C for 30 min, cooled to 0°C, and applied to a second DNA-cellulose column (2.5 × 2.0 cm) equilibrated in TEM buffer. After washing with the same buffer without β-mercaptoethanol, elution of the receptor complex was obtained with 460 mM KCl in thiol-free TEM buffer. The eluted steroid receptor complex was then applied on a 1 ml activated thiol sepharose 4B as described in Fig. 1. Fractions were collected and assayed for both protein and radioactivity. B. Radioactive peaks I (——) and II (▲——▲) obtained in A were analyzed by size exclusion chromatography on a TSK G 3000 SW column and compared to heat activated complex (▽----▽) and DNA-cellulose eluate (●——●).

no receptor was found in the thiol sepharose-excluded peak.

DISCUSSION

The first report of the use of covalent chromatography on a matrix reactive towards thiol compounds in the study of the glucocorticoid receptor was claimed by Harrison[17]. He demonstrated that mouse AtT-20 glucocorticoid receptor, covalently labeled with dexamethasone 21-mesylate would bind to a column of immobilized *p*-(chloromercuri) benzoate. He concluded that there was more than one sulfhydryl group on the receptor since dexamethasone 21-mesylate is believed to bind the receptor through the steroid binding essential sulfhydryl group [18]. However his results were obtained using sodium dodecyl sulfate denaturated receptor and did not afford any information about putative accessible sulfhydryl groups on the native glucocorticoid receptor complex. During the preparation of this paper Bodwell *et al.*[8] published data obtained with two reactive sulfhydryl matrices rather similar to activated thiol sepharose 4B. One matrix, agarose CL 4B diaminoethyl-succinyl-thioethylamine-2-thiopyridyl binds total receptor-bound steroid. The other matrix, agarose CL 4B diaminoethyl-succinyl-cysteinyl-2-thiobenzoic acid binds transformed but not non-transformed complexes. These results were obtained with cytosolic rat thymus glucocorticoid receptors. Retention on the second matrix was also demonstrated in the case of partly purified transformed complex. However no study was performed on highly-purified non-transformed receptor and the physicochemical characteristics of the receptor complexes eluted from the covalent chromatography column were not investigated. Our results obtained with highly purified molybdate stabilized rat liver glucocorticoid receptor clearly demonstrated the presence of at least one accessible sulfhydryl group on the surface of the non-transformed complex. Owing to the polar nature of the spacer arm borne by activated thiol sepharose 4B, and in particular the presence of a negative charge in the surroundings of the sulfhydryl group (the carboxyl group of glycine), this matrix more closely resembles the second matrix described by Holbrook than the first. However in contrast with this last column, it effectively bound both transformed and non-transformed receptor complexes. Moreover HPLC size exclusion chromatography analysis of the complexes obtained by covalent affinity chromatography on activated thiol sepharose 4B clearly demonstrated that both the transformed and non-transformed complexes were obtained in unchanged form. This was important to check since the conditions used during the covalent chromatography step, i.e. removal of sulfhydryl protective compounds followed by elution with very high concentrations of β-mercaptoethanol are known to interfere with the transformation of steroid hormone receptors [19].

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