CYTOSOL PROTEIN AGGREGATION AND DEXAMETHASONE BINDING TO ISOLATED RAT LIVER NUCLEI

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1. Introduction

The current hypothesis that the glucocorticoids exert their specific effects on their target tissues by altering gene expression has received considerable support from demonstrations of the binding of cytosol receptor–glucocorticoid complexes to nuclei in cell-free systems [1–7].

The methods used in these experiments have involved the incubation of isolated nuclei with cytosol containing glucocorticoid–receptor complexes that have been 'activated' either by increasing the temperature or ionic strength of the cytosol [8]. Upon re-isolation of the nuclei by low speed centrifugation the radioactivity bound to the washed pellet is determined and used to calculate the number of nuclear binding sites.

While measuring the binding of the dexamethasone–receptor complex to rat liver nuclei in a cell-free system we have observed aggregation of cytosol proteins. These results show that a substantial proportion of the radioactivity associated with the 'nuclear' pellet obtained by low speed centrifugation is in fact bound to cytosol proteins which have aggregated and coprecipitated independently of the nuclei, leading to an over-estimation of the number of true nuclear acceptor sites.

2. Materials and methods

2.1. *Reagents and buffers*

[1,2-^{3}H]Dexamethasone (specific activity 28 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., UK. Non-radioactive dexamethasone, Norit A activated charcoal, dithiothreitol, crystalline bovine serum albumin, calf thymus DNA and 2,5-diphenyloxazole were obtained from the Sigma Chemical Co., St. Louis, Mo., USA. Dextran T500 was obtained from Pharmacia, Uppsala, Sweden. Buffer A was 50 mM Tris–HCl (pH 7.55) containing 250 mM sucrose, 25 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol.

2.2. *Preparation of labelled cytosol*

Adult male rats of the Wistar albino strain of *Rattus norwegicus* were killed by a blow to the head followed by cervical dislocation and decapitation. Livers were quickly removed, washed with buffer A, blotted dry, weighed and then homogenized in 2 vol of buffer A. The homogenate was centrifuged at 408 000 × g (max.) for 16 min in a Spinco L2-65 ultracentrifuge (SW 56 rotor). After removing the upper lipid layer by suction, 4 vol of the supernatant (cytosol) were incubated at 0°C for 3–4 h with 1 vol of 0.2 μM [^{3}H]dexamethasone in buffer A and with either 1 vol of ethylene glycol or 1 vol of 1 mM non-radioactive dexamethasone in ethylene glycol. The protein bound steroid was determined using the charcoal adsorption technique [9]. All operations were carried out at 0–2°C, unless otherwise specified.

2.3. *Cell free nuclear binding of dexamethasone*

Nuclei were prepared by the method of Blobel and Potter [10]. The nuclei were washed with a 0.05% (w/v) solution (final concentration) of Triton X-100 in 50 mM Tris–HCl (pH 7.5), 25 mM KCl and 5 mM $MgCl_2$. Washed nuclei were resuspended in buffer A

and to 0.4 ml of the suspension was added an equal volume of labelled cytosol which had been preincubated with or without excess non-radioactive dexamethasone, to allow measurement of low affinity and total binding respectively. The solutions were mixed, incubated at 20°C for 1 h then chilled in an ice bath for 10 min and centrifuged at 1500 × *g* for 7.5 min at 2°C. The pellet was washed twice with 1 ml of buffer A and the final pellet was resuspended in 0.1 M NaOH. Aliquots were taken for determination of DNA, protein and radioactivity.

Controls where the labelled cytosol was incubated with buffer A alone instead of with a suspension of nuclei were also included. High affinity binding was calculated by subtracting low affinity binding from total binding.

2.4. *Other assays*

DNA and protein were assayed by the method of Burton [11] and Lowry et al. [12] respectively. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer, using a 10 ml aliquot of Diotol [13]. The channel ratio method was used for quench correction. All dpm were corrected for background radioactivity.

3. Results and discussion

When cytosol that has been preincubated with radioactive dexamethasone at 0°C is incubated for 1 h at 20°C with buffered sucrose in the absence of nuclei, a pellet of protein is obtained on centrifugation at 1500 × *g* for 7.5 min (table 1). This aggregated cytosol protein has associated with it specifically bound dexamethasone as determined by incubation of the cytosol with and without a 5000-fold excess of non-radioactive dexamethasone (table 2).

The amount of aggregation was found to be directly related to the concentration of cytosol protein (fig.1), but independent of the concentration of dexamethasone (table 1) and of magnesium ions in the range 0–40 mM (data not shown). Although the use of ethylene glycol as a solubilizing agent had some effect on the amount of aggregation, it had no significant effect on the binding of dexamethasone to the aggregate (table 2).

Table 1
Aggregation of cytosol protein and the effect of dexamethasone concentration

Dexamethasone concentration (M)	Aggregated protein (mg)
0	0.190 ± 0.005 (3)
3.3×10^{-8}	0.175 ± 0.015 (10)
1.7×10^{-4}	0.175 ± 0.015 (9)

0.4 ml of cytosol (6 mg protein), preincubated for 3–4 h at 0°C with the above concentrations of dexamethasone was diluted with an equal volume of buffer A, incubated at 20°C for 1 h, chilled at 0°C for 10 min then centrifuged at 1500 × *g* for 7.5 min. The pellet was washed twice with buffer A. Values are the mean ± SEM (*n*) determinations.

Table 2
High affinity binding of dexamethasone to aggregated protein and the effect of ethylene glycol

Ethylene glycol	Aggregated protein (mg)	Dexamethasone binding (dpm/mg protein)
+	0.175 ± 0.015 (9)	28 950 ± 3200 (9)
–	0.140 ± 0.010 (6)	24 800 ± 3150 (3)

Cytosol was preincubated for 3–4 h at 0°C with dexamethasone, with or without ethylene glycol. 0.4 ml aliquots (6 mg protein) were treated as described in the legend to table 1.

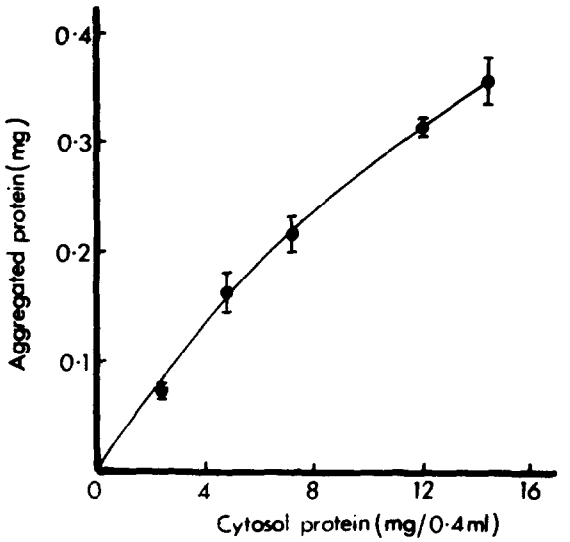

Fig.1. Effect of protein concentration on aggregation. 0.4 ml aliquots of labelled cytosol containing the above amounts of cytosol protein were diluted with 0.4 ml of buffer A, incubated at 20°C for 1 h, chilled at 0°C for 10 min then centrifuged at 1500 × *g* for 7.5 min. The pellet was washed twice and the amount of aggregated protein determined. Values are the mean ± SEM of 3 determinations.

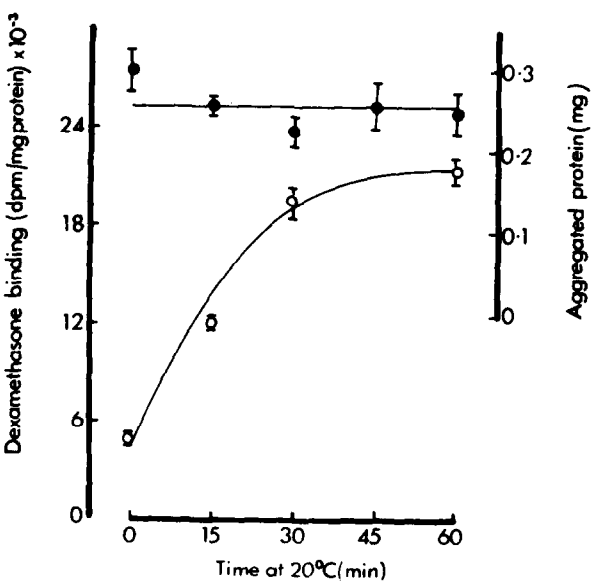

Fig.2. Effect of incubation at 20°C on aggregation and high affinity binding of dexamethasone. After a 3 h preincubation at 0°C, 0.4 ml aliquots of labelled cytosol (5.4 mg protein) were diluted with 0.4 ml of buffer A, incubated at 20°C for between 0–60 min, chilled at 0°C for 10 min, then centrifuged at 1500 × *g* for 7.5 min. The pellet was washed twice and the amount of aggregated protein (•——•) and high affinity binding of dexamethasone (o——o) determined. Values are the mean ± SEM of 3 determinations.

The amount of aggregated protein obtained after preincubation with dexamethasone for 3 h at 0°C was not increased when the cytosol (after dilution with an equal volume of buffer A) was treated for a further hour at 20°C. The increase in temperature did, however, result in an increased specific binding of the steroid to the aggregate (fig.2). After removal of the aggregated protein by centrifugation, incubation of the supernatant for another hour at either 0°C or 20°C resulted in no further aggregation.

To correct for the effect of aggregated cytosol protein on nuclear binding assays, controls where cytosol was incubated with buffered sucrose instead of a suspension of nuclei were routinely included in all experiments. The significance of the controls is summarized in table 3.

Table 3
Significance of cytosol protein aggregation in nuclear binding experiments

Non-radioactive dexamethasone	Bound radioactivity (dpm)		Control/Test
	Cytosol + Nuclei	Cytosol + Buffer A	
–	8650 ± 950	5850 ± 750	0.67 ± 0.04
+	600 ± 100	400 ± 100	0.71 ± 0.07

Cytosol was preincubated with 33 nM [^{3}H] dexamethasone for 3–4 h at 0°C, with or without non-radioactive dexamethasone. 0.4 ml aliquots (6 mg protein) were then diluted with an equal volume of either a suspension of nuclei (0.27 mg nuclear protein, Test) or buffer A (Control), incubated for 1 h at 20°C, chilled at 0°C for 10 min then centrifuged at 1500 × *g* for 7.5 min. The pellets were washed twice with buffer A and the bound radioactivity measured. Values are the mean ± SEM of 12 determinations.

It has been suggested that a property common to all steroid receptor proteins is their marked tendency to aggregate, and that receptor aggregation artifacts are a potential problem in any experiment that defines binding of receptors as co-sedimentation with nuclei, chromatin or DNA [14].

The extent of aggregation that we have observed in our experiments (about 3% of the total cytosol protein) is too great to be attributable merely to steroid receptor aggregation and emphasizes the need for controls to correct for protein aggregation in any experiment investigating the association of protein with isolated nuclei.

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