Structure of the Mouse Glucocorticoid Receptor: Rapid Analysis by Size-Exclusion High-Performance Liquid Chromatography†

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ABSTRACT: Gel-exclusion high-performance liquid chromatography (HPLC) has been used to separate the untransformed from the transformed glucocorticoid receptor (GC-R) extracted from mouse AtT-20 cells. With 200 mM potassium phosphate as the eluent, an efficient separation of the forms of the GC-R is attained in 15–20 min. The untransformed cytosolic GC-R elutes from the column with a Stokes radius (R_s) of 8.2–8.6 nm, as do the molybdate-stabilized GC-R, the purified untransformed GC-R, and the cross-linked cytosolic GC-R. GC-R transformed in vitro by either ammonium sulfate precipitation, KCl treatment, or G-25 chromatography elutes with an R_s of 5.7–6 nm. Also, GC-R extracted from the nucleus with either 0.3 M KCl or 2 mM sodium tungstate, or purified by two cycles of DNA–cellulose chromatography, has an R_s of 5.5–6.3 nm. The data are identical either in the presence or in the absence of 20 mM Na_2MoO_4, suggesting that molybdate is not causing aggregation to produce a larger R_s value than that of the native receptor. Vertical tube rotor sucrose gradient ultracentrifugation of cytosol produces three forms of the GC-R: 9.1 S, 5.2 S, and 3.8 S. Sequential analysis of the GC-R forms by HPLC and vertical tube rotor ultracentrifugation and vice versa allows for the hydrodynamic determination of molecular weight within a very short time period (2–3 h total). The untransformed 9.1S species of GC-R pooled from MoO_4^{2-}-containing sucrose gradients has an R_s of 8.6 nm (M_r 310K–340K) while both the 5.2S and 3.8S forms have an R_s of 6 nm (M_r 115K–140K and 96K–100K, respectively). Conversely, if the 6-nm form of the receptor, generated either by KCl treatment or by G-25 chromatography, is pooled from HPLC, then it subsequently sediments on MoO_4^{2-}-containing gradients as a 3.5–4.6S species. This probably indicates that the 5.2S transformed GC-R is unstable and dissociates into its constituents during HPLC, despite the rapidity of the process. From the calculated molecular weight for each of the three GC-R forms, it is suggested that the untransformed oligomeric GC-R dissociates into monomeric subunits during transformation and that the intermediate form is *not* a dimer of identical monomeric subunits.

Glucocorticoids are physiological regulators which interact with specific receptor proteins in responsive cells (Yamamoto & Alberts, 1976). After the hormone binds to the receptor, the glucocorticoid receptor (GC-R)[1] complex is transformed to a DNA-binding moiety. The transformed receptor interacts with acceptor sites on the DNA to affect specific gene transcription.

The function of the GC-R as a gene regulatory protein is dependent on its structural characteristics. Various models exist to explain the transformation of receptors to DNA-binding forms. These include dissociation of an oligomeric receptor into subunits (Raaka & Samuels, 1983; Vedeckis, 1983b), limited receptor proteolysis (Puca et al., 1972; Sica et al., 1976), and conformational change (Samuels & Tomkins, 1970; Rousseau et al., 1972; Bailly et al., 1980). In addition, covalent modification of the GC-R (e.g., dephosphorylation) may also play a role in transformation (Barnett et al., 1980; Vedeckis & Reker, 1984).

The GC-R from the mouse AtT-20 cell line has been studied extensively by our laboratory (Vedeckis, 1981, 1983a,b; Eastman-Reks et al., 1984; LaPointe & Vedeckis, 1984; Kovačič-Milivojević et al., 1985; Reker et al., 1985). Vertical tube rotor ultracentrifugation resolves the GC-R into three forms: (1) the 9–10S oligomeric untransformed species; (2) the 5.2–6.6S intermediate transformed species; and (3) the 3.8–4.2S transformed monomer. The 5.2–6.6S and 3.8–4.2S forms have been determined to be transformed by DEAE-cellulose and DNA–cellulose chromatography (Reker et al., 1985).

Studies have been done in an effort to determine the composition of the untransformed and intermediate transformed GC-R species. GC-R transformation most likely involves dissociation of the untransformed oligomer either into a dimer of identical hormone-binding subunits, into a monomer bound to a non-hormone-binding molecule(s), or into the 3.8–4.2S monomer alone (Vedeckis, 1983b). It has been most difficult to determine the exact nature of the intermediate transformed GC-R, as this species manifests variable forms depending on assay conditions (e.g., 6.6 S in low salt, molybdate-free sucrose gradients and 5.2 S in Na_2MoO_4-containing gradients). Definitive structural studies are difficult to perform because of problems with verification of the integrity of the receptor form during experimental manipulation. That is, time-dependent dissociation of subunits and/or receptor proteolysis during prolonged analysis may preclude determination of the correct hydrodynamic properties of the GC-R.

This study was undertaken to determine relationships among the three GC-R forms by using very rapid techniques, size-

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[1] Abbreviations: DEAE, diethylaminoethyl; Dex, dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione); GC-R, glucocorticoid receptor(s); HPLC, high-performance liquid chromatography; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); Tris, tris(hydroxymethyl)aminomethane; TEA, triethanolamine; DMS, dimethyl suberimidate dihydrochloride; DCC, dextran-coated charcoal; EDTA, ethylenediaminetetraacetic acid.

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exclusion HPLC and vertical tube rotor ultracentrifugation. In this study, we used HPLC to (1) separate untransformed GC-R from GC-R transformed by KCl, alkaline phosphatase, ammonium sulfate, and G-25 chromatography, (2) determine the effects of Na_2MoO_4 on the Stokes radius (R_s) of the untransformed GC-R, (3) determine the R_s of the untransformed GC-R after covalent cross-linking of subunits, and (4) calculate the molecular weights for the three GC-R forms by using hydrodynamic properties determined by *sequential* analyses.

Materials and Methods

Chemicals. [1,2,4,6,7-^{3}H]Dexamethasone (Dex), 70–110 Ci/mmol, and [6,7-^{3}H]triamcinolone acetonide (TA), 20–37 Ci/mmol, were obtained from Amersham. Ferritin was obtained from Boehringer Mannheim, whereas all other protein standards were purchased from Sigma. Also, sodium molybdate, triethanolamine, and 1-thioglycerol were obtained from Sigma. Dimethyl suberimidate dihydrochloride was a product of Pierce Chemical Co. Sucrose (density gradient grade), ammonium sulfate, and Tris were "Ultra-Pure" grade obtained from Schwarz/Mann. All other chemicals were reagent grade obtained from J. T. Baker.

Cell Culture and Cytosol Preparation. AtT-20 mouse pituitary tumor cells were maintained in culture as described previously (Vedeckis, 1981). Cytosol was prepared from AtT-20 cells in TETg buffer (20 mM Tris, pH 7.4 at 25 °C, 1 mM EDTA, and 12 mM 1-thioglycerol) as described in Vedeckis (1983b). In some experiments, 20 mM sodium molybdate was added to TETg buffer to stabilize the receptor. The GC-R in the cytosol was labeled with either (2.7–5) × 10^{-8} M [^{3}H]TA or 2.4 × 10^{-8} M [^{3}H]Dex overnight. All procedures, unless otherwise noted, were performed at 0–4 °C.

Sucrose Gradient Ultracentrifugation. Vertical tube rotor (VTi 80) ultracentrifugation was carried out as described previously (Eastman-Reks et al., 1984; Reker et al., 1985); 5–20% (w/v) sucrose gradients (5.2 mL total volume) were prepared in TETg buffer (low salt), TETg plus 20 mM Na_2MoO_4, or TETg plus 0.3 M KCl (high salt). Sedimentation properties of the GC-R forms were analyzed by using ^{14}C-methylated protein standards run in parallel tubes (Reker et al., 1985).

Size-Exclusion High-Performance Liquid Chromatography. Samples were chromatographed on an Altex TSK-3000SW column (7.2 × 300 mm), or through two TSK-3000SW columns coupled in series, or through a TSK-4000SW column (7.5 × 300 mm) coupled to a TSK-3000SW column. A TSK-3000SW guard column (7.5 × 100 mm) preceded the size-exclusion columns.

A 250-μL cytosol sample was injected into the column by using a Beckman Model 340 sample injector module. Radiolabeled GC-R was eluted from the columns with either 200 mM potassium phosphate buffer, pH 7 or 7.4 at 25 °C, or potassium phosphate buffer containing 20 mM Na_2MoO_4, or potassium phoshate buffer plus 0.3 M KCl, using a Beckman Model 112 solvent delivery module. The high ionic strength phosphate buffer was used to minimize protein–column interactions (Pavlik et al., 1982; Horiike et al., 1982). GC-R was eluted at a flow rate of 1 mL/min, and 0.5- or 1.0-mL fractions were collected. Aliquots (0.4–1.0 mL) of the fractions were analyzed by liquid scintillation counting with an efficiency of 35%. All buffers and cytosol samples were filtered (0.45-μm pore size membrane filter) prior to use to remove particulate matter. Columns were washed after use with HPLC-grade water and stored in 10% methanol. When necessary, columns were flushed with 10% methanol.

To determine the Stokes radius (R_s) of GC-R forms, ^{14}C-methylated protein standards were run in parallel. A standard curve was drawn by linear regression analysis using the R_s values of the standards. R_s values for GC-R forms were extrapolated from the standard curve of $K_d^{1/3}$ vs. R_s. The profiles presented here are representative of many repeated chromatographies of similar samples. Although the separation between the untransformed and transformed receptor species is not as great as that obtained by using conventional gel filtration, the elution positions of the various receptor forms are highly reproducible for a given set of columns. The untransformed and transformed GC-R species are separated by 1, 1.5, and 2.5 mL for the TSK-3000SW column alone, the TSK-3000SW/TSK-3000SW combination, and the TSK-4000SW/TSK-3000SW combination, respectively. Standard proteins were routinely run to monitor column performance and characteristics.

Cross-Linking of the Untransformed GC-R. Cytosol was prepared from AtT-20 cells in 80 mM triethanolamine (TEA) buffer, pH 8.0 at 4 °C. GC-R was labeled overnight with [^{3}H]Dex. Dimethyl suberimidate was freshly prepared in distilled water at a concentration of 0.1 M, added to cytosol (final concentration 0.01 M), and allowed to incubate for 1 h. Tris was added to a final concentration of 50 mM to inactivate the unreacted DMS. After a 1-h incubation, the solution was filtered through a column of Sephadex G-25 to remove free hormone, unreacted DMS, and excess Tris. KCl was added to the cross-linked cytosol to a final concentration of 0.3 M. After a 30-min incubation, 200-μL samples of cytosol were subjected to vertical tube rotor ultracentrifugation on high-salt sucrose gradients. This procedure separated cross-linked, untransformed GC-R from GC-R that was not cross-linked. Non-cross-linked GC-R sedimented as the 3.8S monomer in high-salt gradients as described previously (Eastman-Reks et al., 1984; Reker et al., 1985), whereas cross-linked GC-R was not subject to subunit dissociation in high salt and sedimented at 9.1 S.

Results

HPLC of [^{3}H]Dex-Labeled Cytosol. The first problem addressed was if HPLC could be used to rapidly separate untransformed GC-R from transformed GC-R and free hormone. Cytosol labeled with [^{3}H]Dex was chromatographed on TSK-4000SW and TSK-3000SW columns coupled in series (Figure 1). From a comparison of the elution positions of protein standards, a peak of radioactivity with an R_s of 8.3 nm is seen, which corresponds to labeled GC-R. This 8.3-nm peak represents specific binding of [^{3}H]Dex to the GC-R, as inclusion of a 100-fold excess of unlabeled Dex eliminates the peak (data not shown). Two additional peaks of radioactivity are also evident. These peaks represent free hormone as they are eliminated by treatment with dextran-coated charcoal (DCC) (Figure 1). The small peak of radioactivity probably represents a somewhat hydrophilic contaminant in the [^{3}H]Dex preparation. The larger peak of free hormone, because of its hydrophobic nature, interacts with the column, and its elution is retarded. A similar effect has been noted when [^{3}H]TA-labeled GC-R is chromatographed on Sephadex LH-20 (Vedeckis, 1981).

Finally, transformation of the GC-R by addition of $(NH_4)_2SO_4$ to 40% saturation produces a sharp peak of labeled GC-R with an R_s of 6.4 nm (Figure 1). The production of this 6-nm form of the GC-R is not likely to be the result of endogenous proteolytic activity in AtT-20 cell cytosol (Vedeckis, 1983a). For example, if GC-R from mouse liver cytosol, which contains high proteolytic activity, is transformed

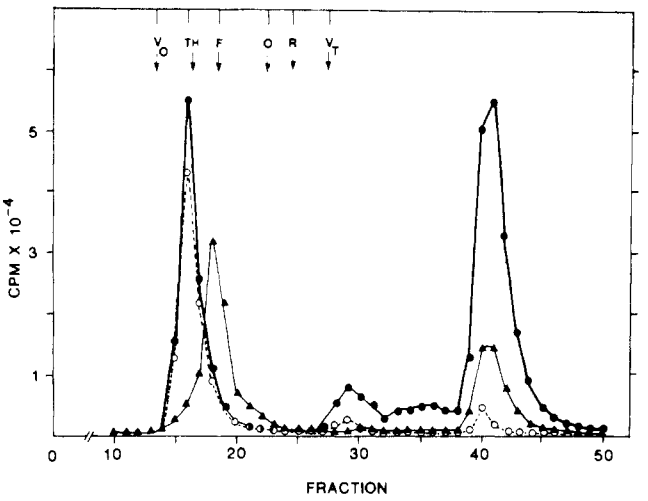

FIGURE 1: HPLC of cytosol labeled with [^{3}H]Dex. Cytosol was prepared from the mouse AtT-20 pituitary tumor cell line and labeled overnight with [^{3}H]Dex. [^{3}H]Dex-labeld cytosol (●), cytosol treated with dextran-coated charcoal (DCC) (○), and cytosol treated with ammonium sulfate (to 40% saturation) (▲) were chromatographed separately on a TSK-4000SW column coupled in series to a TSK-3000SW column. GC-R was eluted with 0.2 M potassium phosphate buffer, pH 7.4, at a flow rate of 1 mL/min. 1.0-mL fractions were collected and then subjected to liquid scintillation counting. Standard proteins used were thyroglobulin (TH, 8.61 nm), iron-free ferritin (F, 6.15 nm), ovalbumin (O, 2.86 nm), and ribonuclease A (R, 1.64 nm). V_0 represents the excluded volume (blue dextran) and V_T the the total column (KCl) volume.

by Sephadex G-25 chromatography and then subjected to HPLC, the transformed GC-R appears as a 6-nm species, while large amounts of a 3.9-nm proteolyzed fragment also appear (Vedeckis et al., 1985). No such fragments are generated when AtT-20 cell GC-R is transformed.

Thus, the data from Figure 1 indicate that untransformed GC-R can be clearly and rapidly separated from transformed GC-R in a matter of 20 min. Also, free hormone can be washed from the columns within an additional 20 min to allow chromatography of multiple GC-R samples in a short period of time.

Effects of Molybdate on Untransformed GC-R. Sodium molybdate has been used to stabilize the GC-R in the untransformed state (Leach et al., 1979; Lee et al., 1981; Holbrook et al., 1983; Dahmer et al., 1984). However, its use has been criticized as a possible cause of nonspecific receptor subunit aggregation [see review by Sherman & Stevens (1984)], which would result in incorrect determinations of the physicochemical properties of the receptor.

To study the effect of MoO_4^{2-}, cytosol was prepared in the presence and absence of MoO_4^{2-} and chromatographed by using potassium phosphate buffer ± 20 mM Na_2MoO_4. As can be seen in Figure 2, the GC-R from cytosol prepared with MoO_4^{2-} has the same elution profile and thus the same R_s (8.3 nm) as cytosolic GC-R analyzed in the absence of MoO_4^{2-}. In addition, untransformed receptor purified in the presence of MoO_4^{2-} by affinity chromatography according to the procedure of Grandics et al. (1984) elutes from a TSK-3000SW column with an R_s of 8.2–8.6 nm (B. Kovačič-Milivojević and W. V. Vedeckis, unpublished results). Thus, the authentic untransformed GC-R extracted from cells in low salt or low salt plus MoO_4^{2-} buffer does not appear to be an artifactual aggregate caused by the extraction procedure.

Cross-Linked GC-R. To determine definitively if the untransformed GC-R exists as an 8.2–8.6-nm species, cross-linking studies were done. Cytosol was prepared in TEA buffer, and the GC-R subunits were cross-linked with dimethyl

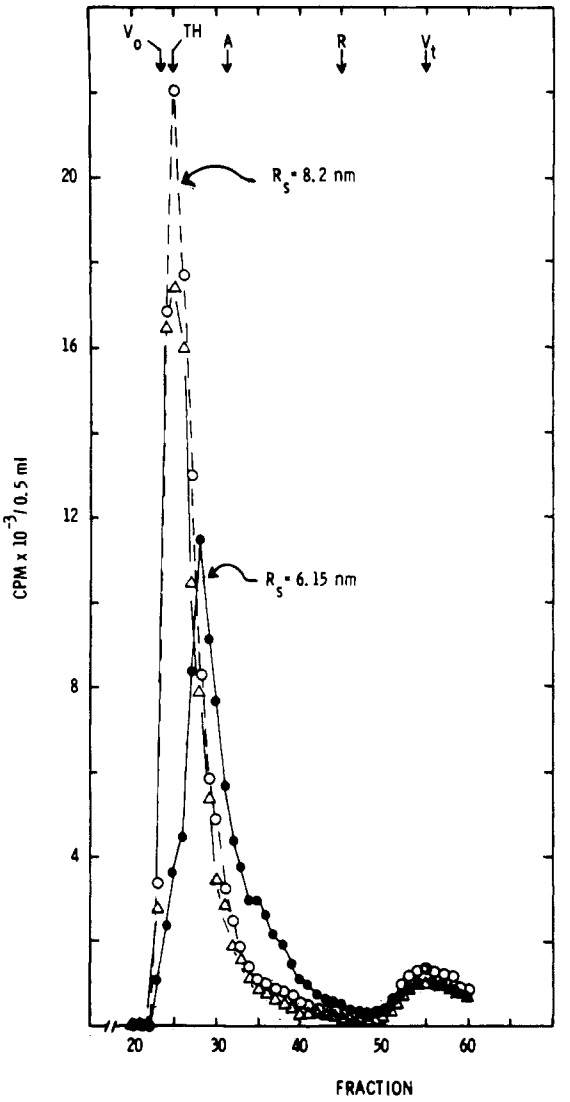

FIGURE 2: HPLC of cytosolic GC-R treated with sodium molybdate. Cytosol was prepared and labeled with [^{3}H]Dex. Untransformed GC-R (○) was eluted from two TSK-3000SW columns coupled in series, with potassium phosphate buffer delivered at a flow rate of 1 mL/min. 0.5-mL fractions were collected and subjected to liquid scintillation counting. Na_2MoO_4 was added to a portion of the cytosol to a final concentration of 20 mM, and the GC-R was eluted in phosphate buffer plus 20 mM Na_2MoO_4 (△). A third aliquot of GC-R was treated with KCl to give a final concentration of 0.3 M. After 3 h, the KCl-treated GC-R was eluted from the columns with phosphate buffer plus 0.3 M KCl (●). Protein standards are described in the legend to Figure 1. A = aldolase (4.5 nm).

suberimidate dihydrochloride (DMS) as described under Materials and Methods. As can be seen in Figure 3A, when cytosolic GC-R is analyzed on high-salt sucrose gradients, receptor that is not cross-linked sediments as the 3.8–4.2S monomer, while roughly half remains as the 9.1S complex and represents the cross-linked GC-R. The peak fractions (19 and 20) of the cross-linked, 9.1S material were then pooled and subjected to sequential analysis by HPLC using potassium phosphate buffer plus 0.3 M KCl. Figure 3B indicates that the cross-linked GC-R has elution characteristics identical with those of [^{3}H]Dex-labeled cytosol chromatographed in potassium phoshate buffer alone ($R_s \simeq 8.2$ nm). In contrast, data in Figure 3C show that non-cross-linked cytosolic GC-R treated with 0.3 M KCl for 4 h and then subjected to high-salt HPLC elutes with an R_s of ~6.4 nm. Thus, incubation of cytosolic GC-R with DMS effectively cross-links receptor subunits to prevent KCl-induced subunit dissociation of the

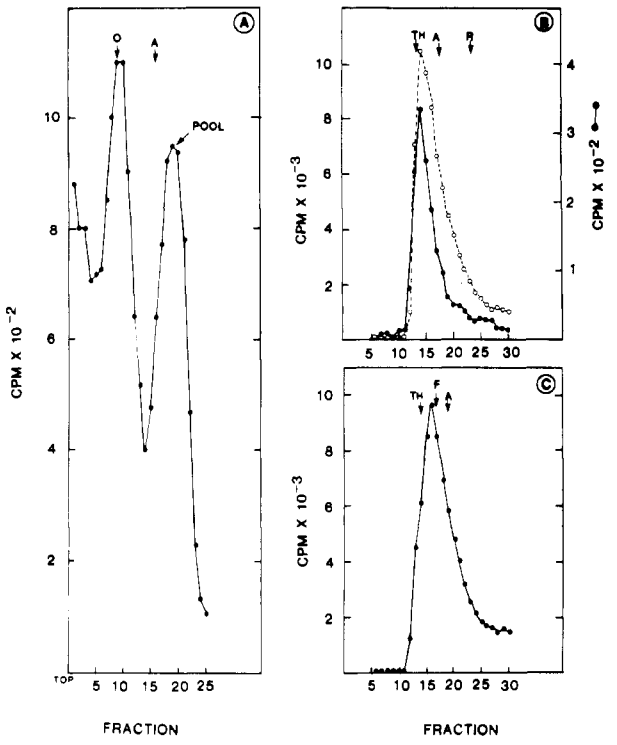

FIGURE 3: HPLC of cross-linked GC-R. Cytosol was treated with the cross-linking agent dimethyl suberimidate as described under Materials and Methods. (A) 200-μL aliquots were subjected to vertical tube rotor ultracentrifugation on high-salt (0.3 M KCl) sucrose gradients. The gradients were fractionated into the same set of vials, from which 50-μL aliquots were removed for scintillation counting. Protein standards used in parallel sucrose gradients were ovalbumin (O; 3.5 S) and aldolase (A; 7.9 S). (B) Fractions 19 and 20 (panel A), representing cross-linked untransformed GC-R, were pooled, and a 250-μL aliquot was subjected to HPLC on a single TSK-3000SW column using 0.2 M potassium phosphate buffer, pH 7.0, plus 0.3 M KCl as the eluent. 0.4 mL of the 0.5-mL fractions was subjected to liquid scintillation counting. The elution position of cross-linked GC-R (●) is compared to that of un-cross-linked cytosolic GC-R (O). In panel C, the elution position of KCl-transformed cytosol (●) is shown to indicate the position of monomeric GC-R (6.4 nm).

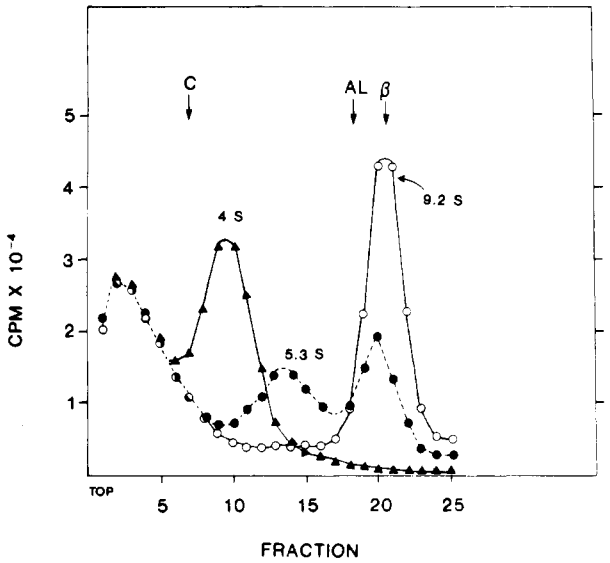

FIGURE 4: Sucrose gradient analysis of GC-R transformed by KCl or alkaline phoshatase. Aliquots of cytosol were treated with 0.3 M KCl for 3 h at 4 °C (▲) or with 107 μg/mL bovine intestinal alkaline phosphatase (Boehringer Mannheim, molecular biology grade) for 3 h at 10 °C (●), or were incubated with no additions for 3 h at 10 °C (untransformed control sample) (O). After the incubations, Na_2MoO_4 was added to each sample to a final concentration of 20 mM. Each sample was then subjected to sucrose gradient ultracentrifugation in TETg buffer plus 20 mM Na_2MoO_4. Protein standards included chymotrypsinogen A (C; 2.6 S), aldolase (A; 7.9 S), and β-amylase (β; 9.4 S).

oligomeric receptor. Also, the R_s of this cross-linked GC-R is identical with that of purified, untransformed GC-R and MoO_4^{2-}-stabilized, cytosolic GC-R.

GC-R Transformed in Vitro. As described in the introduction, previous experiments indicated that the AtT-20 cell GC-R exists in three forms (Vedeckis, 1983b). When the cytosolic GC-R is transformed by several different protocols [alkaline phosphatase treatment, heat (25 °C for 60 min), dialysis, precipitation at 40% saturated $(NH_4)_2SO_4$, 0.3 M KCl treatment for 1–4 h, and chromatography on Sephadex G-25 columns] and then subjected to low-salt sucrose gradient ultracentrifugation, a 5.2S (molybdate-containing buffer) or 6.6S (molybdate-free buffer) intermediate, transformed GC-R is generated (Reker et al., 1985; Kovačič-Milivojević et al., 1985). If these species are centrifuged on high-salt (KCl-containing) sucrose gradients, the 3.8–4.2S transformed monomer results (Eastman-Reks et al., 1984). We were thus curious to determine the elution profiles of GC-R species transformed by different methods.

Cytosol was treated either with 0.3 M KCl for 3 h to generate the monomeric GC-R or with alkaline phosphatase (107 μg/mL; 3 h, 10 °C) to generate the intermediate transformed GC-R. Individual samples were subjected to parallel analysis by sucrose gradient ultracentrifugation and HPLC. Sucrose gradient analysis (Figure 4) indicates that the untransformed GC-R sedimented as a 9.2S species in TETg gradients containing 20 mM Na_2MoO_4, while the KCl-transformed species ran as the 4S monomer in high-salt TETg gradients. Treatment with alkaline phosphatase, as expected, resulted in the generation of the intermediate, transformed GC-R sedimenting at 5.3 S in low-salt, molybdate-containing gradients. As can be seen, only partial GC-R transformation occurred after alkaline phosphatase treatment. When the samples were chromatographed by using HPLC (Figure 5A), the untransformed GC-R eluted with an R_s of 8.2 nm. The elution profile of the alkaline phosphatase treated sample reflected incomplete transformation (Figure 5A). The shoulder preceding the peak corresonds to the untransformed GC-R remaining after enzyme treatment. However, the peak of the transformed GC-R species elutes with an R_s of 6.4 nm, identical with that of the GC-R monomer generated by KCl treatment (Figure 5B).

Studies on the effects of proteases on GC-R structure have shown that the transformed GC-R is readily cleaved to a partially proteolyzed form and to the mero-receptor (Vedeckis, 1983a). All of these forms can be easily separated by using HPLC (Vedeckis et al., 1985). Criticism of the use of alkaline phoshatase transformation of the GC-R has centered on possible contaminating protease activity. It is noteworthy that alkaline phoshatase treatment does not generate any discernible proteolytic fragments during HPLC analysis (Figure 5B) and thus it seems to promote transformation through a dephosphorylation mechanism rather than via contaminating proteases.

To ensure that the elution profile of the cytosolic GC-R transformed in vitro was not being obscured by nonspecific cytosolic components, we compared it with the elution profiles of nuclear GC-R and purified, transformed GC-R. Receptor extracted from the nucleus with either 0.3 M KCl (Vedeckis, 1981) or 2 mM sodium tungstate was subjected to HPLC analysis. The nuclear GC-R eluted from the gel-exclusion columns with an R_s of 6.4 nm (data not shown). Similarly, purification of transformed receptor labeled with [^{3}H]dexa-

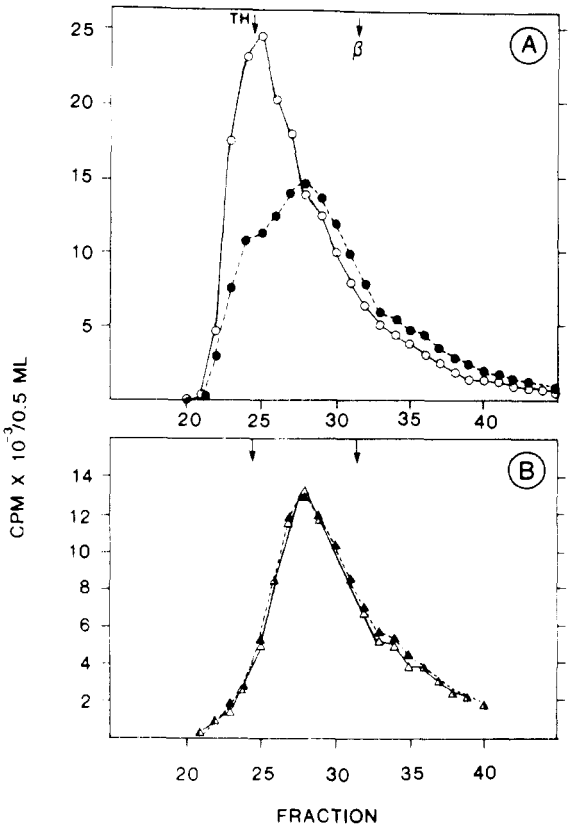

FIGURE 5: HPLC of GC-R transformed by alkaline phosphatase and KCl. Samples prepared according to the legend in Figure 4 were subjected to gel-exclusion HPLC according to the methods described in the legend to Figure 2. (A) Untransformed (○) and alkaline phosphatase transformed (●) GC-R samples were eluted with phosphate buffer plus 20 mM Na_2MoO_4. (B) The KCl-treated samples without (△) or with (▲) prior alkaline phosphatase treatment were run in TETg buffer plus 20 mM Na_2MoO_4 and 0.3 M KCl. Protein standards included thyroglobulin (TH; 8.61 nm) and β-amylase (β; 4.81 nm).

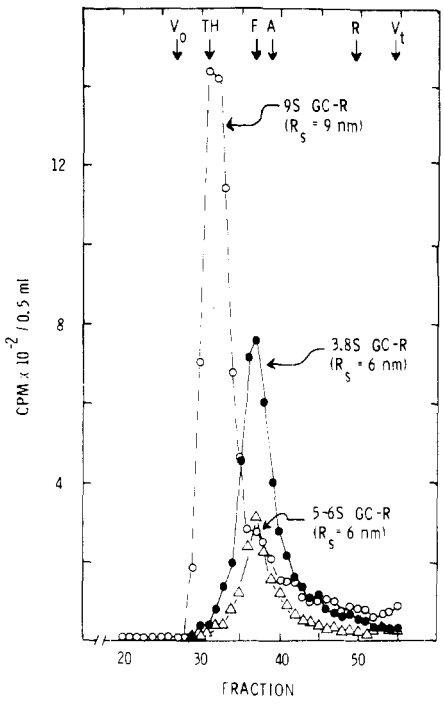

FIGURE 6: HPLC of GC-R forms separated by sucrose gradient ultracentrifugation. Cytosol was prepared and labeled with [^{3}H]Dex. 0.2-mL aliquots of untransformed GC-R (○), G-25-transformed GC-R (△), and 0.3 M KCl-transformed GC-R (●) were sedimented through 5–20% linear sucrose gradients [made in TETg plus 20 mM Na_2MoO_4 (○, △) or TETg plus 0.3 M KCl (●)] as described under Materials and Methods. Gradients were fractionated at 4 °C, and the receptor-containing peak fractions were determined. 0.2-mL aliquots of the 9S, the 5.2S (5–6S), and the 3.8S GC-R forms were separately chromatographed on a TSK-4000SW column coupled in series to a TSK-3000SW column, and 0.5-mL fractions were collected. The eluent was 0.2 M potassium phosphate containing 20 mM Na_2MoO_4 plus (●) or minus (○, △) 0.3 M KCl. Protein standards are the same as those in Figures 1 and 2.

methasone mesylate (Kovačič-Milivojević et al., 1985), using a modification of the method described in Wrange et al. (1979), and gel-exclusion HPLC analysis resulted in an identical elution profile (R_s = 6.4 nm) (data not shown). Thus, the nuclear GC-R and the purified monomer elute from gel-exclusion columns with R_s values identical with that described for cytosolic GC-R species transformed in vitro.

Sequential Analysis of GC-R Forms Using HPLC and Sucrose Gradient Analysis. As mentioned previously, three forms of the GC-R (one untransformed and two transformed) can be clearly differentiated by sucrose gradient analysis. However, in experiments presented here, GC-R transformed in vitro by different methods always elutes from the gel-exclusion columns with the same R_s value. We wanted to ensure that our transformation protocols yielded GC-R forms with the proper *s* values and that these forms were directly comparable to those analyzed by using HPLC. Thus, we ran samples of untransformed and transformed GC-R on sucrose gradients containing molybdate, recovered the peak fractions corresponding to the 9S, 5.2S (5–6S), and 3.8S forms, and analyzed these forms separately by HPLC. Figure 6 indicates that the 9S GC-R elutes as a 9-nm form, which is similar to that of the untransformed cytosolic GC-R. However, both the intermediate, transformed 5.2S GC-R and the 3.8S monomer elute as 6-nm forms.

To study further the problem of how the three forms of the GC-R are related and to get a more valid estimate of molecular weights (M_r), we did sequential analyses of the three receptor forms. We used the untransformed GC-R (cytosolic extract), the intermediate, transformed GC-R (G-25-filtered cytosol), and the monomeric, transformed GC-R (cytosol treated with 0.3 M KCl). Each of these three samples were run both on vertical tube rotor sucrose gradients and on HPLC. The appropriate peak fractions obtained from each of these two techniques were then subjected to the other technique in order to obtain the required hydrodynamic property. Thus, the Stokes radii of all three receptor forms were determined prior to and after sucrose gradient centrifugation, and the sedimentation coefficients were also determined before and after HPLC. This would then give us an unambiguous indication of the hydrodynamic properties of the various receptor forms and show us which forms were altered by these two analytical methods. Figure 7 shows the R_s values of GC-R forms, as determined by HPLC, before and after sucrose gradient analysis. The results are essentially identical with those in Figure 6 and show that the R_s values for untransformed GC-R, G-25-transformed GC-R, and KCl-transformed GC-R are unchanged by sucrose gradient ultracentrifugation. Data in Figure 8 indicate that the *s* values for the untransformed and KCl-transformed GC-R are also unchanged by HPLC. However, the *s* value for the intermediate transformed GC-R generated by G-25 chromatography is diminished following HPLC from 5.6 to 4.6 S. In another similar experiment, G-25-transformed GC-R was chromatographed via HPLC, and the peak fractions were collected and subjected to sucrose gradient analysis. The GC-R sedimented as the 3.5S monomeric form (LaPointe & Vedeckis, 1984). These data in-

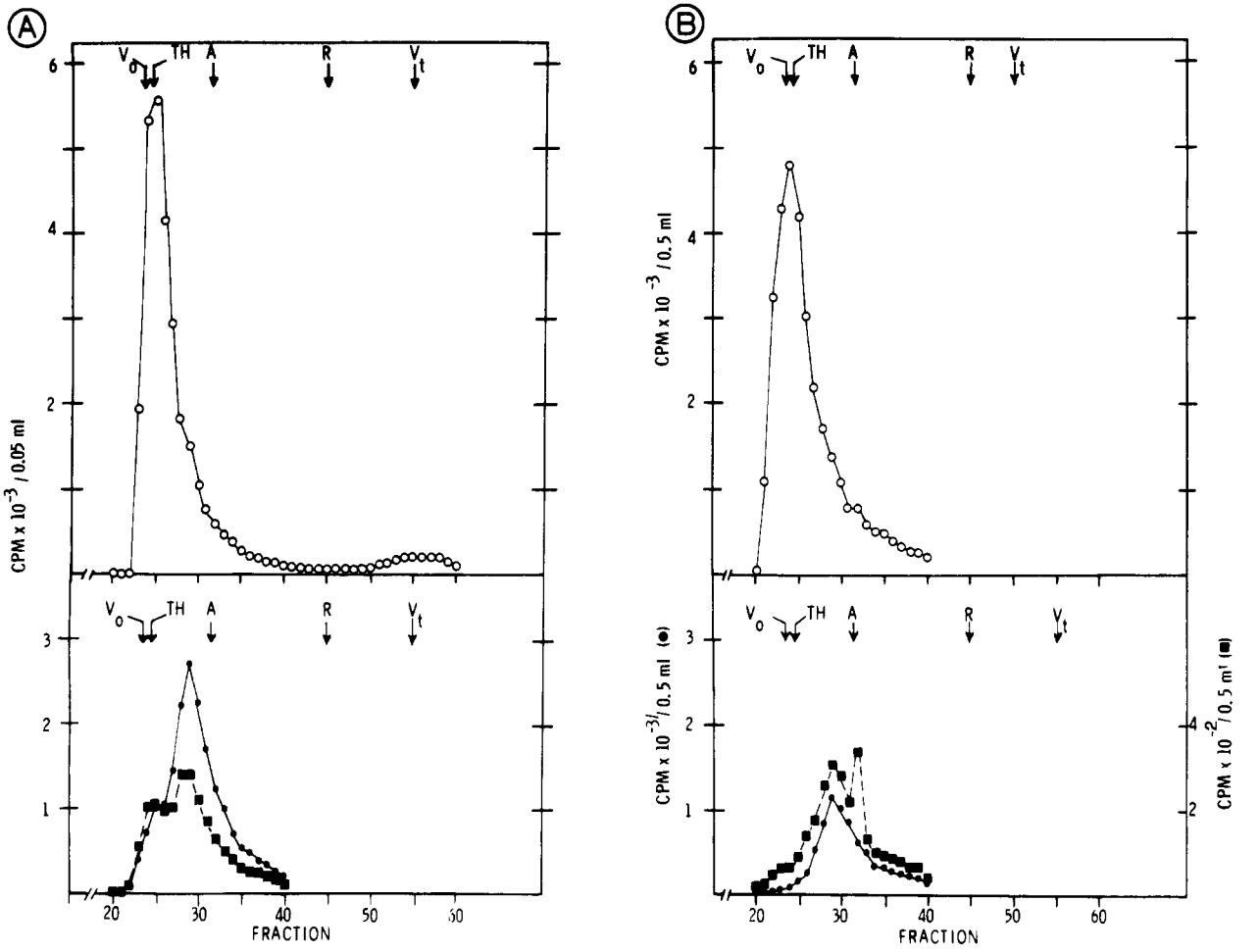

FIGURE 7: Stokes radii of GC-R forms before and after sucrose gradient ultracentrifugation. The data in this figure were derived in conjunction with those in Figure 8. Cytosolic GC-R either was left untransformed (○) or was transformed by KCl treatment (●) or Sephadex G-25 chromatography (■). These samples were analyzed immediately by gel-exclusion HPLC using a TSK-3000SW/TSK-3000SW column combination and the collection of 0.5-mL fractions. (A) The Stokes radii of the untransformed, KCl-transformed, and G-25-transformed GC-R were 8.4, 5.7, and 5.9 nm, respectively. Identical aliquots of the three samples were also centrifuged on sucrose gradients (Figure 8, panels A, C, and E). The 9.1S untransformed peak fractions from the sucrose gradients were pooled and then analyzed on HPLC (panel B, top). Likewise, the 3.9S KCl-transformed and 5.6S G-25-transformed receptor peaks were pooled from sucrose gradients and run on HPLC (panel B, bottom). The 9.1S GC-R has a Stokes radius of 8.6 nm, while the 3.9S and 5.6S forms have an R_s of 5.7 nm.

dicate that the intermediate transformed GC-R is apparently unstable when sequential analysis is performed.

By use of the equation $M_r = 4224sR_s$, the hydrodynamic properties of proteins can be used to estimate molecular weights (Siegel & Monty, 1966). For the untransformed GC-R, an estimate of 310K–340K is determined (R_s = 8.2–8.6 nm; 8.4–9.2 S). The estimated molecular weight of the monomeric GC-R is 94K–100K (R_s = 5.7–5.9 nm; 3.9–4 S), and for the intermediate transformed GC-R, M_r = 115K–140K (R_s = 5.9 nm; 4.6–5.6 S).

The calculated molecular weights allow some important conclusions to be drawn about the structure of mouse AtT-20 cell GC-R. Although there is significant experimental error involved in determining the hydrodynamic properties, no simple stoichiometric relationship exists between the sizes of the untransformed oligomer (310K–340K) and the intermediate transformed oligomer (115K–140K), or between the untransformed oligomer (310K–340K) and the transformed monomer (94K–100K). Thus, it seems that the untransformed, oligomeric GC-R may be composed of hormone-binding subunits and non-hormone-binding macromolecules, as opposed to being a homotetramer of hormone-binding subunits (Vedeckis, 1983b). In addition, it appears that the 5.2–6.6S intermediate transformed GC-R is composed of one 6-nm monomeric subunit plus another non-hormone-binding macromolecule with a molecular weight of 21K–40K. We have suggested elsewhere that this later macromolecule may be a low molecular weight RNA (Kovačič-Milivojević et al., 1985).

Discussion

In this study, gel-exclusion HPLC has been used, for the first time, to analyze the physicochemical properties of the three forms of the mouse AtT-20 cell GC-R. This type of rapid analysis has been employed previously for the study of estrogen and progesterone receptors (Pavlik et al., 1982a,b; Wiehle et al., 1984), the vitamin D receptor (Pike et al., 1982), and the dioxin receptor (Gasiewicz & Rucci, 1984). In all of these studies, HPLC has allowed for quantitative and qualitative analyses of receptor characteristics, while minimizing typical analytical problems, e.g., ligand dissociation and proteolytic modifications of receptor structure.

HPLC of the untransformed GC-R, the molybdate-stabilized cytosolic GC-R, and the purified untransformed GC-R has shown that this macromolecule has an R_s = 8.2–8.6 nm, as has been demonstrated previously by using other gel filtration techniques (Holbrook et al., 1983; Sherman et al., 1983; Vedeckis, 1983b). With regard to the transformed monomeric GC-R (produced in vitro by KCl treatment), a Stokes radius of 5.7–6.4 nm was determined by using HPLC. Again, this

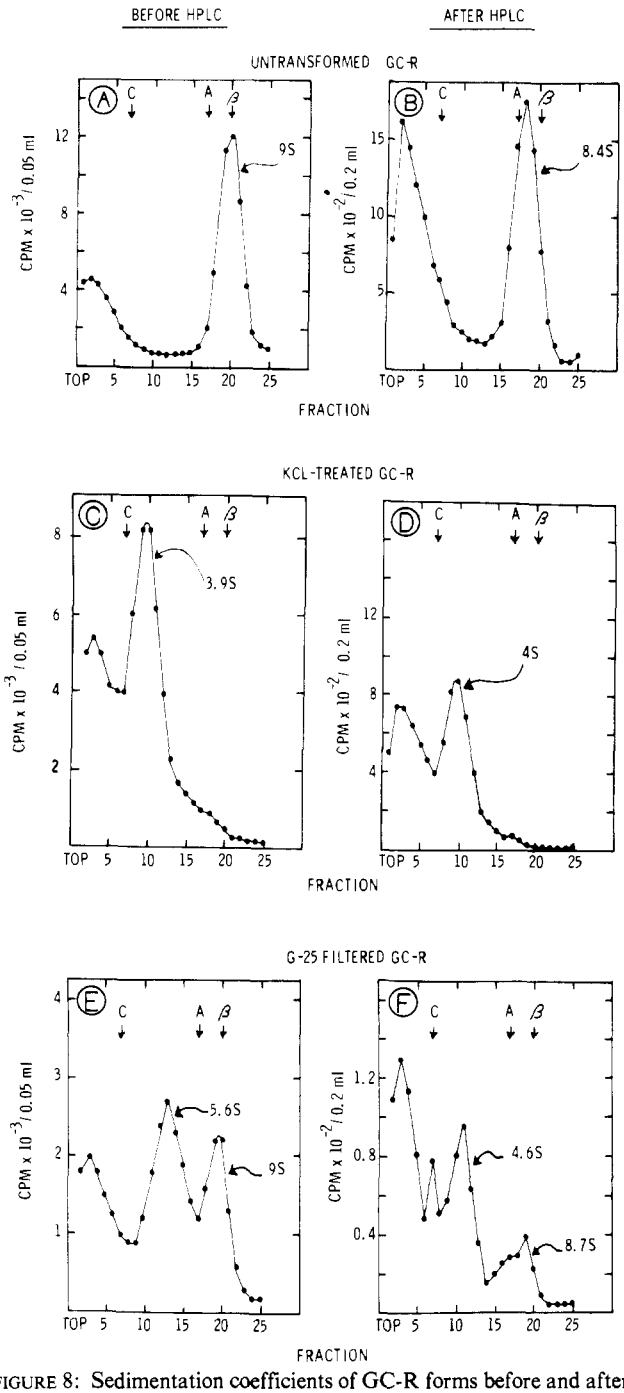

FIGURE 8: Sedimentation coefficients of GC-R forms before and after HPLC. In this part of the experiment, untransformed (A, B), KCl-transformed (C, D), and G-25-transformed (E, F) GC-R were separated on 5–20% linear sucrose gradients to determine *s* values. Panels A, C, and E depict the fractionation profiles of the three receptor forms prior to HPLC. In panels B, D, and F are shown the sedimentation profiles for the three GC-R forms, whose peak fractions were pooled after separate isolation by gel-exclusion HPLC (see Figure 7, panel A for R_s values). Data show that the *s* values for the untransformed and KCl-transformed GC-R species remain essentially unchanged after isolation by HPLC. However, the *s* value for the G-25-transformed GC-R has decreased from 5.6 to 4.6 S.

value is well within the range reported in the literature [see review by Vedeckis (1985)]. Detailed analysis of the intermediate transformed GC-R (5.2–6.6 S) has proven most difficult. A previous study using agarose A-1.5m gel filtration suggested that this GC-R form existed as an 8.3-nm species (Vedeckis, 1983b). However, sequential analysis by HPLC and vertical tube rotor ultracentrifugation reported here indicate that the 5.2–6.6S form has an R_s of 6 nm. It is currently believed that this intermediate form either is a dimer of identical subunits or consists of a monomer plus a non-hormone-binding subunit (Vedeckis, 1983b; Sherman et al., 1983). If this 5.2–6.6S species is composed of a monomer plus a non-hormone-binding, nonproteinaceous macromolecule (Kovačič-Milivojević et al., 1985), then it is not surprising that HPLC analysis results in an R_s of 6 nm, as it is possible that the non-hormone-binding component readily dissociates from the complex by using this technique.

Studies in our laboratory indicate that the 5.2–6.6S form of the receptor can be reconstituted in vitro from the monomeric subunit and a small RNA molecule (Kovačič-Milivojević et al., 1985). The association of receptor with RNA has been previously reported (Chong & Lippman, 1982; Tymoczko & Phillips, 1983; Economidis & Rousseau, 1985). Formation of the 5.2–6.6S species from the GC-R monomer and RNA could occur in two ways. First of all, if the untransformed oligomeric GC-R contains RNA, then during transformation the monomer and RNA could maintain their association as a 5.2–6.6S complex. The alternate possiblity is that RNA is not a component of the untransformed complex. Transformation would then result in the dissociation of the oligomer into monomeric subunits, which could bind to the cytosolic RNAs. Data from our laboratory indicate that RNA is not a component of the untransformed oligomeric GC-R (B. Kovačič-Milivojević and W. V. Vedeckis, submitted for publication). Thus, transformation of the GC-R probably involves dissociation of the oligomer into monomeric subunits, as has been suggested by dense amino acid labeling experiments (Raaka & Samuels, 1983). Formation of the 5.2–6.6S GC-R form would then follow via binding of the monomer to a small RNA species.

The hydrodynamic properties of the three GC-R forms and the calculated molecular weight for each determined in this study would certainly support the hypothesis of an oligomeric receptor dissociating into monomeric subunits during transformation. Our data fit well with the concept that the untransformed GC-R is an oligomer of hormone-binding subunits, non-hormone-binding species, and perhaps low molecular weight inhibitors of transformation to yield a complex with a molecular weight of ~320K (8.2 nm; 9.2 S) [see reviews by Vedeckis (1985), Dahmer et al. (1984), and Sherman & Stevens (1984)]. During transformation, this oligomeric complex probably dissociates into monomeric hormone-binding subunits (M_r 94K–100K; R_s = 6 nm; 4 S), which could subsequently bind small RNA molecules to form the 5.2S–6.6S complex. This complex is not stable during rapid analysis by HPLC. At present, the exact role of RNA in GC-R structure and function is unclear. Further studies using covalently cross-linked, dexamethasone mesylate labeled GC-R and denaturing polyacrylamide gel electrophoresis are under way to further elucidate the structure of the oligomeric GC-R forms.

Registry No. Dex, 50-02-2; MoO_4^{2-}, 14259-85-9; $(NH_4)_2SO_4$, 7783-20-2; KCl, 7447-40-7.

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Role of the 50-Kilodalton Tryptic Peptide of Myosin Subfragment 1 as a Communicating Apparatus between the Adenosinetriphosphatase and Actin Binding Sites

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ABSTRACT: Limited glutaraldehyde modification of tryptic myosin subfragment 1, which mainly consists of 26-, 50-, and 20-kilodalton (kDa) peptides, resulted in the selective cross-linking of the 20- and 50-kDa peptides. The cross-linking pattern was altered by nucleotides, depending on the base structure. Neither the reactive thiols on the 20-kDa peptide nor the reactive lysyl residue on the 26-kDa peptide was modified with the reagent, regardless of the presence or absence of nucleotide. Glutaraldehyde treatment of the protein resulted in marked increases in its Mg^{2+}-ATPase activity and affinity for actin. High ATPase activity and actin affinity were not produced if the treatment was conducted in the presence of ATP. These ATPase and actin binding properties of the protein derivatives are explained by assuming that glutaraldehyde "freezes" the existing interactions between the 20- and 50-kDa peptides in the activated and nonactivated conformational states, respectively. Taking into account the previous reports that the ATPase site resides between the 26- and 50-kDa peptides, and the 50-kDa peptide binds either ATP or actin, the present results suggest that the 50-kDa peptide acts as a communicating apparatus between the ATPase and actin binding sites of myosin. A simple model for the intersite communication is also proposed.

The interaction of actin with myosin heads and the actin-dependent activation of the Mg^{2+}-ATPase of the myosin molecule are crucial events of the mechanochemical transduction process in muscle and other motile systems. The

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