Interaction of the $M_r \approx 90,000$ Heat Shock Protein with the Steroid-biding Domain of the Glucocorticoid Receptor*

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We have investigated the physicochemical characteristics of tryptsin-treated, molybdate-stabilized glucocorticoid-receptor complexes from rat liver in the presence of 10 mM sodium molybdate by high performance size-exclusion chromatography, high performance ion-exchange chromatography, and sedimentation analysis. Trypsin treatment was performed under conditions previously reported to degrade the monomeric $M_r \approx 94,000$ steroid-binding protein to an $M_r \approx 27,000$ ligand-binding entity (Wrange, O., and Gustafsson, J.-Å. (1983) J. Biol. Chem. 258, 856-865). Also in the presence of molybdate, an $M_r \approx 27,000$ steroid-binding fragment was obtained by limited trypsinization. However, no major differences in the tested physicochemical parameters were seen when trypsin-treated glucocorticoid-receptor complexes were compared with crude cytosolic complexes. Furthermore, the $M_r \approx 27,000$ steroid-binding fragment generated in the presence of molybdate could be immunoprecipitated by antibodies specific for the glucocorticoid receptor-associated $M_r \approx 90,000$ heat shock protein. These results provide direct evidence for an interaction of the $M_r \approx 90,000$ heat shock protein with the steroid-binding domain of the glucocorticoid receptor, known to correspond to the C-terminal third of the receptor protein.

Sodium molybdate is known to have major effects on structural characteristics of the glucocorticoid receptor in vitro. For instance, molybdate has been shown to stabilize the ligand-binding properties of the glucocorticoid receptor and to inhibit the activation of the receptor to a DNA-binding state (1). In its molybdate-stabilized form, the glucocorticoid receptor exists as a heteromeric complex ($M_r \approx 300,000$) which contains a single $M_r \approx 94,000$ steroid-binding unit (2) in association with an $M_r \approx 90,000$ non-steroid-binding protein (3, 4). This $M_r \approx 90,000$ protein has been identified as a heat shock protein (5,6) and has also been shown to associate with other steroid receptors (7-10). We have recently been able to purify the rat liver glucocorticoid receptor-associated $M_r \approx 90,000$ heat shock protein (hsp90)$^1$ to near homogeneity (11).

Characterization of the purified complex has led us to suggest that the molybdate-stabilized glucocorticoid receptor might be composed of one $M_r \approx 94,000$ steroid-binding unit and a dimer of hsp90 (12).

As earlier reported (13, 14), the $M_r \approx 94,000$ glucocorticoid receptor can be converted to smaller fragments by limited proteolysis. Among the proteases tested, trypsin has been shown to degrade both crude (15) and purified (16) preparations of glucocorticoid receptor to an $M_r \approx 27,000$ fragment to which the steroid remains bound if binding has occurred prior to proteolysis.

In this report, we first show that trypsin treatment of the glucocorticoid receptor performed in the presence of molybdate also generates an $M_r \approx 27,000$ ligand-binding entity and that this fragment remains associated with hsp90.

EXPERIMENTAL PROCEDURES

RESULTS

Behavior of Molybdate-stabilized Glucocorticoid Receptor on Mono Q Column after Trypsin Treatment—We have previously reported (19) that the molybdate-stabilized rabbit liver glucocorticoid receptor is negatively charged and that a relatively high salt concentration (~0.32 M NaCl) is necessary to elute it from an anion-exchange Mono Q column. In analogy, a single peak of specifically bound (not shown) tritiated triamcinolone acetonide was eluted with 0.32 M NaCl from a Mono Q column when rat liver glucocorticoid receptor was chromatographed in the presence of molybdate (Fig. 1, open circles). Following trypsin treatment of labeled rat hepatic glucocorticoid receptor under conditions described to generate an $M_r \approx 27,000$ steroid-binding fragment (15), samples were analyzed by ion-exchange chromatography under the same conditions on a Mono Q column. After treatment with trypsin, the molybdate-stabilized glucocorticoid receptor became slightly more negatively charged than the intact receptor and was eluted with ~0.35 M NaCl (Fig. 1, closed circles). Similar experiments were performed where cytosol was incubated with the affinity label dexamethasone mesylate (20). The peaks of specifically bound radioactivity eluted from the Mono Q column were concentrated by precipitation with trichloroacetic acid, and the proteins were analyzed by SDS-PAGE and autoradiography. When untreated glucocorticoid-receptor complexes were analyzed, a major species of $M_r \approx 94,000$ and additional fragments of lower molecular weight were covalently labeled by dexamethasone mesylate (Fig. 2, lane 1). Following trypsin treatment performed in the pres-

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1 Recipient of a research fellowship from the Swedish Medical Research Council.

2 Portions of this paper (including "Experimental Procedures" and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
The glucocorticoid receptor contains a dimer of hsp90. This non-steroid-binding protein is negatively charged (11) and is probably responsible for the acidic character of the molybdate-stabilized glucocorticoid receptor and its behavior on anion-exchange matrices (21, 22). It has been shown that the $M_i$ of 27,900 proteolytic fragment of the glucocorticoid receptor, commonly called mero-receptor (23), does not bind to DEAE (24, 25). Thus, its behavior on a Mono Q column in the presence of molybdate suggested an association with an acidic protein(s), possibly hsp90.

Effect of Trypsin Treatment on Hydrodynamic Properties of Molybdate-stabilized Glucocorticoid Receptor—The molybdate-stabilized $[^{3}H]$triamcinolone acetonide-labeled glucocorticoid receptor from rat hepatic cytosol eluted as a single peak of saturable (not shown) radioactivity from the Superose 12 column (Fig. 3, open circles). By combination of the experimentally determined Stokes radius and sedimentation coefficient, a relative $M_i$ of $-290,000$ was calculated (12). This complex could be precipitated by specific anti-hsp90 antibodies (7).

The hydrodynamic parameters of labeled glucocorticoid receptors subjected to mild proteolysis with trypsin were determined under similar experimental conditions. The Stokes radius was found to be $-7.4$ nm. When similar preparations were analyzed by sucrose density centrifugation in a vertical rotor, sedimentation coefficient values of $-9.2$ S were calculated (Fig. 4A, open circles). By combination of the experimentally determined Stokes radius and sedimentation coefficient, a relative $M_i$ of $-290,000$ was calculated (12). This complex could be precipitated by specific anti-hsp90 antibodies (7).

The effect of trypsin treatment on physicochemical properties of the glucocorticoid receptor is summarized in Table I.

## DISCUSSION

It has previously been shown (26) that although the glucocorticoid receptor from rat thymus cytosol, stabilized by molybdate, was extensively degraded by endogenous proteases, the Stokes radius of the glucocorticoid-receptor complexes determined in the presence of 20 mM sodium molybdate was not significantly modified ($-8$ nm). In analogy, mild chymotrypsin treatment of the wild-type glucocorticoid receptor from S49.1 lymphoma cells has been shown to have little effect on the hydrodynamic properties of the molybdate-stabilized receptor, although the molecular weight of the ligand-binding entity, determined by photoaffinity labeling and SDS-PAGE, was decreased from 94,000 to $-40,000$ (27). Finally, mutant receptors of the increased nuclear transfer type, which lack the N-terminal half of the glucocorticoid receptor and contain a steroid-binding polypeptide of $M_i$ of $40,000$, have been shown to be stabilized by molybdate in an $-7$-nm form (27, 28). Taken together, these results indicate that the N-terminal half of the glucocorticoid receptor molecule is not required for the receptor to be stabilized in a larger form by sodium molybdate. However, not all the individual components of this larger glucocorticoid-receptor complex were defined in the cited studies.

The results we present here show that it is possible to further digest the receptor molecule to an $M_i$ of $27,000$ protein which is still maintained within a larger complex. Furthermore, we show that this complex is recognized by specific antibodies to hsp90.

Recently, the domain structure of the glucocorticoid receptor has been defined at the protein level (29). Limited proteolysis and amino acid sequence analysis of the purified rat liver glucocorticoid receptor have indicated that the $M_i$ of $27,000$ proteolytic fragment corresponds to residues 518–795 of the intact molecule and includes the steroid-binding domain.

Subtraction of the molecular weight of the steroid-binding unit determined under denaturing conditions after covalent labeling ($M_i$ of $27,000$) from the molecular weight of the trypsinized molybdate-stabilized glucocorticoid-receptor complex ($M_i$ of $220,000$) gives a resulting $M_i$ of $-200,000$ (27). This is consistent with the existence of at least a partly intact hsp90 in its dimeric form (11), within the remaining complex. In this context, it should be mentioned that under the conditions we used, hsp90 is much less sensitive to trypsin than the $M_i$ of $27,000$ fragment, corresponding to the ligand-binding domain of the receptor, with hsp90, they do not exclude an interaction of

## TABLE I

<table>
<thead>
<tr>
<th>+Molybdate</th>
<th>−Molybdate</th>
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<tr>
<td>Elution from Mono Q (m NaCl)</td>
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<td>$R_i$ (nm)</td>
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<tr>
<td>$s_{20,w}$ (S)</td>
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</tr>
<tr>
<td>Native $M_i$</td>
<td>$-290,000$</td>
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<tr>
<td>$M_i$ from SDS-PAGE</td>
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<td>Precipitation with anti-hsp90 antibodies</td>
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<table>
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<th>−Trypsin</th>
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<tr>
<td>$s_{20,w}$ (S)</td>
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<tr>
<td>Native $M_i$</td>
<td>$-5.8$</td>
</tr>
<tr>
<td>$M_i$ from SDS-PAGE</td>
<td>$-30,000$</td>
</tr>
</tbody>
</table>

$^a$ M. Denis, unpublished data.
$^b$ Denis et al. (12).
$^c$ Carlstedt-Duke et al. (29).
$^d$ Denis et al.

Glucocorticoid Receptor and Heat Shock Protein

hsp90 with other domain(s) of the receptor molecule in the intact 9S complex.

In a recent report, Picard and Yamamoto (30) have studied the subcellular distribution of β-galactosidase-receptor fusion derivatives and have described two signals which mediate hormone-dependent nuclear localization of the rat glucocorticoid receptor. One of them (NL2) resides within the C-terminal third of the receptor (residues 540–795). In the absence of hormone, both the wild-type receptor and the recombinant protein 407–715 (residues 407–740 of the receptor fused to β-galactosidase) were found in the cytoplasm of the transfected cells. On the other hand, the recombinant protein 407–615 (residues 407–615 of the receptor fused to β-galactosidase) was localized in the nucleus without dexamethasone treatment. Bearing our results in mind, it is tempting to explain this difference in localization by the fact that the 407-615.Z protein does not associate with hsp90. This may result in exposure of the DNA-binding domain of the protein, allowing the receptor to accumulate in the nucleus, where it could bind to its target sites, even in the absence of hormone. The demonstration that deletions performed in the C-terminal region of the mouse (31), the rat (32, 33), and the human (34) glucocorticoid receptors and also of the chicken progesterone receptor (35) give rise to mutant receptors that are constitutive activators supports this idea. It could be speculated that NL2 contains site(s) of the receptor molecule which interacts with hsp90, thereby repressing the transcriptional activity of the receptor in the absence of hormone.

During the completion of this manuscript, the work by Pratt et al. (36) was published. In that study, the sedimentation coefficients of mutants of the human glucocorticoid receptor expressed in COS-7 cells were determined. Deletion of residues 532–697 (corresponding to residues 550–715 of the receptor gene) produced in cells transfected with a deleted mutant of the human glucocorticoid receptor complex after limited trypsinization, we have been able to show that the steroid-binding domain indeed does interact with hsp90. Interestingly, coinciding with our findings, it has recently been shown that the temperature-mediated dissociation of hsp90 from the receptor molecule represents a hormone-dependent event (22, 37). The hormone binding has also been proposed to induce a conformational change of the steroid-binding protein provoking the dissociation of hsp90 (22, 38).

REFERENCES

Glucocorticoid Receptor and Heat Shock Protein

EXPERIMENTAL PROCEDURES

Chemicals - [3H]triamcinolone acetonide (25.3 Ci/mmol) was from the Radiochemical Centre (Amersham, Bucks, UK) and [1,2-3H]dexamethasone mesylate (42.2 Ci/mmol) was from New England Nuclear (Boston, MA). Trypsin (from bovine pancreas) was obtained from Calbiochem (La Jolla, CA). Nonradioactive triamcinolone acetonide, bovine trypsin inhibitor, and all other analytical grade reagents were from Sigma (St Louis, MO).

Preparations of Cytochrome C Labeling of Glucocorticoid Receptor - Cytochrome c from ammonium sulfate was solubilized in 1 M sodium phosphate, 1 mM EDTA, 10 mM sodium metobate, pH 7.4, as previously described (11). Labeling of glucocorticoid receptor was performed by incubation for 2 h at 0-4°C with 300 nCi [3H]triamcinolone acetonide or 200 nCi [1H]dexamethasone mesylate in the absence or presence of 10 μM unlabeled triamcinolone acetonide. Following incubation, free steroid was removed by chromatography on Q-Sepharose columns (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M buffer.

Elution - Fresh solutions of trypsin in distilled water (1 mg/ml) were prepared immediately prior to use. Limited proteolysis of glucocorticoid receptor complexes was carried out by incubating cytochrome c with trypsin (5.6 μg of trypsin per 1 mg of cytochrome c) for 3 h at 10°C (11). The reaction was stopped by the addition of a 10-fold excess (by weight) of bovine trypsin inhibitor.

High Performance Liquid-Phase Chromatography - Samples (300 μl) of labeled cytochrome c were chromatographed on a Mono Q HR 5/5 column (Pharmacia) equilibrated in pHM buffer (20 mM sodium phosphate, 1 mM EDTA, 10 mM NaCl, pH 7.4). Retained proteins were eluted with a 0-1 M NaCl linear gradient in pHM buffer at a flow rate of 1 ml/min at 4°C. Fractions (1 ml) were collected and counted for radioactivity.

High Performance Liquid-Ethanol Chromatography - Samples (300 μl) of labeled cytochrome c were applied to a Superose 12 HR 10/30 column (Pharmacia). Chromatography was performed at a flow rate of 0.5 ml/min with pHM buffer supplemented with 0.15 M NaCl. One-minute fractions were collected and aliquots were assayed for radioactivity.

Sucrose gradient ultracentrifugation - Linear (5-20%, w/v) sucrose gradients were prepared in pHM buffer (unless otherwise indicated) containing 0.1 M NaCl. Samples were layered on to the gradients, and the tubes were centrifuged in a VTi 80 rotor (Beckman) at 48,000 g for 90 min at 4°C. Five-drop fractions were collected and counted.

Anti-hapten Antibodies - Polyclonal antibodies against the glucocorticoid receptor-associated hapten purified from rabbit serum (12) were raised in rabbits (13). Specific antibodies were isolated by chromatography of the antiserum on a column of CR-activated Sepharose (Pharmacia) to which had been coupled -1 μg of purified hapten. After washing, bound antibodies were eluted with 50 mM acetic acid, 50 mM NaCl, pH 3. Following neutralization with Tris, the eluates were dialyzed with pHM buffer containing 0.1 M NaCl. Each fraction was assayed using a pHM buffer pH 7.4.

Fig. 1. HPLC analysis of limited-label glucocorticoid receptor treated with trypsin. Cytochrome c samples (300 μl), labeled with [3H]triamcinolone acetonide and subjected to mild proteolysis (○) or not (□), were applied to a Mono Q column. The column was washed for 30 min with pHM buffer. Elution was performed with a 0-1 M linear NaCl gradient (←→) in the equilibrating buffer. Aliquots (10 μl) were assayed for total radioactivity.

Fig. 2. HPLC analysis of fully-label glucocorticoid receptor eluted from the Mono Q column. Cytochrome c samples, labeled with [3H]dexamethasone mesylate, were treated and chromatographed on a Mono Q column as described in Fig. 1. Fractions were collected and assayed for radioactivity. Lane 1, untreated cytochrome c; lane 2, cytochrome c treated with trypsin; lane 3 contains [3H]-labeled standard proteins (mol. wt x 10^-3); rat hep, 90; bovine serum albumin, 66; ovalbumin, 45; bovine carbonic anhydrase, 29; lactalbumin, 14.

Fig. 3. HPLC analysis of partially-label glucocorticoid receptor treated with trypsin. Cytochrome c samples (300 μl), labeled with [3H]triamcinolone acetonide and subjected to mild proteolysis (○) or not (□), were applied to a Mono Q column. The column was washed for 30 min with pHM buffer. Elution was performed with a 0-1 M linear NaCl gradient (←→) in the equilibrating buffer. Aliquots (10 μl) were assayed for total radioactivity.

Fig. 4. Effect of trypsin on the sedimentation of the limited-label glucocorticoid receptor. Aliquots (10 μl) of cytochrome c labeled with [3H]triamcinolone acetonide were either treated with trypsin (○) or not (□), and incubated in a total volume of 100 μl with buffer (○) or buffer containing 5 μg of purified anti-hapten antibodies for 60 min at 4°C (□). The incubates were loaded on top of 5-20% (w/v) sucrose gradients prepared in pHM buffer containing 0.15 M NaCl. Following centrifugation, 5-drop fractions were collected and counted. Aliquots (1, 7.6 kDa) and ovalbumin (12, 3.0 S) were used as standards and run on separate gradients.

Fig. 5. Effect of trypsin on the sedimentation of the fully-label glucocorticoid receptor. Aliquots (10 μl) of cytochrome c labeled with [3H]dexamethasone mesylate were either treated with trypsin (○) or not (□), and incubated in a total volume of 100 μl with buffer (○) or buffer containing 5 μg of purified anti-hapten antibodies for 60 min at 4°C (□). The incubates were loaded on top of 5-20% (w/v) sucrose gradients prepared in pHM buffer containing 0.15 M NaCl. Following centrifugation, 5-drop fractions were collected and counted. Aliquots (1, 7.6 kDa) and ovalbumin (12, 3.0 S) were used as standards and run on separate gradients.

Fig. 6. Effect of trypsin on the sedimentation of the partially-label glucocorticoid receptor. Aliquots (10 μl) of cytochrome c labeled with [3H]triamcinolone acetonide and subjected to mild proteolysis were either treated with trypsin (○) or not (□), and incubated in a total volume of 100 μl with buffer (○) or buffer containing 5 μg of purified anti-hapten antibodies for 60 min at 4°C (□). The incubates were loaded on top of 5-20% (w/v) sucrose gradients prepared in pHM buffer containing 0.15 M NaCl. Following centrifugation, 5-drop fractions were collected and counted. Aliquots (1, 7.6 kDa) and ovalbumin (12, 3.0 S) were used as standards and run on separate gradients.