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Transformed Mouse Glucocorticoid Receptor: Generation and Interconversion of the 3.8S, Monomeric and 5.2S, Oligomeric Species†

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ABSTRACT: Recent studies have implicated subunit dissociation as a possible mechanism of glucocorticoid receptor transformation [Vedeckis, W. V. (1983) *Biochemistry 22*, 1983-1989; Raaka, B. M., & Samuels, H. H. (1983) *J. Biol. Chem. 258*, 417-425]. While it is becoming increasingly evident that the untransformed (non-nuclear-binding and non-DNA-binding) glucocorticoid receptor from mouse AtT-20 cells is a 9.1S oligomeric species (M_r 290 000-360 000), two transformed species have been described for this receptor. One of these has a sedimentation coefficient of 5.2 S (on molybdate-containing gradients), while the smallest nonproteolyzed, monomeric subunit is 3.8 S. The present study was undertaken to determine which is the most common form generated both in vitro and in vivo and the structural relationship between these two forms. A wide variety of in vitro transformation protocols all yielded the 5.2S form when analyzed on molybdate-containing sucrose gradients by using a vertical tube rotor. Kinetic studies showed that the appearance of the 5.2S form coincided precisely with the appearance of transformed receptor, as defined by DEAE-cellulose elution. Furthermore, when the 3.8S and 5.2S peaks were collected from sucrose gradients directly, they were transformed receptors as defined by both DEAE-cellulose and DNA-cellulose chromatography, while the 9.1S sucrose gradient peak was untransformed when the same criteria were used. The 3.8S monomer, when isolated from high-salt sucrose gradients and then desalted, reverted to the 5.2S form (molybdate-containing gradients) or a 6.6S form (low-salt, molybdate-free gradients). Additionally, when the receptor was transformed in vivo by incubating mouse AtT-20 cells with radioactive hormone at 37 °C, the transformed receptor obtained was the 5.2S species. Treatment of the in vivo generated transformed receptor with ribonuclease A caused a decrease in sedimentation coefficient of the 5.2S form to the 3.8S species, suggesting that RNA may be a component of the intermediate, 5.2S transformed receptor complex. The appearance of this 5.2S, intermediate-transformed receptor species under a wide variety of experimental conditions suggests that it is a discrete, and perhaps physiologically relevant, entity.

After steroid hormone ligands are bound, steroid receptor proteins undergo a process called transformation,[1] which results in a conversion of the protein from a non-DNA-binding and non-nuclear-binding form to a species that binds to genomic constituents. Although it has been studied for many years, the molecular mechanism of steroid receptor transformation has remained obscure. Recent studies on transformation of the mouse and rat glucocorticoid receptors (GC-R)[2] have suggested that this process may involve the dissociation of an oligomeric, 9-10S, untransformed complex into transformed receptor subunits (Vedeckis, 1981, 1983a,b; Holbrook et al., 1983; Raaka & Samuels, 1983; Eastman-Reks et al., 1984).

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[1] We have previously used the more traditional term, activation, to describe this process. However, as is shown here and elsewhere (Holbrook et al., 1983; Raaka & Samuels, 1983; Vedeckis, 1983b; Eastman-Reks et al., 1984), a physicochemical alteration in structure ("change in form") does occur in this process. Therefore, as suggested by Wheeler et al. (1981), we will use the term transformation to indicate the change in receptor form accompanying its conversion to a species which binds to nuclear constituents. Activation will be reserved for the conversion of the receptor from a form which is incapable of binding ligand to one which can bind hormone.

[2] Abbreviations: GC-R, glucocorticoid receptor; Tris, tris(hydroxymethyl)aminomethane; Dex, dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione); Na_2EDTA, disodium ethylenediaminetetraacetate; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; RBF, receptor binding factor; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone).

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In spite of these studies, the nature of the authentic, physiologically relevant, transformed GC-R has not yet been determined. Raaka & Samuels (1983) have postulated that transformation involves the dissociation of a homotetramer into identical, 4S monomeric subunits. On low-salt sucrose gradients they also observed a "4–5S" species, which they believed to be somehow related to (and interconvertible with) the 4S monomer. Additional information on this form was not presented in this study. In a more detailed series of studies (Vedeckis, 1981, 1983a,b; Eastman-Reks et al., 1984), we determined the physicochemical characteristics of the untransformed, transformed, and proteolyzed mouse GC-R. It was found that the 9.1S untransformed receptor could be converted into two transformed species. One of these transformed receptor species has a sedimentation coefficient of about 5.2 S on molybdate-containing sucrose gradients, while the other has an *s* value of 3.8 S. Thermal transformation studies have suggested that the normally occurring, physiologically relevant, transformed GC-R may be the 5.2S form (Vedeckis, 1983b; Eastman-Reks et al., 1984).

Presented below is a series of experiments demonstrating that the 5.2S mouse GC-R is the predominant transformed species obtained after either in vivo or in vitro transformation. Thus, we suggest that GC-R transformation results in the conversion of a 9.1S, oligomeric, untransformed receptor into a 5.2S, oligomeric, transformed species. Although it is possible that this process involves the dissociation of an untransformed homotetramer into transformed homodimers, preliminary results suggest that a small RNA may be a component of the intermediate, transformed 5.2S species.

Materials and Methods

Chemicals. Sucrose (density gradient grade), ammonium sulfate, and Tris were "Ultra Pure" grade obtained from Schwarz/Mann. The protein standards, bovine pancreatic ribonuclease (RNase) (type XII-A) and calf intestinal alkaline phosphatase (type VII-L), were all obtained from Sigma Chemical Co. Ferritin was purchased from Boehringer-Mannheim. Sodium molybdate and 1-thioglycerol were also obtained from Sigma. [1,2,4,6,7-^{3}H]Dexamethasone (Dex), 82 and 77 Ci/mmol, was from Amersham, and [1,2,4-^{3}H]-triamcinolone acetonide (TA), 37 Ci/mmol, was from New England Nuclear. All other chemicals were reagent grade obtained from J. T. Baker.

Cell Culture and Cytosol Preparation. The culturing of the mouse pituitary tumor AtT-20 cell line in spinner flasks was as described previously (Vedeckis, 1981). In addition, the preparation of cytosol from these cells in TETg buffer (20 mM Tris-HCl, pH 7.4 at 25 °C; 1 mM EDTA; 12 mM 1-thioglycerol, added fresh daily) has been described in detail elsewhere (Vedeckis, 1983b). Unless otherwise noted, all subsequent procedures were performed at 0–4 °C.

In Vivo Transformation. AtT-20 cells were pelleted at $10000g_{av}$ for 15 min at 4 °C, and the supernatant medium was decanted. Packed AtT-20 cells (2.25 mL) were suspended in 3 mL of Dulbecco's modified Eagles medium (DME; KC Biological) to which was added [^{3}H]TA to a final concentration of 5.4×10^{-8} M. After a 1-h incubation at 0 °C, these cell suspensions were divided into three tubes. At the appropriate times, tubes were transferred to a water bath and incubated for 5 or 15 min at 37 °C. The last tube (control, untransformed) was not warmed at all. The cells were pelleted at 0–4 °C for 5 min and washed twice in 10 mL of cold Tris–saline (10 mM Tris-HCl, pH 7.4 at 25 °C, and 0.148 M NaCl). Each cell pellet was suspended in 3 volumes of TETg buffer and swelled for 30 min at 0–4 °C. Cytoplasmic extracts were then prepared as described previously (Vedeckis, 1983b).

Sucrose Gradient Ultracentrifugation. Swinging-bucket and vertical tube rotor sucrose gradient ultracentrifugations were carried out as described previously (Eastman-Reks et al., 1984). Briefly, swinging-bucket rotor sucrose gradients (5 mL) were centrifuged at 45 000 rpm ($190000g_{av}$), at 2 °C, for 16 h. Most vertical tube rotor sucrose gradients (5.2 mL) were spun at 80 000 rpm ($463000g_{av}$) to a preset cumulative centrifugal effect (ω^2t) of 2.40×10^{11} rad^2/s. Subsequent studies showed that using a preset ω^2t of 3.0×10^{11} rad^2/s gives a somewhat better separation between the 3.8S and 5.2S forms. This setting was used in some of these studies as indicated and is now used exclusively in our laboratory. The total length of the run for the vertical tube rotor (including acceleration and deceleration times) was 65 min with a final ω^2t of 2.44×10^{11} rad^2/s or 79 min with a final ω^2t of 3.04×10^{11} rad^2/s. Vertical tube rotor centrifugation was performed with the brake and slow acceleration on. Eleven-drop (swinging-bucket rotor) or 12-drop (vertical tube rotor) fractions were collected from the bottom of the tubes by gravity flow. The protein standards used were as described in Eastman-Reks et al. (1984).

Labeling of Receptor and Removal of Unbound Hormone. Receptor was labeled by incubating cytosol overnight with 2.4×10^{-8} M [^{3}H]Dex. In some instances, the method of transformation used [Sephadex G-25 or Sephadex LH-20 chromatography, $(NH_4)_2SO_4$ precipitation, dialysis] resulted in the removal of free hormone. In other experiments, excess unlabeled hormone was removed by dextran–charcoal adsorption. This was carried out essentially as described previously (Eastman-Reks et al., 1984), with the exception that the volume of charcoal suspension which was prepelleted was reduced to half the volume of cytosol to be treated.

During the course of these experiments, we noted that the transformed GC-R was unstable to dextran–charcoal treatment, resulting in preferential loss of hormone-binding activity from this species. Thus, for those experiments in which quantitation of all receptor forms was critical, an LH-20 slurry adsorption procedure was developed. This was based upon the fact that LH-20 will adsorb the hydrophobic unbound Dex, but not adsorb the hormone bound to receptor. In addition, this procedure does not promote receptor transformation, as does LH-20 column chromatography (Eastman-Reks et al., 1984). Four volumes (typically 1.6 mL) of a 50% LH-20 slurry were centrifuged for 5 min in an Eppendorf microfuge. The supernatant liquid was removed and 0.4 mL of cytosol added to the LH-20 pellet. After suspension of the LH-20 resin and a 5-min adsorption period, the sample was centrifuged as before; 0.2 mL of the LH-20-treated cytosol was then applied to the sucrose gradients. It should be noted that this method is not ideal, since the removal of unbound hormone is not as complete as with dextran–charcoal. The use of larger amounts of LH-20 slurry resulted in a more dilute receptor preparation, as well as exceeding the volume which can be placed in currently available microfuge tubes. Nevertheless, this technique was invaluable for the success of the experiments described below.

DEAE-cellulose, DNA–Cellulose, and Phosphocellulose Chromatography. Cytosol or pooled sucrose gradient fractions were applied to 4-mL (packed volume) columns of DEAE-cellulose. After the columns were washed with TETg buffer to a drop-through volume of 25 mL, the transformed receptor was step eluted with 10 mL of TETg–0.12 M KCl, while the untransformed receptor was subsequently eluted with 10 mL

of TETg–0.3 M KCl or TETg–0.5 M KCl. Previous studies (Vedeckis, 1981, 1983a,b) had shown that the transformed receptor elutes at 0.08 M KCl on this resin, while the untransformed species elutes at about 0.20 M KCl. DNA–cellulose chromatography was carried out in exactly the same manner through the drop-through step. Adsorbed (transformed) GC-R was then step eluted with 10 mL of TETg–0.5 M KCl. Phosphocellulose chromatography was carried out as for the DNA–cellulose columns except that the TETg wash volume was 20 mL and receptor was step eluted with TETg–0.3 M KCl.

Liquid Scintillation Counting. Four milliliters of Beckman Ready-Solv EP was added to each sample. Counting was performed at an efficiency of about 35% in a Beckman LS 7500 liquid scintillation spectrometer.

RESULTS

Sedimentation Coefficients of Mouse Glucocorticoid Receptor Forms. Previous studies (Vedeckis, 1983b) identified three differently sedimenting species of the mouse GC-R. These studies were performed by using swinging-bucket rotor sucrose gradients, and the sedimentation position of the protein standards was determined by using their absorbance at 280 nm. Subsequent studies using vertical tube sucrose gradients (Eastman-Reks et al., 1984) have shown that the long times associated with conventional swinging-bucket rotor centrifugation may lead to artifactual results due to subunit or hormone dissociation during the run. Therefore, we reevaluated the sedimentation coefficients of the untransformed and transformed mouse GC-R by using vertical tube rotor sucrose gradients and ^{14}C-methylated protein standards (Eastman-Reks et al., 1984).

In all cases (data not shown), a slight increase in the values for the sedimentation coefficients of the receptor forms was obtained by using the vertical tube rotor ($\omega^2 t$ = 3.00 × 10^{11} rad^2/s; total run time = 79 min). Thus, the untransformed, molybdate-stabilized receptor had an s value of 9.1 S (±0.1 S; n = 3) instead of 9.0 S. The intermediate, transformed species was 5.2 S (±0.1 S; n = 17) instead of 5.0 S in molybdate-containing gradients, and 6.6 S (±0.04 S; n = 20) in low-salt, molybdate-free gradients. The sedimentation coefficient of the monomeric GC-R increased to 3.8 S (±0.1S; n = 17), instead of being 3.2 S. We presume that these increases are due to the rapidity of analysis afforded by the vertical tube rotor. That is, a constant dissociation of the hormone from the GC-R during the 16-h swinging-bucket rotor centrifugation could result in a shift of the radioactive peak toward a lower sedimentation coefficient. Therefore, we will subsequently use our revised s values (9.1, 5.2, and 3.8 S) in discussing the various GC-R forms. It should be noted that for the monomeric receptor subunit (3.8 S; R_s = 6 nm) this will result in a higher calculated molecular weight (M_r ~96 000), than that obtained previously by using a value of 3.2 S (M_r 81 000). This higher calculated molecular weight is similar to those obtained by others (Eisen et al., 1981; Nordeen et al., 1981; Gehring & Hotz, 1983; Housley & Pratt, 1983; Simons et al., 1983) for covalent affinity-labeled rat and mouse GC-R run on denaturing (SDS) polyacrylamide gels (M_r ≃90 000).

Receptor Obtained after Various Modes of Transformation. It has been found that a wide variety of experimental manipulations can promote receptor transformation [reviewed by Grody et al. (1982) and Schmidt & Litwack (1982)]. We had previously shown (Eastman-Reks et al., 1984) that thermal transformation (25 °C, 1 h) resulted primarily in the formation of the 5.2S transformed receptor. In an attempt to determine whether the 5.2S form or the 3.8S form was the predominant

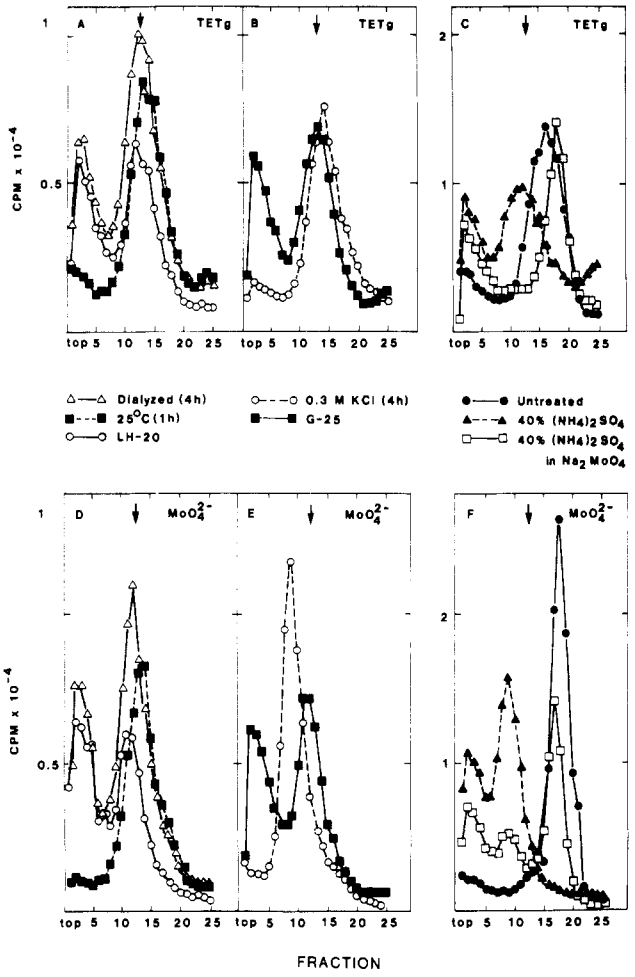

FIGURE 1: Vertical tube rotor sucrose gradients of the AtT-20 cell glucocorticoid receptor transformed by various methods. Cytosol labeled with [^{3}H]dexamethasone was subjected to various in vitro treatments to promote receptor transformation. The procedures employed included the following: dialysis (4 h), warming at 25 °C (1 h), and LH-20 chromatography (panels A and D); treatment with 0.3 M KCl (4 h) and G-25 chromatography (panels B and E); precipitation at 40% saturated $(NH_4)_2SO_4$ (panels C and F). Controls included untreated cytosol and receptor precipitated at 40% saturated $(NH_4)_2SO_4$ in the presence of 20 mM Na_2MoO_4 (panels C and F). Sucrose gradients were run in either TETg buffer (panels A–C) or TETg–20 mM Na_2MoO_4 (panels D–F). The key for each curve is presented between the TETg and MoO_4^{2-} gradient panels. The vertical arrows designate the sedimentation position of iron-free human transferrin (4.9 S).

in vitro transformed GC-R species obtained from mouse AtT-20 cytosol, we utilized a number of alternative types of in vitro transformation protocols. The transformed GC-R and suitable controls were then analyzed on either low-salt (TETg) or MoO_4^{2-}-containing sucrose gradients on the VTi 80 vertical tube rotor. These results are shown in Figure 1.

On TETg sucrose gradients, the transformed receptor obtained in each case had a sedimentation coefficient of about 6.6 S. The in vitro transformation protocols used included warming (25 °C, 1 h), dialysis vs. TETg buffer (4 h), precipitation at 40% saturated ammonium sulfate, incubation at 0.3 M KCl (1–4 h), and chromatography on Sephadex G-25 or Sephadex LH-20 columns (followed by a 4-h incubation at 0–4 °C). Controls included untreated cytosol and receptor precipitated with ammonium sulfate in the presence of 20 mM Na_2MoO_4. These controls ran, as expected, as 8–10S peaks (Figure 1C).

Identical samples were also run on MoO_4^{2-}-containing sucrose gradients (Figure 1D–F). Most of the untransformed

Table I: Kinetics of Mouse Glucocorticoid Receptor Transformation[a]

transformation method	$t_{1/2}$ of 9.1S (min)	k ($\times 10^{-3}$ min^{-1})
thermal (25 °C)	28	25
G-25 filtration	146	4.7
dialysis	172	4.0

[a] Mouse AtT-20 cell GC-R was transformed by the indicated protocol, as described under Materials and Methods and Results. Unbound hormone was removed from the thermally transformed and dialyzed cytosol samples by dextran–charcoal adsorption, while the LH-20 slurry method was used for the G-25-transformed sample. After vertical tube rotor sucrose gradient centrifugation, the decrease of the untransformed (9.1S) GC-R was determined by linear regression analysis of the semilogarithmic plot of the percent of 9.1S receptor vs. time. The points used for these calculations were those that demonstrated the best linearity at early times [thermal (25 °C) = 0–30 min; G-25 = 15–180 min; dialysis = 15–180 min). Half-lives of the 9.1S receptor were determined from the plots, and the rate constant was determined from the formula $k = 0.693/t_{1/2}$.

GC-R samples migrated as a sharp peak in the 5.2S region of the gradient. In addition, a sharp 9.1S receptor peak was obtained for the untransformed receptor in both untreated cytosol and the sample precipitated with $(NH_4)_2SO_4$ in 20 mM Na_2MoO_4 (Figure 1F). However, in contrast with the results obtained on the TETg gradients, when KCl-treated or $(NH_4)_2SO_4$-precipitated receptors were analyzed on MoO_4^{2-}-containing gradients, the 3.8S, transformed species was obtained. We have previously seen the 3.8S receptor after centrifuging cytosol on sucrose gradients containing 0.3 M KCl and 20 mM Na_2MoO_4 (Eastman-Reks et al., 1984) and after DEAE-cellulose isolation of the transformed receptor (see below). Perhaps these treatments disrupt ionic interactions between the subunits and a factor necessary for the stabilization of the 5.2S species or disrupt an interaction between the subunit themselves. In most subsequent experiments we have utilized sucrose gradients containing 20 mM Na_2MoO_4, because this agent acts as a stopping reagent for the transformation reaction, preserves hormone-binding activity, and yields sharper and more distinct receptor peaks when mixtures of forms are present.

Kinetics of Receptor Transformation. Having shown that the predominant in vitro transformed AtT-20 cell GC-R is 5.2 S, we wished to evaluate the kinetics of the transformation reaction. In particular, we wished to determine if a precursor–product relationship existed between the 9.1S and 5.2S species. Initial experiments involved the use of elevated temperature (25 °C) and dialysis to study the kinetics of GC-R transformation (data not shown). The amount of 9.1S receptor decreased, while the 5.2S form concomitantly increased, with increasing times of transformation. This seemed to indicate that the 9.1S GC-R was converted to the 5.2S species during receptor transformation. In addition, the rate of thermal receptor transformation was much more rapid than dialysis-induced transformation (Table I). In these experiments the amounts of 5.2S and 9.1S GC-R present at the various times of transformation were expressed as the percent of total receptor recovered (that is, bound hormone). A closer inspection of the gradients, however, revealed a decrease in total recovered receptor at the later time points (2–4 h). This made interpretation of the kinetic data more difficult, since it was not known if the apparent 9.1S to 5.2S conversion was due merely to the preferential loss of hormone binding (inactivation) of the 9.1S species. This is particularly likely for the dialysis study, since the dissociation rate of hormone from the untransformed GC-R is significantly faster than that from the transformed species (McBain et al., 1981). Therefore, we developed a method to study in vitro GC-R transformation which minimized the loss of bound hormone. In addition, we

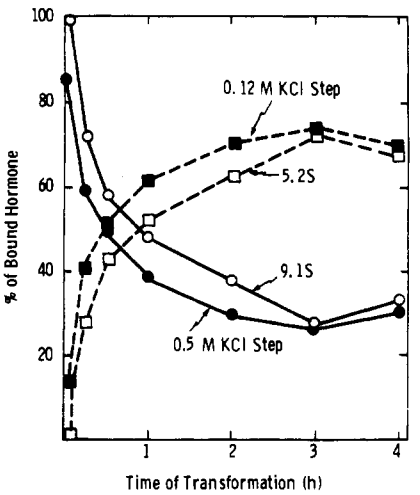

FIGURE 2: Correlation between the time course of glucocorticoid receptor transformation and the sedimentation coefficient of the protein. Aliquots of the samples transformed by G-25 chromatography were chromatographed on DEAE-cellulose columns as described under Materials and Methods. The transformed receptor was step eluted with TETg–0.12 M KCl, while the untransformed protein was subsequently step eluted with TETg–0.5 M KCl. The transformed, 0.12 M KCl step (■) and untransformed, 0.5 M KCl step (●) are expressed as the percent of the total adsorbed receptor (0.12 plus 0.5 M KCl steps). The radioactivity appearing in the 5.2S (□) and 9.1S (○) peaks were summated and expressed as the percent of total receptor-bound hormone (5.2S plus 9.1S).

wished to compare the apparent 9.1S and 5.2S conversion with another method for estimating receptor transformation.

Mouse AtT-20 cell GC-R was transformed by chromatography over a Sephadex G-25 column, which is believed to remove an endogenous, low molecular weight inhibitor of transformation [reviewed by Litwack (1979), Sekula et al. (1981), and Schmidt & Litwack (1982)]. The position of the receptor-bound hormone was determined by liquid scintillation counting of aliquots of the fractions. To prevent the loss of bound hormone during the subsequent period of spontaneous transformation at 0–4 °C, we added 5×10^{-9} M [^{3}H]Dex to the peak receptor fractions. To stop the transformation reaction at the various times, 20 mM Na_2MoO_4 was added to each fraction. Finally, the excess free [^{3}H]Dex was removed by the LH-20 slurry procedure described under Materials and Methods, and the samples were analyzed on vertical tube rotor sucrose gradients. The 9.1S peak decreased, while the 5.2S peak concomitantly increased, with increasing time of transformation (Figure 2). The loss of total hormone-binding activity with increasing time of transformation was minimal (17% at 3 h). Thus, it appears that the 9.1S GC-R gives rise to the transformed 5.2S form.

Identical, companion samples of G-25-transformed receptor were analyzed simultaneously via DEAE-cellulose chromatography. Numerous studies have shown that the first peak eluting from the DEAE-cellulose column (at 0.06–0.11 M salt) is the transformed species, while the untransformed receptor elutes at a higher salt concentration (0.20–0.22 M) [reviewed in Vedeckis (1985)]. In our studies, we step eluted the transformed mouse AtT-20 cell GC-R with 0.12 M KCl, while the untransformed species was subsequently eluted with 0.5 M KCl. We then expressed the amounts of transformed and untransformed receptor as the percentage of the total recovered receptor. These results are shown in Figure 2. An excellent correspondence can be seen between the appearance of the 5.2S species and the transformed (0.12 M KCl step) receptor, as well as the disappearance of the 9.1S peak and the untransformed (0.5 M KCl step) moiety, with increasing times of

Table II: Transformation State of the 9.1S, 5.2S, and 3.8S Forms of the Mouse Glucocorticoid Receptor[a]

	% of total receptor		
	DNA–cellulose	DEAE-cellulose	
receptor form	transformed (adsorbed)	transformed (0.12 M KCl step)	untransformed (0.5 M step)
9.1S	7	11	89
5.2S	80	77	23
3.8S	74	78	22

[a] The 9.1S, 5.2S, and 3.8S forms were isolated directly from sucrose gradients as described under Results. Identical aliquots of each were analyzed on both DEAE-cellulose and DNA–cellulose columns.

transformation. This strongly supports our contention that the 9.1S to 5.2S conversion represents the physical changes occurring upon AtT-20 cell GC-R transformation.

The rates of transformation and half-lives of the untransformed GC-R were determined by using a semilogarithmic plot. The values obtained are presented in Table I. As can be seen, the most rapid method of in vitro transformation was obtained with warming of the cytosol followed by Sephadex G-25 gel filtration and dialysis. At longer times of transformation linearity was lost, and the rate of 9.1S receptor disappearance decreased (data not shown). This could represent the attainment of an equilibrium between the formation of the transformed receptor and the reversal of the transformation reaction.

Confirmation of the Transformation State of the Sucrose Gradient Receptor Forms. Although the excellent correlation between the appearance of the 5.2S GC-R and that of the transformed receptor (0.12 M KCl step) strongly suggested their identity, a direct analysis of the receptor form obtained by one method using the other would be ideal. Previous attempts to perform these studies were hampered by the length of time required to perform the sucrose gradient analysis with swinging-bucket rotors (16–18 h). However, the use of the VTi 80 rotor allowed this study to be accomplished.

The 9.1S form was obtained by centrifuging MoO_4^{2-}-treated cytosol into molybdate-containing sucrose gradients using the vertical tube rotor ($\omega^2 t = 3.00 \times 10^{11}$ rad^2/s; run time = 79 min). The 5.2S form was generated by warming cytosol at 25 °C for 1 h followed by sedimentation on molybdate-containing sucrose gradients, while the 3.8S receptor was obtained from 0.3 M KCl-treated cytosol (3 h; 0–4 °C) centrifuged on high-salt sucrose gradients. Each set of gradients was fractionated at 0–4 °C. Fifty-microliter aliquots of each fraction were subjected to liquid scintillation counting, and the peak fractions were used for DEAE-cellulose and DNA–cellulose chromatography. For the 3.8S GC-R, the KCl was first removed by passage of the pooled 3.8S peak fractions over a Sephadex G-25 column equilibrated in TETg. Table II summarizes the results of these experiments.

As can be seen, the 9.1S form of the receptor did not adsorb to DNA–cellulose, and it eluted predominantly (89%) with the 0.5 M KCl step from DEAE-cellulose. Both of these are characteristics of the untransformed GC-R. In contrast to this, both the 5.2S and 3.8S GC-R species bound well to DNA–cellulose and eluted predominantly in the 0.12 M KCl step from DEAE-cellulose. These are properties of a transformed receptor species. Therefore, these experiments show directly that the 9.1S GC-R is untransformed and that both the 5.2S and 3.8S species are transformed (DNA-binding) receptors.

The converse experiment was also performed. Transformed GC-R and untransformed GC-R were collected from the DEAE-cellulose column by step eluting with 0.12 and 0.3 M KCl, respectively. Molybdate was added to inhibit any further transformation, and the samples were analyzed by using vertical tube rotor sucrose gradients. The untransformed receptor (0.3 M KCl step) eluted almost exclusively as the 9.1S form (Figure 3). However, the transformed receptor, obtained as the 0.12 M KCl step from DEAE-cellulose, sedimented as the 3.8S form. This is reminiscent of the results presented earlier (Figure 1E,F) on the sedimentation of the KCl- and $(NH_4)_2SO_4$-transformed receptor on MoO_4^{2-}-containing gradients, when the 3.8S species was also obtained. It is possible that the salt elution and/or DEAE-cellulose chromatography in some way alters the structure of the transformed GC-R, causing its conversion from 5.2S to 3.8S (see Discussion). Finally, receptor transformed by warming (25 °C, 1 h) was applied to a phosphocellulose column and subsequently step eluted with TETg–0.3 M KCl. This was then diluted with an equal volume of TETg and subjected to sucrose gradient centrifugation. This sample sedimented at about 5.2 S (Figure 4), further supporting the concept that the 5.2S form is a transformed species.

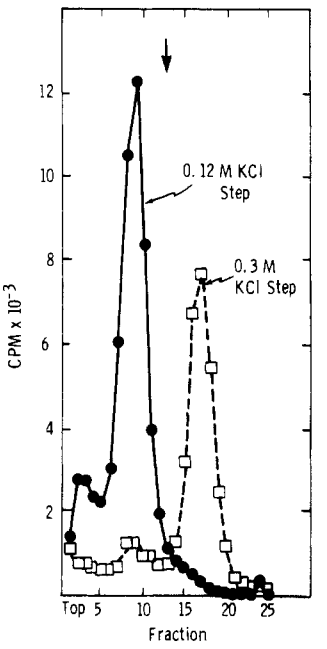

FIGURE 3: Sedimentation properties of the transformed and untransformed glucocorticoid receptors obtained after DEAE-cellulose chromatography. Cytosol (5 mL) was chromatographed over a 40-mL (packed volume) G-25 column and allowed to transform for 2 h at 0–4 °C. Three milliliters of the pooled, receptor-containing fractions were applied to a 4-mL (packed volume) DEAE-cellulose column. After the column was washed with 25 mL of TETg buffer, the transformed receptor was step eluted with 0.12 M KCl and collected in 0.5-mL fractions. The fractions containing receptor-bound hormone were determined by counting aliquots (20 μL) of each fraction. The highest receptor-containing peak fraction was made 20 mM in Na_2MoO_4. The untransformed receptor was then step eluted from the same column by using 0.3 M KCl, localized by counting aliquots, and the peak fraction made 20 mM in Na_2MoO_4. Aliquots (0.2 mL) of the transformed (●) and untransformed (□) receptors were then analyzed on 5–20% MoO_4^{2-}-containing low-salt sucrose gradients by using the vertical tube rotor. The vertical arrow denotes the sedimentation position of iron-free human transferrin (4.9 S).

Interconvertibility of the 3.8S and 5.2S Forms. It has been suggested previously (Vedeckis, 1983b) that the 5.2S mouse GC-R is a complex of the 3.8S monomer with either an additional monomeric subunit or with a non-hormone-binding, receptor binding factor. Thus, we wished to determine if the 5.2S receptor could be reconstituted from the 3.8S subunits. The 3.8S receptor was prepared by centrifuging cytosol pretreated with salt (0.3 M KCl; 3 h) on vertical tube rotor,

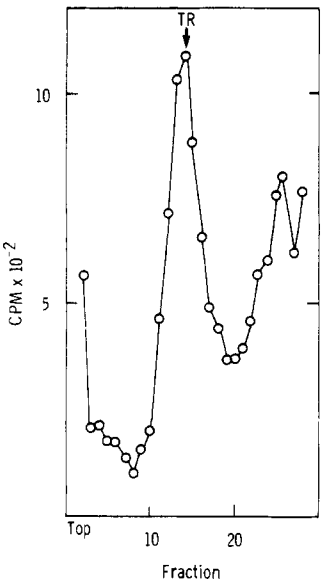

FIGURE 4: Sucrose gradient ultracentrifugation of receptor eluted from phosphocellulose. AtT-20 cell cytosol (3.4 mL) was transformed by warming at 25 °C for 1 h. The sample was applied to a 4-mL phosphocellulose column, followed by washing with 20 mL of TETg buffer. Absorbed (transformed) receptor was step eluted with TETg–0.3 M KCl and collected in 0.5-mL fractions. One hundred and fifty microliters of the peak was diluted with an equal volume of TETg buffer, and 200 μL of this was centrifuged on 5–20% sucrose gradients for 16 h by using an SW 50.1 swinging-bucket rotor. The vertical arrow denotes the sedimentation position of iron-free human transferrin (4.9 S).

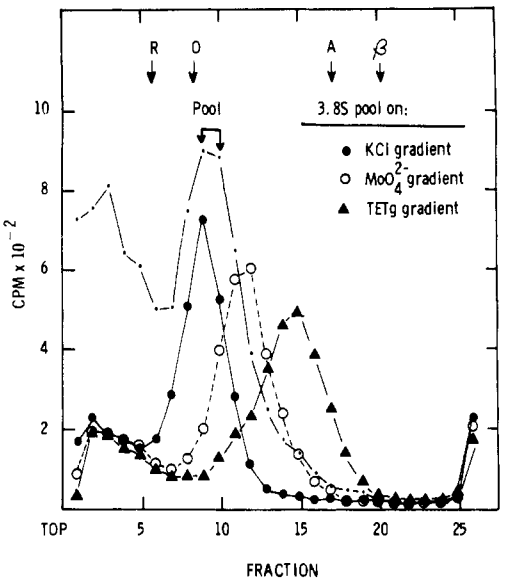

FIGURE 5: Interconversion of the 3.8S monomeric, transformed glucocorticoid receptor and the 5.2S and 6.6S oligomeric receptor species. KCl-treated (0.3 M; 3 h) AtT-20 cell cytosol was sedimented on six separate vertical tube rotor sucrose gradients containing 0.3 M KCl (---). These were collected into a single set of vials (at 0–4 °C) and the two peak fractions (3.8S) pooled ("Pool"). The pool was desalted on a Sephadex G-25 column, and the peak receptor-containing excluded volume fractions were identified. Two hundred microliters of this desalted monomeric receptor were then centrifuged on 5–20% sucrose gradients (vertical tube rotor) made up in TETg alone (▲), TETg containing 0.3 M KCl (●), or 20 mM sodium molybdate (○). Standard proteins run on parallel gradients were ribonuclease A (R; 1.9 S), ovalbumin (O; 3.5 S), aldolase (A; 7.9 S), and β-amylase (β; 9.4 S).

high-salt sucrose gradients. Six separate gradients were fractionated into a single set of vials and aliquots counted to determine the sedimentation position of the 3.8S receptor. The 3.8S peak fractions were then filtered over Sephadex G-25 equilibrated in TETg buffer alone, TETg–0.3 M KCl, or TETg–20 mM Na_2MoO_4. Each of the gel-filtered samples was then centrifuged on TETg, TETg–0.3 M KCl, and TETg–20 mM Na_2MoO_4 vertical tube rotor sucrose gradients. Since the results obtained were virtually identical regardless of the buffer used to equilibrate the Sephadex G-25 column, only data from the samples gel filtered in TETg buffer are shown (Figure 5).

When the gel-filtered 3.8S GC-R was sedimented in 0.3 M KCl containing sucrose gradients, it continued to sediment at about 3.8 S. When similar samples were centrifuged on MoO_4^{2-}-containing sucrose gradients, an increase in sedimentation rate was observed to about 4.9 S, while sedimentation into TETg gradients yielded a 6.6S form. These forms are quite similar to the 5.2S and 6.6S species observed when transformed receptor is sedimented directly on molybdate-containing and low-salt gradients, respectively. Thus, the 5.2S intermediate, transformed receptor can be reconstituted from 3.8S monomers isolated directly from sucrose gradients by salt removal. The possible composition of the 5.2S intermediate species is dealt with under Discussion.

Alkaline Phosphatase Promotion of the 9.1S to 5.2S Conversion. Dephosphorylation of steroid receptor proteins has been implicated as a possible necessary step for receptor transformation [reviewed in Grody et al. (1982) and Schmidt & Litwack (1982)]. This is based upon the inhibition of receptor transformation by a variety of phosphatase inhibitors (John & Moudgil, 1979; Leach et al., 1979; Nishigori & Toft, 1980; Schmidt et al., 1980; Barnett et al., 1980; Shyamala & Leonard, 1980; Wolfson et al., 1980; Vedeckis, 1981, 1983a,b) and the increase in the amount of material that elutes early from DEAE-cellulose after addition of alkaline phosphatase

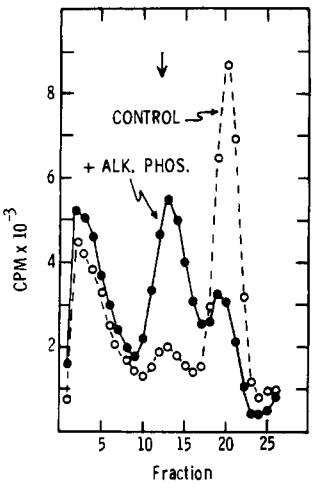

FIGURE 6: Promotion of glucocorticoid receptor transformation by calf intestinal alkaline phosphatase. Alkaline phosphatase (77 μg/mL) was added to an aliquot of AtT-20 cell cytosol, while the control sample received an equivalent volume of distilled water. Both samples were incubated for 1 h at 10 °C. The unbound hormone was removed by using the LH-20 slurry adsorption procedure, and 200 μL of each sample was centrifuged on MoO_4^{2-}-containing, vertical tube rotor sucrose gradients. Control (○); alkaline phosphatase treated (●). The vertical arrow shows the sedimentation position of iron-free human transferrin (4.9 S).

to cytosol (Barnett et al., 1980). We have undertaken a detailed study on the possible role of dephosphorylation in receptor transformation (C. E. Reker and W. V. Vedeckis, unpublished results). A typical result obtained in these studies is shown in Figure 6.

AtT-20 cell cytosol was incubated for 1 h at 10 °C with or without 77 μg/mL calf intestinal alkaline phosphatase; 20 mM Na_2MoO_4 was added, and the samples were centrifuged on

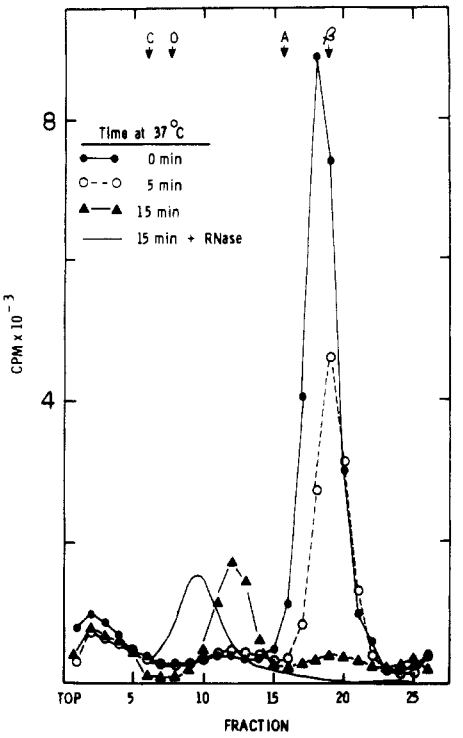

FIGURE 7: Sedimentation properties of the glucocorticoid receptor transformed in vivo. AtT-20 cells were collected, incubated at 0 °C for 1 h with [^{3}H]dexamethasone, and then warmed at 37 °C for 5 (○) or 15 min (▲) as described under Materials and Methods. An additional sample of cells (●) was not warmed at all and served as a control. The cells were washed in cold Tris–saline, and cytosol was prepared in TETg buffer as described under Materials and Methods. Aliquots (0.2 mL) were then centrifuged on low-salt, 5–20% sucrose gradients containing 20 mM Na_2MoO_4 on the vertical tube rotor. An aliquot of the transformed receptor obtained from the cytosol of cells incubated at 37 °C for 15 min was stored overnight at −20 °C. After being thawed, 90 units/mL bovine pancreatic ribonuclease A was added for 1 h at 0 °C. This RNase-treated sample was then centrifuged on a molybdate-containing sucrose gradient (—). Standard proteins used are the same as indicated in the legend to Figure 5, except for cytochrome *c* (C; 1.7 S).

MoO_4^{2-}-containing vertical tube rotor sucrose gradients. As is clearly seen, alkaline phosphatase promoted the conversion of the 9.1S GC-R to the 5.2S form. Thus, dephosphorylation of some substance in cytosol (perhaps the GC-R itself) appears to be required for receptor transformation, and again, the predominant transformed species obtained has a sedimentation coefficient of 5.2 S.

Structure of the Receptor Transformed in Vivo. A primary goal of the current study was to elucidate the identity of the physiologically relevant, transformed mouse GC-R. The studies presented above suggest that the 5.2S species is the predominant in vitro form. However, it would be advantageous to analyze the transformed receptor under more physiological circumstances. Thus, intact cells were incubated with 5.4×10^{-8} M [^{3}H]TA for 1 h at 0 °C. In vivo receptor transformation was then initiated by incubating the cells for 5 or 15 min at 37 °C and the cytosolic receptor prepared as described under Materials and Methods. Cells that had not been incubated at 37 °C served as a source of untransformed cytosolic receptor. These three samples were then centrifuged on molybdate-containing sucrose gradients (Figure 7).

The GC-R labeled in intact cells at 0 °C alone sedimented as untransformed receptor, that is, with a sedimentation coefficient of 9.1 S. With increasing time of incubation at 37 °C, the 5.2S intermediate receptor form appeared, and no 3.8S monomeric species was evident. In vivo receptor transformation was virtually complete after incubating cells at 37 °C for 15 min. The overall level of cytoplasmic receptor decreased during the 15-min incubation time, and this may be due to an increase in the amount that was bound to the nuclear pellet. Indeed, it has been suggested that, in the intact cell, steroid receptors either partition freely between the nuclear and cytoplasmic compartments or are primarily nuclear localized (Sheridan et al., 1979; Walters et al., 1980; King & Green, 1984; Welshons et al., 1984). In any event, under the most physiological conditions currently available, it appears that the intermediate, transformed GC-R (5.2S) is the species generated after in vivo transformation.

Also shown in Figure 7 is the result of treating in vivo transformed GC-R with bovine pancreatic ribonuclease A. Cytosol receptor that was obtained from cells incubated at 37 °C for 15 min was stored overnight at −20 °C. This sample was then slowly thawed at room temperature and incubated with 90 units/mL of RNase A. Vertical tube rotor sucrose gradient centrifugation on molybdate-containing sucrose gradients revealed that the 5.2S GC-R was converted to the monomeric, 3.8S species. This suggests that the 5.2S GC-R is comprised of a monomeric receptor subunit and a low molecular weight RNA molecule.

Discussion

We have previously provided evidence that mouse glucocorticoid receptor transformation involves subunit dissociation (Vedeckis, 1983b; Eastman-Reks et al., 1984). This is consistent with data showing that rat GC-R transformation is a first-order reaction (Atger & Milgrom, 1976). Independently, Raaka & Samuels (1983) and Holbrook et al. (1983) have reached the same conclusion. Also, an additional series of experiments favors subunit dissociation as the mechanism of rat GC-R transformation, although the latter process was not assessed directly (Sherman et al., 1983). Since we had identified two transformed mouse GC-R species, 5.2S and 3.8S, we wished to determine the relationship between these two transformed receptors.

The experiments presented above suggest that mouse GC-R transformation involves the dissociation of an oligomeric, untransformed, 9.1S species into transformed 5.2S subunits. The evidence for this includes the ubiquity of 5.2S receptor generation using different modes of in vitro transformation (Figure 1), the kinetic correlation between 5.2S and transformed (0.12 M KCl eluting DEAE-cellulose material) receptor appearance (Figure 2), and the identification (by reciprocal sedimentation and DEAE-cellulose and DNA–cellulose elutions) of the 3.8S and 5.2S forms as transformed species and the 9.1S receptor as untransformed (Figures 3 and 4). Significantly, the sole transformed species obtained when a more physiological, in vivo (whole cell) transformation was performed was the 5.2S GC-R (Figure 7).

A detailed understanding of the molecular mechanism of GC-R transformation, and the identity of the true gene regulatory form of this protein, is not yet available. Raaka & Samuels (1983) postulated that a homotetramer (8–10S) dissociates into monomeric (4S) subunits upon transformation. It should be noted that this was based, to some extent, on their obtaining a 3.5S form of the receptor on high-salt (0.4 M KCl) sucrose gradients. On low-salt gradients, however, they noted a 4–5S receptor. Upon resedimentation they showed that these two forms were either identical with each other or derived from each other. We suggest that their 4–5S form is identical with our 5.2S species which is, indeed, interconvertible by salt treatment with the authentic 3.5S (our 3.8S) monomeric form (Figure 5).

The composition of the 5.2S transformed complex has not yet been unequivocally elucidated. It has been suggested (Raaka & Samuels, 1983; Vedeckis, 1983b) that the untransformed 9–10S GC-R complex is a homotetramer of identical M_r ~90 000 subunits and that the 5.2S form of the receptor is an intermediate, homodimer of 3.8S subunits (Vedeckis, 1983b). Although not definitive, recent electron microscopic evaluation of the binding of purified, transformed GC-R to a hormone-regulated gene fragment (the long terminal repeat of mouse mammary tumor virus) also supports the concept of a dimeric (Govindan et al., 1982) or larger, oligomeric (Payvar et al., 1983) receptor. Our initial suggestion for a dimeric receptor (Vedeckis, 1983b) was based upon molecular weight estimates of the AtT-20 cell GC-R analyzed in buffers containing both 0.3 M KCl and 20 mM Na_2MoO_4. When these samples were analyzed *in parallel* on sucrose gradients and a gel filtration column, a calculated molecular weight of about 176 000 was obtained (5 S; 8.3 nm). We have recently begun an in-depth study on the components of the 5.2S receptor species. In particular, we wished to determine the Stokes radius of the 5.2S form by performing gel filtration on this form isolated directly from sucrose gradients. Surprisingly, *sequential* analyses of the 5.2S GC-R either on conventional agarose A-1.5m chromatography (C. E. Reker and W. V. Vedeckis, unpublished results) or on high-performance gel permeation liquid chromatography (M. C. LaPointe, C. E. Reker, and W. V. Vedeckis, unpublished results) have yielded a Stokes radius of 5.7–6 nm (as opposed to 8.3 nm). One possibility for these discrepancies as compared to results of our earlier study may reflect an instability of the 5.2S species. That is, perhaps the 5.2S form does have a Stokes radius of 8.3 nm (is a dimer of 3.8S subunits) but that, even when rapid (20 min) high-performance liquid chromatography is used, it is very unstable after isolation from sucrose gradients and dissociates into 3.8S, 5.7–6-nm monomers.

Alternatively, the 5.2S form may actually have a Stokes radius of 5.7–6 nm. In previous studies (Vedeckis, 1983b) the 5.2S form was obtained by centrifuging cytosol on swinging-bucket rotor sucrose gradients containing 0.3 M KCl and 20 mM Na_2MoO_4. However, perhaps dissociation to the 5.2S form did not occur when the same sample was run in parallel on the agarose A-1.5m column in the same buffer (that is, the receptor remained as the 8.3-nm, 9.1S complex). If the correct Stokes radius of the 5.2S GC-R is 5.7–6 nm, then the calculated molecular weight would be 125 000–132 000; this would more closely fit the alternative hypothesis (Vedeckis, 1983b) that the 5.2S complex consists of one 3.8S (M_r 96 000) monomeric, hormone-binding subunit and a non-hormone-binding, receptor binding factor (M_r 29 000–36 000). The existence of non-hormone-binding, receptor binding factors (RBF) has been suggested previously for the estrogen, progesterone, androgen, and glucocorticoid receptors (Murayama et al., 1980a,b; Colvard & Wilson, 1981; Barnett et al., 1983). Interestingly, an RBF with a molecular weight of 29 000–36 000 would have a sedimentation coefficient of about 3–5 S. As was shown here, the 3.8S GC-R, when isolated from a high-salt sucrose gradient, underwent an increase in sedimentation rate when centrifuged on MoO_4^{2-}-containing (4.9S) or TETg (6.6S) sucrose gradients. It is, thus, possible that an excess of the putative RBF cosedimented with the 3.8S monomer on the high-salt gradients and then reassociated with the monomer when the salt was removed. Preliminary experiments have shown that the 3.8S receptor isolated with a 0.12 M KCl step elution from DEAE-cellulose continues to sediment in that position when recentrifuged in MoO_4^{2-}-containing (Figure 6) or TETg gradients (C. E. Reker and W. V. Vedeckis, unpublished results). However, the addition of the 0.3 M KCl step elution from the same DEAE-cellulose column back to the 3.8S (0.12 M KCl step) GC-R causes a shift in sedimentation of the receptor to 5.2 S on MoO_4-containing gradients and 6.6 S on TETg gradients. In view of the fact that the 5.2S species is converted to the monomeric form by RNase treatment (Figure 7), it is possible that a low molecular weight (3–5S) RNA is separated from the 3.8S monomer when the 5.2S species is chromatographed on DEAE-cellulose. Thus, at present, it seems possible that the 5.2S intermediate, transformed receptor may be composed of a 3.8S monomer plus a low molecular weight RNA (M_r 29 000–36 000). A more detailed study supports a role for RNA in the structure of the 5.2S intermediate, transformed GC-R (B. Kovačič-Milivojević and W. V. Vedeckis, unpublished results).

In conclusion, we have shown that an intermediate, 5.2S transformed GC-R is generated by a wide variety of in vitro transformation protocols, as well as during in vivo transformation. The ubiquitous appearance of this species suggests that it may be a physiologically relevant form of this eukaryotic, gene regulatory protein.

Registry No. Dex, 50-02-2; TA, 76-25-5; ribonuclease A, 9001-99-4; alkaline phosphatase, 9001-78-9.

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Biodehalogenation: Reactions of Cytochrome P-450 with Polyhalomethanes†

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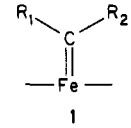
ABSTRACT: The products, stoichiometry, and kinetics of the oxidation of the enzyme cytochrome P-450 cam by five polyhalomethanes and chloronitromethane are described. The reactivity of the enzyme is compared with that of deuteroheme and with the enzyme in its native cell, *Pseudomonas putida* (PpG-786). In all cases, the reaction entails hydrogenolysis of the carbon–halogen bond: $2Fe^{II}P + RCX_n \rightarrow 2Fe^{III}P + RCHX_{n-1}$ (P = porphyrin or P-450 cam in vitro and in vivo). Trichloronitromethane was the fastest reacting substrate, and chloroform was the slowest. The results establish that *P. putida* is a valid whole cell model for the reductase activity of the P-450 complement in these reactions. The reactions of cytochrome P-450 with polyhaloalkanes proceed in a manner quite analogous to other iron(II) proteins in the G conformation. The chemistry observed for the enzyme parallels that of its iron(II) porphyrin active site. Iron-bonded carbenes are not intermediates, and hydrolytically stable iron alkyls are not products of these reactions.

The reactions of cytochrome P-450 with organic halides can be an important means of both activation and detoxification of these xenobiotics in vivo (U.S. Department of Health, Education and Welfare, 1977; Jakoby, 1980; Ullrich, 1977). It is generally held that unsaturated halides are reactivated by conversion to the corresponding epoxides (Henschler, 1977), though the epoxides are not necessary intermediates in the production of halocarbonyl compounds obtained in these processes (Miller & Guengerich, 1982, Liebler & Guengerich, 1983). The nature of the reaction of saturated aliphatic polyhalides with this class of enzyme (Uehleke et al., 1973; Bini et al., 1975; Mansuy et al., 1974; Nastainczyk et al., 1978; Wolf et al., 1977a,b; Nastainczyk et al., 1982; Ahr et al., 1982) is less clear. Of particular interest are the reactions of polyhalomethanes ($R_{4-n}CX_n$, n = 2–4) because the bonding in this class of substrate is typical of that found commonly in anesthetics, solvents, and pesticides. Moreover, visible spectral studies of such reactions (Mansuy et al., 1974; Nastainczyk et al., 1978; Wolf et al., 1977a,b) have suggested the generation of unique "carbene complexes" of the P-450 heme (**1**). Such



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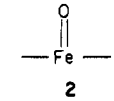

2

structures are analogous to the "iron–oxene" formulation (**2**) for the activated oxygen intermediate in P-450-catalyzed oxygen insertion reactions (Ullrich, 1977; Sligar et al., 1977; Griffin et al., 1975; Guengerich & MacDonald, 1984). The unusual bonding in structures like **1** and the "halothane carbene" in particular (**1**, $R_1 = CF_3$ and $R_2 = H$) has received serious attention by theoreticians (Loew & Goldblum, 1980). While the enzyme functions primarily as an oxidase, clearly it has reductase capacity vested in its iron(II) porphyrin form. The reduction of *N*-oxides, epoxides, hydroperoxides, and polyhalomethanes has been noted (Guengerich & Macdonald, 1984). Isolated cases of the reactivity of primarily reduced liver fragments to polyhalomethanes have been reported (Uehleke et al., 1973; Bini et al., 1975; Mansuy, et al., 1974; Nastainczyk et al., 1978, 1983; Wolf et al., 1977a,b; Ahr et al., 1982), but product work is sketchy and contradictory. Thus, the conversion of carbon tetrachloride and bromotri-

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