Purification of the Glucocorticoid Receptor from Rat Liver Cytosol*

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The [\(^{1}H\)triamcinolone acetonide-labeled glucocorticoid receptor from rat liver cytosol was purified to 85% homogeneity according to sodium dodecyl sulfate gel electrophoresis. It consisted of one subunit with a molecular weight of 89,000 and had one ligand-binding site per molecule. The purification involved sequential chromatography on phosphocellulose, DNA-cellulose twice, and Sephadex C-200. Between the two chromatography steps on DNA-cellulose, the receptor was heat activated. The receptor was affinity eluted from the second DNA-cellulose column with pyridoxal 5'-phosphate. The purification achieved in the first three chromatography steps varied between 60 and 95% homogeneity in different experiments. After chromatography on the second DNA-cellulose column, the steroid-receptor complex had a Stokes radius of 6.0 nm and a sedimentation coefficient of 3.4 S in 0.15 M KCl. In the absence of KCl, the sedimentation coefficient was 3.6 S.

After concentration on hydroxylapatite, the steroid-receptor complex was analyzed by isoelectric focusing in polyacrylamide gel. The radioactivity was shown to focus together with the major protein band with pl 5.8. Following limited proteolysis with trypsin, the radioactivity, together with the major protein band, focused at pl 6.2 as previously described for the unpurified steroid-receptor complex.

Several investigations have shown that glucocorticoid hormones, in similarity to other steroid hormones, bind to specific receptor proteins in the target cells (for reviews see Refs. 1-4). The resulting steroid-receptor complex is the first step in the series of events that lead to the biological effects induced by these hormones. A detailed knowledge of the structure and function of the receptor proteins would be of great help for the further understanding of the mechanism of action of steroid hormones. The glucocorticoid receptor protein from rat liver cytosol has been characterized in crude or partially purified receptor preparations (5-13). In order to better characterize the structure and function of the receptor, a pure preparation of the receptor is required. This report describes a simple and rapid purification procedure for the glucocorticoid receptor from rat liver cytosol.

MATERIALS AND METHODS

All glassware was either disposable and discarded after one use or nondisposable but used solely for the purification of the glucocorticoid receptor. All glassware was siliconized with Sigmacote before use. All columns were siliconized each time the matrix was packed.

Buffers—Buffer A: 1 mM EDTA, 20 mM sodium phosphate, pH 7.0, 10% (w/v) glycerol, 2 mM 2-hydroxyethylmercaptan, and 50 mM NaCl.
Buffer B: 1 mM EDTA, 20 mM sodium phosphate, pH 7.8, 10% (w/v) glycerol, 2 mM 2-hydroxyethylmercaptan, and 50 mM NaCl.
Buffer C: 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, 10% (w/v) glycerol, 2 mM 2-hydroxyethylmercaptan, and 50 mM NaCl.
Buffer D: 20 mM sodium borate, pH 8.1, 3 mM MgCl₂, and 10% (w/v) glycerol.
Buffer E: 1 mM EDTA, 20 mM Tris-Cl, pH 7.8, 10% (w/v) glycerol, and 2 mM 2-hydroxyethylmercaptan.
Buffer F: 1 mM EDTA, 20 mM Tris-HCl, pH 7.4, 10% (w/v) glycerol, 2 mM 2-hydroxyethylmercaptan, 150 mM KCl, and 0.02% (w/v) sodium azide.

Cytosol Preparation—The animals were killed by cervical dislocation. The livers were perfused in situ via the inferior vena cava with 60 ml of ice-cold Buffer A, removed, and placed in ice-cold Buffer A.

Four livers were finely minced together with 45 ml of Buffer A and homogenized in a Teflon/glass Potter-Elvehjem homogenizer using a type 35 rotor. The time elapsed from the killing of the first rat to the start of the centrifugation was approximately 30 min. The temperature was kept as close to 0°C as possible throughout the procedure. After centrifugation, the floating lipid layer was carefully removed by aspiration and the resulting clear supernatant used as cytosol. The animals were given a standard pellet diet supplemented with 9 g of NaCl/liter of solution to drink following the operation.

Colon Chromatography—The conditions for the various column chromatographies can be seen in Table I. The columns used for phosphocellulose and the first DNA-cellulose column contained silica gel developed in the solvent system chloroform/ethanol, 9:1 (v/v). The radioactive isotope was always at least 96% pure, and no contaminating components were found in the unlabeled steroid.

Tritium and tritiated albumin were used in the following experiments: 9a-fluoro-11b,21-dihydroxy-16a,17a-(1-methylthyl)-didehydro-5 pregnan-14,21-diene-3,20-dione; pyridoxal 5'-phosphate, 3-hydroxy-2-methyl-5-(phosphonoxy) methyl-4-pyridinecarboxaldehyde; SDS, sodium dodecyl sulfate.

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1 The following abbreviations and trivial names are used: triamcinolone acetonide, 9α-fluoro-11β,21-dihydroxy-16α,17α-(1-methylthyl)-didehydro-5-pregnan-14,21-diene-3,20-dione; pyridoxal 5'-phosphate, 3-hydroxy-2-methyl-5-(phosphonoxy)methyl-4-pyridinecarboxaldehyde; SDS, sodium dodecyl sulfate.
terred glass filters that were covered with a nylon net. Sephadex G-200 columns were packed in Pharmacia K 26/70 columns. The matrix in all other columns was packed on a loosely packed small plug of silanized glass wool covered with 5 to 10 mm of Sephadex G-25 ( coarse).

Phosphocellulose was washed as described by Burgess (14) and the fines removed by decanting several times before the packing of the matrix. The hydrostatic pressure over the column was raised by attaching 1 m of plastic tubing (internal diameter 3 mm) after the column in order to increase the flow.

Double-stranded DNA-cellulose was prepared essentially as described by Alberts and Herrick (15) with the exception that the DNA-cellulose mixture was dried in an evacuated desiccator at room temperature over silica gel for 1 to 2 weeks as recommended by Potuzak and Dean (16). The resulting DNA-cellulose contained 300 to 500 µg of DNA/ml of packed matrix. Fines were removed by repeated decanting.

Samples were concentrated by chromatography on hydroxylapatite. The receptor was eluted from the column with Buffer E containing 0.2 M sodium phosphate, pH 7.8, and the eluate collected in fractions of 0.3 ml. The fractions containing the receptor were pooled and desalted on Sephadex G-75 columns.

SDS-Polyacrylamide Gel Electrophoresis—Electrophoresis was carried out in slab gels (140 x 80 x 2 mm) containing 9% (w/v) acrylamide in a discontinuous buffer system (17). Staining was performed at 60°C with 0.25% (w/v) Coomassie brilliant blue R-250 in H₂O:ethanol:acetic acid, 5:5:1 (by volume) and destaining at 60°C with H₂O:ethanol:acetic acid, 40:23 (by volume).

Samples to be analyzed by SDS-gel electrophoresis were first concentrated by acid precipitation together with 10 µl of 2% (w/v) serum albumin as standard. The precipitation was carried out twice as described by Bensadonn and Weinstein (19) was used, with bovine

Radioactivity Measurement—Radioactivity was measured in duplicate samples in an Intertechnique SL34 liquid scintillation spectrometer and 7 ml of Lumagel (Lumac AG, Basel, Switzerland) per sample. Correction for quenching was carried out using the external standard technique, and disintegrations per min were calculated directly. The efficiency for tritium was 40 to 45%.

Steroid-binding Assay—The specific binding of radioactivity was determined using dextran-coated charcoal as previously described (11). The binding was measured using double samples both without and with 10 µM triamcinolone acetonide. All four incubations contained 100 nM [³H]triamcinolone acetonide. Specifically bound radioactivity was calculated as the difference between the charcoal-resistant bound radioactivity in the presence or absence of unlabeled triamcinolone acetonide.

RESULTS

In order to prevent partial or total degradation of the glucocorticoid receptor by protease present in the rat liver (10, 11, 13), it was important that the preparation of cytosol...
Fig. 3. Glycerol gradient centrifugation of the steroid-receptor complex eluted from the second DNA-cellulose column. Aliquots (0.2 ml) of a purified receptor preparation eluted from the second DNA-cellulose column (purity 34%) were incubated at 0°C for 30 min with or without 0.15 M KCl followed by centrifugation for 20 h at 49,000 rpm on 5 ml of 12 to 25% (w/v) glycerol gradients containing 1 mM EDTA, 20 mM Tris-HCl, pH 7.8, and 2 mM 2-hydroxyethylmercaptan, with or without 0.15 M KCl (10). Fresh human hemoglobin, 4.13 S (21), was used as standard for the calculation of sedimentation coefficients (22). Duplicate samples were analyzed simultaneously.

Fig. 4. Sephadex G-200 chromatography of the steroid-receptor complex eluted from the second DNA-cellulose column. Four milliliters of the purified receptor preparation described in Fig. 3 were chromatographed on a calibrated Sephadex G-200 column (Table I). The column was previously calibrated with blue dextran, ferritin, yeast alcohol dehydrogenase, bovine serum albumin, and hemoglobin (10, 23). The void volume is marked V0 and the elution volume for pyridoxal 5'-phosphate, Pyr-P.

was carried out as fast as possible and that the temperature throughout the procedure was as close to 0°C as possible.

Atger and Milgrom (8) have reported that the exposure of the glucocorticoid-receptor complex to phosphocellulose accelerates the activation procedure, probably due to the high ionic strength. The [3H]triacrinolone acetoxime-labeled cytosol (about 240 ml) was, therefore, chromatographed on phosphocellulose at a maximal flow rate. The receptor complex eluted in the flow through volume (about 400 ml of yellow-colored solution). After adjustment of the pH to 7.8 with 1 M NaOH, this fraction was immediately chromatographed on the first DNA-cellulose column. Optimal conditions were achieved at a flow rate of 7.5 ml/cm²/h. A faster flow rate resulted in the contamination of the flow through volume, in which the receptor complex eluted, with proteins that normally bind to DNA-cellulose. After elution of the flow through volume, the column was washed with two column volumes of Buffer B and then eluted with two column volumes of 1 M NaCl in order to control how much of the receptor complex had become activated during the second chromatographic step.

The flow through volume from the first DNA-cellulose column was incubated at 25°C for 30 min (activation) and then cooled on ice for 10 min. Following activation, the pool was chromatographed on the second, 40 ml DNA-cellulose column at a flow rate of 9.5 ml/cm²/h. This flow rate allowed the receptor complex to bind to the matrix. An increased flow rate resulted in a decreased recovery of the receptor complex.

After the sample had run into the matrix, the walls of the
FIG. 7. SDS-polyacrylamide gel electrophoresis of fractions from the DEAE-Sepharose chromatogram. Selected fractions from the chromatogram shown in Fig. 6 were analyzed by SDS-gel electrophoresis as described under "Materials and Methods." All samples except the first standard on the top left gel were acid precipitated. The samples from the DEAE-Sepharose chromatogram column were carefully washed repeatedly with Buffer C. The column was eluted with 2 volumes of Buffer C followed by 2 volumes of Buffer D (20). Finally, the steroid-receptor complex was eluted from the DNA-cellulose column containing 10 mM pyridoxal 5'-phosphate (Fig. 1) as described by Dolan and Litwack (20). No radioactivity could be further eluted by an increase of the ionic strength to 1 M NaCl. The successive purification of the receptor from cytosol using this procedure is shown for a typical experiment in Table II. The recovery of the receptor, using this procedure, varied between 20 and 45% and the purity of the receptor preparation from 60 to 95% (mean 74%) for the three most recent experiments (calculated as described in Table II). The radioactivity in the purified receptor preparation was shown to be macromolecular bound by chromatography on Sephadex G-75 (Fig. 2). Gel filtration on Sephadex G-75 was found to be much more efficient for the desalting of preparations containing the purified receptor complex than chromatography on Sephadex G-
The steroid receptor complex in the purified preparation was analyzed by gel-slab gradient centrifugation (Fig. 3) and gel filtration on Sephadex G-200 (Fig. 4). The sedimentation coefficient in the absence of KCl and in 0.15 M KCl was 3.6 and 3.4 S, respectively (Fig. 3). Gel filtration on calibrated columns gave a single peak with a Stokes radius of 6.0 nm (Fig. 4). An approximation of the molecular weight was calculated from these data (in the presence of 0.15 M KCl) as described previously (22, 24). Assuming the partial specific volume to be 0.725 cm$^3$/g and the solvation factor 0.2 g of solvent per g of solute (21), the molecular weight was calculated to be 85,000 and the frictional ratio 1.9.

After concentration of the purified receptor complex on hydroxylapatite and desalting by gel filtration on Sephadex G-75, the receptor was analyzed by isoelectric focusing (Fig. 5). The radioactivity in the concentrated purified receptor preparation focused at pH 5.8 together with the major protein band as determined by staining of the gel corresponding to the same sample. Incubation of the receptor preparation with trypsin resulted in a shift of the pI for the peak of radioactivity as well as for the major protein band to 6.2.

Further purification of the receptor preparation from the second DNA-cellulose column on DEAE-Sepharose resulted in a single peak of radioactivity eluting at 0.16 ml of solvent (al, the molecular weight was calculated from the radioactivity and protein concentration to be 95,000 and the frictional ratio 1.9. The sedimentation coefficient was 4.2 S and the Stokes radius 6.0 nm (Fig. 4). Densitometric scanning of the stained gel from Fraction 20 gave a relative concentration of Band A in this fraction of 69%. Calculation of the purity of the receptor in this fraction from the radioactivity and protein concentration gave a purity of 80%. The overall purity of the receptor in Fractions 16 to 24 was 76% (cf. Table II).

The ratio of Band B to Band A was determined by densitometry and increased successively from 0.14 in Fraction 17 to 0.42 in Fraction 24 of the DEAE-Sepharose chromatogram. Band B, with a mobility corresponding to a molecular weight of 75,000, constitutes 14.7% of the protein in Fraction 20.

Fraction 20 contains 9.2 μg of protein/ml and 84 pmol of [3H]triamcinolone acetonide/ml. Assuming a molecular weight of 89,000 for Band A and assuming that Band A is the receptor, this would result in 1.2 ligand-binding sites per receptor molecule. The same calculations carried out for Band B, if the receptor, result in 4.3 ligand-binding sites per molecule.

**FIG. 8. Densitometric scan of the peak fraction from a Sephadex G-200 chromatogram of the purified steroid-receptor complex.** The steroid-receptor complex eluted from the second DNA-cellulose column (95% pure according to the protein determination) was chromatographed on Sephadex G-200 (Table I). Fractions corresponding to the first peak of radioactivity (cf. Fig. 4) were analyzed by SDS-gel electrophoresis, and the stained gel was scanned in a Beckman R-112 densitometer at 500 nm with slit 2 x 0.3 mm. The scan of the fraction containing most radioactivity (6 μg of receptor, 105% pure according to the protein determination) is presented here.

In another experiment, the pool of radioactivity after the second DNA-cellulose chromatography was chromatographed on Sephadex G-200, and the fractions eluted corresponding to the first peak of radioactivity (cf. Fig. 4) were analyzed by SDS-gel electrophoresis. Approximately 50% of the radioactivity applied was recovered in this first peak. The major band seen had an electrophoretic mobility corresponding to a molecular weight of 89,000. The occurrence of this band in the fractions followed closely the distribution of radioactivity in the chromatogram (not shown). Two minor bands with electrophoretic mobilities corresponding to molecular weights of 77,000 and 65,000, respectively, were also visible in the peak fractions. Densitometric scanning of the stained gel corresponding to the fraction with the highest concentration of radioactivity showed that the band with a molecular weight of 89,000 constituted 85% of the total protein (Fig. 8). The purity of the receptor preparation as determined by protein and radioactivity measurement was 95% before and 105% after the Sephadex G-200 chromatography. In another experiment carried out in a similar manner, the purity before Sephadex G-200 chromatography was 70%, and after, 110% as determined from the protein and radioactivity measurements.

**DISCUSSION**

The purification procedure reported in this paper is based on the ability of the steroid-receptor complex to interact with DNA following an activation step. The differential affinity of the nonactivated and the activated forms of the steroid-receptor complexes for DNA-cellulose has proved a useful tool in the characterization or the partial purification (or both) of the androgen receptor in rat prostate (27), the A subunit of the progesterin receptor in chick oviduct (28), the glucocorticoid receptor in rat liver (11, 29) and in mouse lymphoma cells (30), and the estrogen receptor in rat uterus (31, 32). Kalimi et al. (29) have shown that phosphocellulose has similar properties to DNA-cellulose with regard to the separation of the activated complex from the nonactivated complex.

Several groups have reported the partial purification of the glucocorticoid receptor by a two-step chromatographic procedure on either phosphocellulose (33, 34) or DNA-cellulose (35) with an intermediate activation step. However, these previous reports have not resulted in either a sufficiently pure receptor preparation or in sufficient amounts of the purified receptor to allow further characterization of the protein by, for example, SDS-gel electrophoresis. Phosphocellulose has a much larger protein-binding capacity than DNA-cellulose. The use of this matrix as the first chromatographic step reduces the amount of DNA-cellulose required. However, an DNA-cellulose has other binding properties than phosphocellulose, the pool from the phosphocellulose column must first be chromatographed on DNA-cellulose before activation takes place. The importance of the first DNA-cellulose column is illustrated by the binding of 9.2 mg of protein to this column (Table II).

The technique of affinity elution of the glucocorticoid receptor from DNA-cellulose with pyridoxal 5'-phosphate (20, 36, 37) has proved to be of great value for the purification of the receptor. We have not compared the purification using this method directly with other methods of elution, but our previous experience with elution of the receptor with NaCl indicates that the affinity elution method is superior. In addition, the eluted steroid-receptor complex is more stable in pyridoxal 5'-phosphate solution than in buffer containing the high concentrations of salt that are required to elute the complex from DNA-cellulose (20). Furthermore, when eluted with pyridoxal 5'-phosphate, the resulting receptor preparation can be further purified directly by ion exchange chro-
matography on DEAE-Sepharose, as the ionic strength is low.

We have previously reported that the glucocorticoid receptor in crude rat liver cytosol has a Stokes radius of 6.1 nm and a sedimentation coefficient of 4.0 S in high salt (10). This gave a calculated molecular weight of 102,000 and a fractional ratio of 1.84 when calculated as described before (10, 23, 24; see also under "Results"). The failure of the purified glucocorticoid receptor complex to form aggregates in low salt and the decrease in the sedimentation coefficient of the purified complex indicate that contaminating proteins in crude cytosol interact with the receptor and interfere with the sedimentation coefficient.

The Stokes radius of 6.0 nm and the sedimentation coefficient of 3.4 S for the purified receptor complex in 0.15 M KCl gave a molecular weight of 85,000 and a fractional ratio of 1.9 when calculated as above. This was in acceptable agreement with the molecular weight of 89,000 as determined by SDS-gel electrophoresis, which indicates that the receptor molecule contains one single polypeptide chain. The relatively good agreement between the measurement of the purity of the receptor by protein determination and SDS-gel electrophoresis strongly indicates that there is only one hormone-binding site per receptor molecule.

After the final Sephadex G-200 chromatography, a higher purity on SDS-gel electrophoresis than calculated by protein and radioactivity measurement would be expected, as some of the radiolabeled hormone dissociates from the receptor (cf. Fig. 4). However, this was not the case, and it would seem, therefore, that free receptor molecules are either adsorbed to the gel matrix or degraded by proteolysis. The latter possibility could account for the contaminating components seen in Fig. 8 which could be degradation products of the receptor. Further studies on the limited proteolysis of the purified receptor are in progress.

The purified receptor complex was found to be sensitive to limited proteolysis by trypsin (Fig. 5). In crude cytosol, the receptor complex shows a heterogeneous electrofocusing pattern following limited proteolysis with trypsin with a double peak at pH 5.9 to 6.1 and 6.3 to 6.5 (12). The nontrypsinized receptor complex shows a heterogeneous electrofocusing pattern indicating that contaminating proteins in crude cytosol interact with the receptor and interfere with the sedimentation coefficient.

that small amounts of lysosomal enzymes (13), trypsin, papain, or a-chymotrypsin can convert the receptor into a form with a Stokes radius of 3.6 nm and a calculated molecular weight of 44,000, it would seem likely that the 45,000 form reported by Govindan and Sekeris is a proteolytic fragment of the 90,000 form. Climent et al. (6) reported that the partially purified glucocorticoid receptor was much smaller than previously reported, 33,500 in molecular weight. It would seem likely, therefore, that they have purified a proteolytic fragment of the receptor.

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REFERENCES

26. Application Note 250 (1977) p. 8, LKB-Produkter, Bromma, Sweden
Purification of the glucocorticoid receptor from rat liver cytosol.
O Wrange, J Carlstedt-Duke and J A Gustafsson


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