The Transformed Glucocorticoid Receptor Has a Lower Steroid-Binding Affinity Than the Nontransformed Receptor†

Takayuki Nemoto, Yuko Ohara-Nemoto, Marc Denis,‡ and Jan-Åke Gustafsson*

Department of Medical Nutrition and Center for BioTechnology, Karolinska Institute, Huddinge University Hospital F-60, Novum, S-141 86 Huddinge, Sweden

Received June 2, 1989; Revised Manuscript Received October 2, 1989

ABSTRACT: High-salt treatment of cytosolic glucocorticoid receptor (GR) preparations reduces the steroid-binding ability of the receptor and induces the conversion of the receptor from a nontransformed (non-DNA-binding) 9S form to a transformed (DNA-binding) 4S entity. Therefore, we decided to investigate the possible relationship between these two phenomena. Steroid-free GR was converted from a 9S to a 4S form by exposure to 0.4 M NaCl. The binding of [^{3}H]triamcinolone acetonide ([^{3}H]TA) to the 9S form was almost saturated at a concentration of 20 nM, whereas [^{3}H]TA was hardly bound to the 4S form at this concentration. The 4S form was efficiently labeled at 200 nM. Scatchard analysis of the GR exposed to 0.4 M NaCl in the presence of 10 mM molybdate showed the presence of two types of binding sites with apparent dissociation constants of 0.52 ± 0.07 and 64.1 ± 16.2 nM, respectively. In the absence of molybdate, the ratio of the lower affinity site was increased, but the total number of binding sites was not modified. The GR with the low [^{3}H]TA-binding affinity bound to DNA–cellulose even in its unliganded state, whereas the form with the high affinity did not. Immunoblot analysis using anti-GR monoclonal antibody revealed no difference in molecular size (M_r 94 000) between the high- and low-affinity entities. These results indicate that the transformed GR has a reduced [^{3}H]TA-binding affinity as compared to the nontransformed GR. The steroid-binding domain (amino acids 477–777) and the DNA- and steroid-binding domains (amino acids 415–777) of the human GR were expressed in *Escherichia coli* as protein A fused proteins. The apparent dissociation constant of these proteins (70 nM) was similar to that of the native transformed rat GR. Taken together, these results suggest that the component(s) associating with the nontransformed GR, possibly the heat shock protein hsp 90, play(s) an important role in stabilizing the GR in a high-affinity state for steroids.

Glucocorticoid hormones exert their effects through binding to a specific intracellular protein to form glucocorticoid–receptor complexes [see Schmidt and Litwack (1982) and Gustafsson et al. (1987) for a review], which are then converted to a DNA-binding or transformed state (Atger & Milgrom, 1976; Sherman et al., 1983). The nontransformed glucocorticoid receptor (GR)[1] appears to be an oligomer containing a receptor monomer and a dimer of hsp 90 (Denis et al., 1987), which dissociates from the GR during the transformation process[2] (Mendel et al., 1986). This phenomenon occurs in vivo (Munck & Foley, 1979) and can also be achieved in vitro, e.g., by increasing the temperature or the ionic strength (Schmidt & Litwack, 1982). The transformed glucocorticoid–receptor complexes bind to specific DNA regions (glucocorticoid responsive elements) upstream of or within the regulated genes and enhance or reduce their transcription rate (Chandler et al., 1983).

The loss of the ligand-binding ability of GR, called inactivation,[2] has been observed in intact cells (Munck & Brinck-Johnsen, 1968) and under cell-free conditions (Nielsen et al., 1977). Addition of sulfhydryl reducing reagents to the cytosol partially prevents this process (McBlain & Shyamala, 1980), probably through the protection of sulfhydryl groups essential for steroid binding. On the other hand, the inactivation occurring during exposure to salt, ATP, or elevated temperature is not prevented by reducing reagents (Nielsen et al., 1977; McBlain & Shyamala, 1980). Sodium molybdate, a potent inhibitor of transformation, partially prevents this inactivation. Interestingly, McBlain et al. (1981) noted the similarity of conditions for receptor inactivation and transformation.

In this study, assuming that the inactivation of GR is correlated with the transformation process, we investigated the effect of high salt on the transformation and the steroid-binding characteristics of steroid-free GR. Salt treatment of the nontransformed GR caused its conversion from the 9S to the 4S form even in the absence of hormone. The salt-induced transformed GR was characterized by glycerol gradient centrifugation, DNA–cellulose chromatography, Scatchard analysis, and protein immunoblot. Our results consistently indicated that the transformed GR had a lower steroid-binding affinity than the nontransformed GR. Furthermore, we expressed human GR in *Escherichia coli* and compared its steroid-binding characteristics with the GR in rat liver.

† Supported by grants from the Swedish Medical Research Council (13X-2819) and Karo Bio AB.

* Address correspondence to this author.

‡ Present address: Department of Medical Biochemistry, U.F.R. of Medicine, 1 Rue Gaston Veil, F-44035 Nantes Cedex, France.

[1] Abbreviations: GR, glucocorticoid receptor; hsp 90, M_r ~90 000 heat shock protein; GR415 and GR477, protein A fused to amino acids 415–777 and 477–777 of human GR, respectively; TA, triamcinolone acetonide, 9α-fluoro-11β,21-dihydroxy-16α,17α-[(1-methylethylidene)-bis(oxy)]pregna-1,4-diene-3,20-dione; dexamethasone, 9α-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione; DCC, dextran-coated charcoal; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

[2] In this study, we will use the word "transformation" to describe the conversion from the 9S form of GR that does not bind to DNA to the 4S, DNA-binding form and the word "inactivation" to describe the apparent loss of ligand-binding activity of GR.

0006-2960/90/0429-1880$02.50/0

Materials and Methods

Materials. [6,7-^{3}H]Triamcinolone acetonide (29 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Hydroxylapatite HTP, sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebis(acrylamide), ammonium persulfate, and reagents for immunoblot analysis were purchased from Bio-Rad (Richmond, CA). Sephacryl S300 and Sephadex G25 were purchased from Pharmacia (Uppsala, Sweden). Phosphocellulose P11 was from Whatman (Maidstone, England), DEAE-Toyopearl 650S was from Toso (Tokyo, Japan), and leupeptin was from Protein Research Foundation (Osaka, Japan). Restriction enzymes, T4 DNA polymerase, and ligase were from Boehringer Mannheim (Mannheim, West Germany). Calf thymus double-stranded DNA–cellulose and all other analytical grade reagents were obtained from Sigma (St. Louis, MO).

Buffers. The following buffers were prepared at 23 °C and stored at 4 °C prior to use. Buffer A: 20 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, pH 7.3. Buffer B: buffer A containing 10 mM sodium molybdate. Buffer C: 20 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.3.

Preparation of Liver Cytosol. Sprague-Dawley rats, 8–10 weeks of age, were adrenalectomized and kept for 3–5 days with 0.9% NaCl before sacrifice. The animals were killed by cervical dislocation. The livers were perfused in situ with ice-cold 0.9% NaCl and placed in ice-cold buffer B. All following experiments were performed at 0–4 °C unless stated otherwise. The tissues were finely minced and homogenized in 2 volumes of buffer B containing 0.5 mM leupeptin in a Teflon–glass homogenizer. The homogenate was centrifuged at 10000*g* for 10 min, and the supernatant was centrifuged at 150000*g* for 45 min. The resulting clear supernatant was passed through one-fourth volume of phosphocellulose equilibrated with buffer B, and the flow-through fraction was used for subsequent receptor purification.

Partial Purification of Steroid-Free GR. Phosphocellulose flow-through fraction (about 100 mL) was applied on a DEAE-Toyopearl column (2.5 × 10 cm) equilibrated with buffer B. The column was washed with buffer B containing 80 mM NaCl. After the absorbance at 280 nm had reached the base line, the column was connected to a Sephacryl S300 column (2.5 × 60 cm) equilibrated with buffer A. Proteins retained on the DEAE column were eluted with 0.2 M NaCl in buffer B and subsequently chromatographed on Sephacryl S300. Sixty-drop fractions were collected. A 20-fold purification of the unliganded receptor was achieved by using this technique with a 60–70% recovery.

Steroid-Binding Measurement. An aliquot (80 μL) of sample was incubated with 20 or 200 nM [^{3}H]TA in the presence and absence of a 200-fold molar excess of radioinert TA in a final volume of 100 μL. After 4–6 h, the sample was mixed with an equal volume of hydroxylapatite suspension [50% (v/v) in buffer B] for 10 min with occasional vortexing. The precipitate was washed 3 times with 1 mL of buffer B, and [^{3}H]TA–GR complexes were extracted with 0.5 mL of 0.4 M potassium phosphate buffer (pH 7.3) for 10 min and counted for radioactivity. The specific binding was determined by subtracting the binding in the presence of unlabeled TA (nonspecific binding) from the total binding. Nonspecific binding usually represented 2–5% at 20 nM [^{3}H]TA and 15–20% at 200 nM [^{3}H]TA of total binding.

Scatchard (1949) Analysis. The sample (80 μL) was incubated with 1–200 nM [^{3}H]TA in the presence or absence of a 200-fold molar excess of radioinert TA to a final volume of 100 μL. After 14–16 h, the incubation was mixed with an equal volume of hydroxylapatite suspension and centrifuged. An aliquot (50 μL) of the supernatant was counted to quantitate free [^{3}H]TA concentration. Then, [^{3}H]TA bindings were measured as described above. Apparent dissociation constants were calculated from the SCATCHARD PLOTTING program (Okubo Maikon Co., Tokyo, Japan), which has been developed for the analysis of two types of binding sites.

Glycerol Gradient Centrifugation. Glycerol gradients (12.5–40% v/v) were prepared in buffer B. Samples were treated with dextran-coated charcoal (DCC, 0.5% activated charcoal and 0.05% dextran T70 in buffer A) as described previously (Nemoto et al., 1987) and loaded onto the gradients. Gradients were centrifuged at 300000*g* for 15 h. Four-drop fractions were collected and counted.

Plasmid Constructions. The plasmid containing the human GR cDNA, pRSVHGR (Hollenberg et al., 1985), was first digested with *Sph*I, blunt-ended with T4 DNA polymerase, and then redigested with *Xba*I. The *Sph*I–*Xba*I fragment coding for amino acids 477–777 was inserted into the pRIT32, *Sma*I/*Xba*I site, giving the plasmid pEHGR4770. *E. coli* (MZ1) cells were transformed with the plasmid, and colonies producing the protein A fused steroid-binding domain were selected. The plasmid containing the sequence of DNA- and steroid-binding domains (amino acids 415–777) was constructed as described previously (Bonifer et al., 1989).

Expression of Human GR. Cells were grown in LB medium supplemented with 1% (w/v) glucose, 1% (w/v) casamino acids, and 0.1 mg/mL ampicillin. Expression of the fusion proteins was induced at mid-logarithmic growth by addition of an equal volume of the same medium warmed at 55 °C, and incubation was continued at 42 °C. After 2 h, cells were collected by centrifugation and washed with cold phosphate-buffered saline (pH 7.0). The pellet was frozen in a dry ice–ethanol bath and stored at −70 °C until use.

Preparation of Cell Lysates. The frozen cell paste was suspended in buffer A supplemented with 1 mM PMSF, 0.5 mM leupeptin, 1 μM pepstatin, and 10 μg/mL soybean trypsin inhibitor. Lysozyme was added to a final concentration of 1 mg/mL, and the suspension was incubated at 0–4 °C for 30 min followed by freezing and thawing. The suspension was centrifuged at 150000*g* for 30 min, and the soluble extract fraction was obtained.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblot Analysis. Polyacrylamide gel electrophoresis was run with a discontinuous system (4% stacking gel and 10 or 12.5% separating gel) according to Laemmli (1970). Following electrophoresis, proteins were stained with Coomassie Brilliant Blue or transferred to a nitrocellulose sheet. Immunodetection was then carried out by incubating the anti-GR monoclonal antibody 7 (Okret et al., 1985) followed by reaction with alkaline phosphatase conjugated anti-mouse immunoglobulins. The epitope recognized by the monoclonal antibody was located between amino acids 119 and 273 of the rat GR (Rusconi & Yamamoto, 1987). The fusion proteins expressed in *E. coli* were immunoblotted in the same way since the protein A portion reacts with the antibody as described previously (Bonifer et al., 1989).

Protein Determination. Protein concentrations were measured according to the method of Bradford (1976) using bovine serum albumin as standard.

Results

It has been reported that liver cytosol contains an endogenous transformation inhibitor (Bodine & Litwack, 1988) and

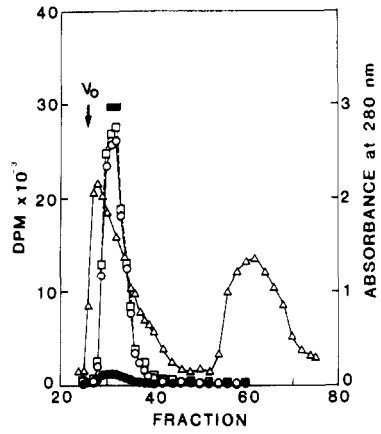

FIGURE 1: Partial purification of the unliganded GR. PC-through was separated on DEAE-Toyopearl and Sephacryl S300 combined columns as described under Materials and Methods. After collection of the fractions eluted from Sephacryl S300, each fraction was incubated with 20 nM [^{3}H]TA in the presence or absence of a 200-fold excess of cold TA. The specific binding (○) was determined by subtracting the nonspecific binding (●) from the total binding (□). Absorbance at 280 nm (△). V_0; void volume. The fractions indicated by a bar were pooled and used for following experiments.

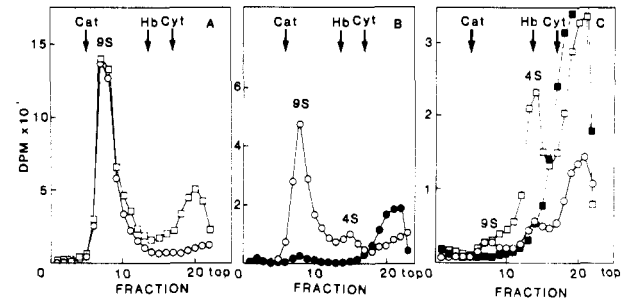

FIGURE 2: Glycerol gradient centrifugation of the GR. (A) The partially purified GR in Figure 1 was incubated with 20 nM (○) or 200 nM [^{3}H]TA (□) in the presence of 10 mM molybdate. After 4 h, the samples (200 μL) were treated with DCC and centrifuged on the gradients. (B) The partially purified GR was exposed to 0.4 M NaCl in the presence of 10 mM molybdate for 3 h. The sample was then incubated with 20 nM [^{3}H]TA in the presence (●) or absence (○) of cold TA for 4 h. After DCC treatment, 200-μL aliquots were layered on the gradients and centrifuged. (C) The partially purified GR was exposed to 0.4 M NaCl in the absence of molybdate for 3 h. The sample was then supplemented with 10 mM molybdate and incubated with 20 nM [^{3}H]TA in the absence of cold TA (○) or with 200 nM [^{3}H]TA in the presence (■) or absence (□) of cold TA for 4 h. After DCC treatment, 200-μL aliquots were centrifuged on the gradients. Catalase (Cat, 11.3 S), hemoglobin (Hb, 4.2 S), and cytochrome *c* (Cyt, 1.7 S) were used as standards and run on separate gradients.

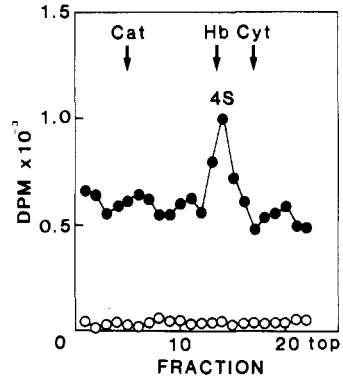

FIGURE 3: Glycerol gradient centrifugation of the unliganded GR exposed to 0.4 M NaCl. The partially purified GR was exposed to 0.4 M NaCl for 3 h. After addition of 10 mM molybdate, a 400-μL sample was run on the gradient. After collection of 200-μL fractions, 100-μL aliquots were incubated with 20 (○) or 200 nM [^{3}H]TA (●), and the bindings were determined. Markers are as in Figure 2.

that the transformed GR forms 6–7S aggregates with tRNA in the cytosol (Ali & Vedeckis, 1987). In order to avoid interference with these components, we first partially purified steroid-free nontransformed GR from rat liver. After the cytosol was passed through phosphocellulose, the nonretained fraction was loaded onto a DEAE-Toyopearl column, from which the GR elutes at a lower salt concentration as compared to other anion exchangers. Preliminary experiments showed that both [^{3}H]TA-bound and steroid-free GR eluted at the same NaCl concentration (about 0.15 M) in the presence of 10 mM molybdate and the [^{3}H]TA-binding ability of the GR was not significantly decreased after chromatography (data not shown). To eliminate both NaCl and molybdate from the sample, a Sephacryl S300 column was connected to the DEAE-Toyopearl column, resulting in a rapid removal of these components. Bound GR was eluted from the DEAE column with buffer B containing 0.2 M NaCl and chromatographed on the Sephacryl S300 column equilibrated in buffer A. Eluted fractions were assayed for the presence of GR by incubation with 20 nM [^{3}H]TA. As shown in Figure 1, the GR eluted from Sephacryl S300 as a single peak just after the void volume fraction, and the nonspecific binding was very low. Polyacrylamide gel electrophoresis showed that hsp 90 was most predominant in this fraction (see Figure 6B, lane P). The physicochemical characteristics of hsp 90 are similar to those of the nontransformed GR (Denis et al., 1987; Nemoto et al., 1987; Ohara-Nemoto et al., 1988). Therefore, hsp 90 nonassociated with GR appeared to virtually comigrate with the nontransformed GR under these conditions. When the fraction was supplemented with 10 mM molybdate and incubated with 20 nM [^{3}H]TA, the GR sedimented in the 9S region (9.1 ± 0.1 S, n = 18) on glycerol gradients (Figure 2A), indicating that the GR was in a nontransformed state. This fraction was used as the partially purified steroid-free GR to study the salt-induced inactivation process of GR.

The GR labeled with 200 nM [^{3}H]TA in the presence of 10 mM molybdate also sedimented at 9 S on gradients, and the amount of radioactivity contained in the 9S peak was almost identical with the one obtained following incubation with 20 nM [^{3}H]TA (Figure 2A). On the other hand, when the GR was exposed to 0.4 M NaCl for 3 h in the presence of 10 mM molybdate and then labeled with 20 nM [^{3}H]TA, the 9S peak was reduced, and a small peak was obtained at 4 S (4.0 ± 0.01 S, n = 8, Figure 2B). Furthermore, in the absence of molybdate, the 9S peak was dramatically reduced on exposure to 0.4 M NaCl, and no increase of the 4S peak was observed following incubation with 20 nM [^{3}H]TA (Figure 2C). These results indicated the inactivation of GR under high-salt conditions. However, when the same sample was incubated with 200 nM [^{3}H]TA, the 4S peak significantly increased, while the 9S peak remained small (Figure 2C). The specificity of the 4S peak was confirmed by displacement with a 200-fold molar excess of cold TA (Figure 2B,C). These results suggested that the 4S form of GR had a lower [^{3}H]-TA-binding affinity than the 9S form. Recent studies have indicated the requirement of hormone for GR transformation (Groyer et al., 1987; Denis et al., 1988). Since labeled GR was centrifuged on the gradients (Figure 2), it was equivocal whether the GR dissociated to the 4S form on exposure to 0.4 M NaCl or during the following incubation with the ligand. To answer this question, [^{3}H]TA-binding ability was measured after glycerol gradient centrifugation of steroid-free GR treated with 0.4 M NaCl. The 4S peak was observed when the fractions were incubated with 200 nM [^{3}H]TA, whereas no

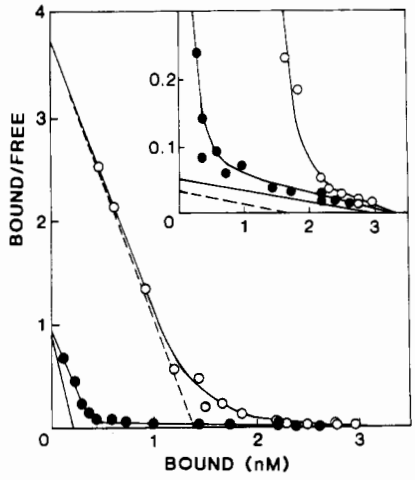

FIGURE 4: Scatchard analysis of the GR exposed to 0.4 M NaCl. The partially purified GR was exposed to 0.4 M NaCl in the presence (○) or absence (●) of 10 mM molybdate. After 3 h, 10 mM molybdate was supplemented to the sample without molybdate, and then both samples were incubated with 1–200 nM [^{3}H]TA in the presence or absence of a 200-fold molar excess of cold TA for 14 h. The specific [^{3}H]TA binding was determined by subtracting the binding with cold TA from the binding without cold TA. An insert shows the same data expanding the values on the y axis. Straight lines represent the low- and high-affinity binding sites calculated by SCATCHARD PLOTTING [(○), +molybdate; (●), −molybdate, the lower affinity sites are shown in the insert]. Solid curves are obtained from calculated values of the two binding sites.

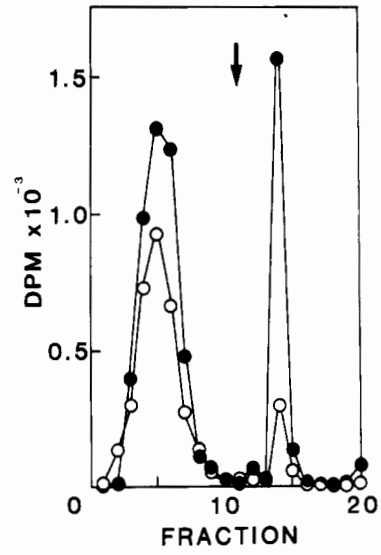

FIGURE 5: DNA–cellulose chromatography of unliganded GR. The partially purified GR (2 mL) was exposed to 0.4 M NaCl for 3 h. After the sample was desalted by passing through Sephadex G25 equilibrated in buffer B containing 50 mM NaCl, it was applied on a DNA–cellulose column (1.5 × 2 cm) equilibrated with buffer B plus 50 mM NaCl. After the column was washed with 10 mL of the same buffer, the bound proteins were eluted with buffer B containing 0.4 M NaCl (arrow). Thirty-drop fractions (1.5 mL) were collected. The specific bindings after incubation of each fraction was determined with 20 (○) and 200 nM (●) [^{3}H]TA.

peak was detected after incubation with 20 nM [^{3}H]TA (Figure 3). This result clearly demonstrated that the GR dissociated to the 4S form by salt treatment in the absence of hormone.

In order to quantify changes in steroid-binding affinity, we performed Scatchard analysis of the GR treated with 0.4 M NaCl in the presence or absence of 10 mM molybdate. To prevent secondary transformation following salt treatment, all samples were supplemented with molybdate to a final concentration of 10 mM following treatment with 0.4 M NaCl, and, therefore, both samples were labeled under identical conditions. The number of binding sites and the apparent dissociation constants were calculated with SCATCHARD PLOTTING program. The results indicated the presence of two independent binding sites as represented in Figure 4. The apparent dissociation constants were 0.52 ± 0.07 and 64.1 ± 16.2 nM (n = 4) in the presence of 10 mM molybdate and 74.2 ± 11.6 nM (n = 4) in the absence of molybdate. The apparent dissociation constant of the high-affinity binding sites in the absence of molybdate was 0.1–1 nM but could not be determined accurately since its ratio was very low (4–8%). Importantly, the total number of binding sites did not differ between the two conditions (Figure 4).

We tested the DNA–cellulose-binding ability of steroid-free GR and compared the steroid-binding affinities of DNA–cellulose-bound and unbound entities. Following chromatography on DNA–cellulose of steroid-free GR, an aliquot of each fraction was incubated with [^{3}H]TA (Figure 5). When the ligand was used at a concentration of 20 nM, most of the specific binding sites were recovered in the flow-through fractions (fractions 2–9), and only 10% of the specific binding sites bound to DNA–cellulose. When the same fractions were incubated with a 10-fold higher concentration of [^{3}H]TA, the number of detectable specific binding sites eluted at 0.4 M NaCl was increased 5-fold. By contrast, only a 1.4-fold increase was found in the flow-through fractions. These results indicate that the steroid-free GR is able to interact with DNA–cellulose and that the low-affinity binding sites correspond to the transformed GR. In contrast, the GR which is unable to interact with DNA–cellulose, i.e., the nontransformed GR, has a higher [^{3}H]TA-binding affinity.

The transformed GR is known to be susceptible to proteolysis (Sherman et al., 1983). Therefore, the alteration in the binding affinity we observed might be related to a proteolytic degradation of GR occurring in 0.4 M NaCl. A proteolyzed GR may have a slower sedimentation rate and a lower steroid-binding affinity than an intact GR molecule. In order to examine this hypothesis, the partially purified GR samples, nontreated or exposed to 0.4 M NaCl in the absence or presence of 10 mM molybdate, were subjected to electrophoresis under denaturing conditions and immunoblotted with the anti-GR monoclonal antibody. A major band was observed at M_r 94 000 (Figure 6A). An M_r 79 000 band was also detected, which may correspond to a proteolytic product of the receptor (Wrange et al., 1984) or, alternatively, might represent a separation artifact due to the fact that the very abundant hsp 90 migrates just slightly faster than the GR and might disturb the resolution of GR. As shown in Figure 6C, the M_r 94 000 band was observed both in the flow-through fractions (fractions 4–8) and in the 0.4 M NaCl eluate (fraction 14) of DNA–cellulose chromatography. In this case, the smaller molecular band was not detected, probably reflecting a difference in the amount of proteins applied on the polyacrylamide gel. Hence, we concluded that, although partial proteolysis of GR could be observed, the alteration from the high-affinity [^{3}H]TA-binding form to the low-affinity form was not related to proteolytic degradation of GR.

In order to further investigate the changes in steroid-binding affinity of GR, two kinds of truncated human GR fused to protein A were expressed in *E. coli*;[3] GR415 contained the DNA- and the steroid-binding domains (amino acids 415–777), whereas GR477 only contained the steroid-binding

[3] Y. Ohara-Nemoto, P.-E. Strömstedt, K. Dahlman, J. Carlstedt-Duke, and J.-Å. Gustafsson, unpublished results.

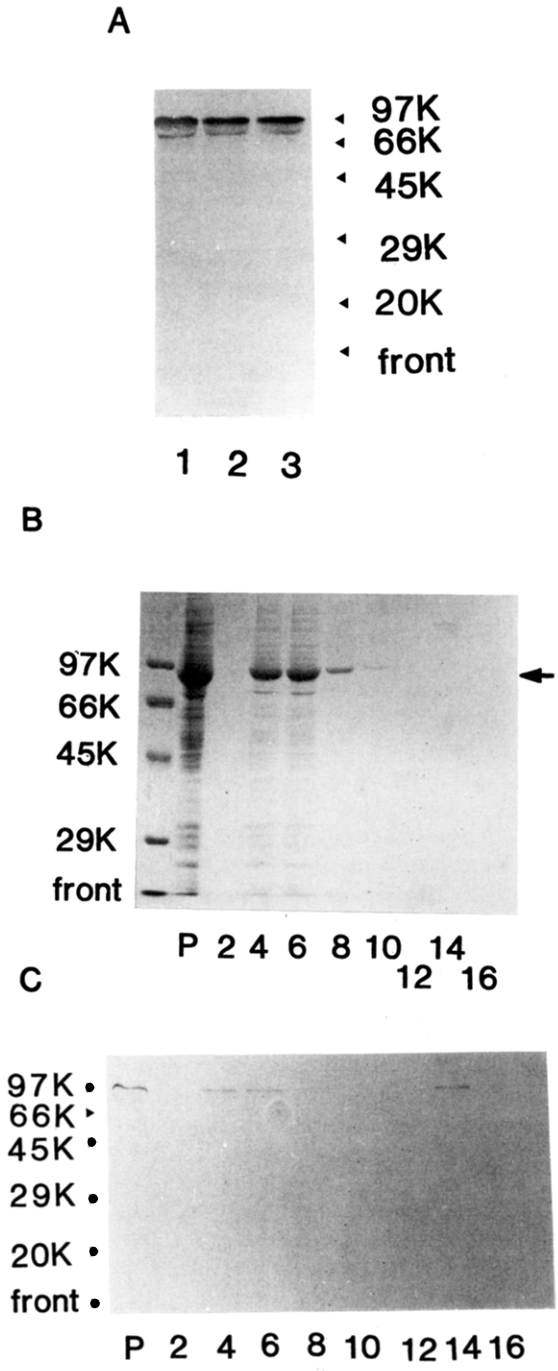

FIGURE 6: Polyacrylamide gel electrophoresis and immunoblot analysis of GR. (A) The partially purified GR [nontreated (lane 1) or treated with 0.4 M NaCl for 3 h in the absence (lane 2) or presence (lane 3) of 10 mM molybdate] were subjected to polyacrylamide gel electrophoresis and immunoblotted with anti-GR monoclonal antibody. (B and C) The fractions separated on DNA–cellulose chromatography (Figure 5) were electrophoresed and (B) stained with Coomassie Blue or (C) immunoblotted. P, the partially purified GR passed through Sephadex G25 following salt exposure. Numbers 2–16 correspond to fractions 2–16 in Figure 5. An arrow indicates the position of hsp 90. Molecular weight standards are phosphorylase *b* (97K), bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20K), and α-lactalbumin (14K).

domain (amino acids 477–777). The expressed fusion proteins were detected by immunoblot analysis. As shown in Figure 7B, an M_r 65 000 band was the main detected component in both lysates. For GR477, the value was in agreement with that calculated from amino acid sequences deduced from cDNA sequences (68 kDa). On the other hand, the molecular weight of expressed GR415 was smaller than the expected

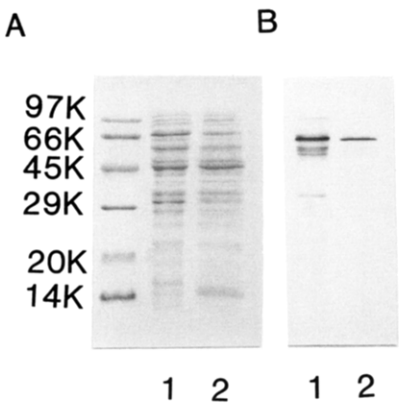

FIGURE 7: Polyacrylamide gel electrophoresis and immunoblot analysis of the fusion proteins. The lysates of GR415 and -477 were subjected to electrophoresis under denaturing conditions. The proteins were (A) stained with Coomassie Blue or (B) transferred to a nitrocellulose sheet and immunoblotted by the mouse monoclonal antibody followed by anti-mouse immunoglobulin G (alkaline phosphatase conjugate). Lane 1, GR415; lane 2, GR477. Molecular weight markers are as in Figure 6.

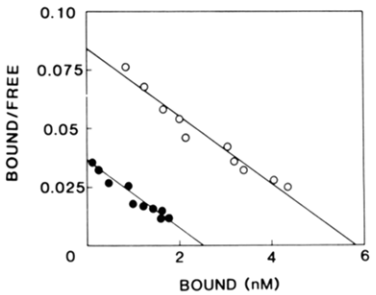

FIGURE 8: Scatchard analysis of the GR expressed in *E. coli*. The lysates of GR415 and -477 were incubated with 1–200 nM [^{3}H]TA in the presence or absence of a 200-fold molar excess of radioinert TA. After 16 h, [^{3}H]TA bindings were measured, and specific bindings were calculated as described under Materials and Methods. GR415 (●) and -477 (○).

76-kDa value. This may be due to the unusual charge property of the DNA-binding domain. Abnormal mobility of purified recombinant DNA-binding domain on electrophoresis has also been reported (Dahlman et al., 1989). For GR415, several proteolytic fragments were also detected. Most of the expressed proteins were recovered in an insoluble fraction as reported previously (Bonifer et al., 1989). However, specific [^{3}H]TA binding was found in a soluble fraction. No specific [^{3}H]TA binding was detected in a control strain with or without the parent vector (pRIT32 and -33) (data not shown). Scatchard analysis showed a single binding site with an apparent dissociation constant of 68.5 nM for GR415 (n = 2) and 75.3 ± 2.9 nM for GR477 (n = 3) (Figure 8). Hence, the [^{3}H]TA-binding affinities of the two fusion proteins were virtually identical with and similar to that of the transformed GR from rat liver.

Discussion

The results presented here show that partially purified GR is transformed in 0.4 M NaCl even in the absence of hormone and that, surprisingly, the transformed GR has approximately a 100-fold lower steroid-binding affinity than the nontransformed GR. These findings strongly indicate that the salt-induced inactivation is caused by the decrease of steroid-binding affinity accompanied by the transformation of steroid-free GR.

Since the experiments in Figures 2 and 4 were done with partially purified GR, we cannot exclude the possibility that

unknown factor(s) is (are) involved in the alteration of the steroid-binding affinity induced by salt treatment. However, the results presented in Figures 3 and 5 show that the low-affinity binding site is also detected after glycerol gradient centrifugation and DNA–cellulose chromatography. Since most proteins including hsp 90 are not retained on the column (Figure 6B), DNA–cellulose chromatography resulted in further purification of the unliganded GR. The experiment performed using the purified GR fractions strongly suggests that the low-affinity binding of [^{3}H]TA is an intrinsic property of the transformed GR. The demonstration that the truncated GR expressed in *E. coli* has a similar [^{3}H]TA-binding affinity supports this conclusion. These results suggest that a non-hormone-binding component associated with the nontransformed GR, probably hsp 90, is important for maintaining GR in the high [^{3}H]TA-binding affinity state.

In contrast to this study, Moudgil et al. (1986) reported an absolute requirement of the hormone for the transformation of rat liver GR. The cited and most previous studies (Nielsen et al., 1977; McBlain & Shyamala, 1980; McBlain et al., 1981) have used a single concentration of the ligand (20–50 nM). We show here, however, that a higher concentration of [^{3}H]steroid is needed to label the transformed GR. In addition, it seems important to use the hydroxylapatite adsorption assay to determine [^{3}H]steroid binding. This method was time-consuming, but accurate determination of low-affinity binding was possible since nonspecific binding was low. Using immunochemical techniques, we recently showed that the thermal transformation of the cytosolic receptor is hormone-dependent, but we also demonstrated that transformation of steroid-free GR bound to an immunosorbent could be achieved by extensive washing with 0.15 M NaCl (Denis et al., 1988).

Most expression studies of GR have been done with reticulocyte lysate (Rusconi & Yamamoto, 1987) or mammalian cells (Pratt et al., 1988; Hollenberg et al., 1987; Denis & Gustafsson, 1989). Rusconi and Yamamoto (1987) reported that the rat GR expressed in reticulocyte lysate had a high [^{3}H]dexamethasone-binding affinity (apparent dissociation constant $\approx$7 nM). We recently described that the GR expressed in reticulocyte lysate is associated with endogenous hsp 90 (Denis & Gustafsson, 1989). In contrast, GR expressed in *E. coli* appears not to be associated with the protein homologous to eukaryotic hsp 90, since GR477 sedimented at 5.4–5.5 S on glycerol gradients irrespective of the presence of molybdate.[3] Thus, the apparent difference in the steroid-binding affinities of the GR expressed in reticulocyte versus GR expressed in *E. coli* (GR415 and -477; the dissociation constant $\approx$70 nM), respectively, supports the notion that hsp 90 is important for a higher steroid-binding affinity of GR.

The deduced amino acid sequence of hsp 90 in *E. coli* is 42% identical with human hsp 90 (Bardwell & Craig, 1987). Thus, it is not surprising that several amino acids essential for the interaction with GR may not be present in the bacterial protein, thereby not permitting any interaction with GR. For instance, a highly charged stretch of 50 amino acids present in the eukaryotic protein is deleted in the bacterial analogue. Interestingly, this very region has been proposed to interact with steroid receptors (Catelli et al., 1988). In fact, ionization of acidic amino acid residues in the nontransformed GR stabilizes the 9S form of GR (Nemoto et al., 1988). In vitro mutagenesis analysis of hsp 90 is needed to further characterize the interaction of hsp 90 with GR.

Hsp 90 directly interacts with the steroid-binding domain of GR (Denis et al., 1988). This interaction may alter the fine structure of the steroid-binding domain, maintaining the domain in the high-affinity steroid-binding state.

Acknowledgments

We thank Dr. Kenn-ichi Nakamura (Hiroshima Women University, Hiroshima, Japan) for calculation of apparent dissociation constants with the program he developed, Dr. Jan Carlstedt-Duke and Karin Dahlman for constructive discussions during this study, and Scott Cuthill for proofreading of the manuscript.

Registry No. NaCl, 7647-14-5; triamcinolone acetonide, 76-25-5.

References

Ali, M., & Vedeckis, W. V. (1987) *J. Biol. Chem. 262*, 6771–6777.

Atger, M., & Milgrom, E. (1976) *Biochemistry 15*, 4298–4304.

Bardwell, J. C. A., & Craig, E. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5177–5181.

Bodine, P. V., & Litwack, G. (1988) *J. Biol. Chem. 263*, 3501–3512.

Bonifer, C., Dahlman, K., Lanzer, M., Strömstedt, P.-E., Flock, J.-I., & Gustafsson, J.-Å. (1989) *J. Steroid Biochem. 32*, 5–11.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Catelli, M. G., Radanyi, C. Renoir, J. M., Binart, N., & Baulieu, E.-E. (1988) *J. Cell. Biochem., Suppl. 12D*, 276.

Chandler, V. L., Maler, B. A., & Yamamoto, K. R. (1983) *Cell 33*, 489–499.

Dahlman, K., Strömstedt, P.-E., Rae, C., Jörnvall, H., Flock, J.-I., Carlstedt-Duke, J., & Gustafsson, J.-Å. (1989) *J. Biol. Chem. 264*, 804–809.

Denis, M., Wilkström, A.-C., & Gustafsson, J.-Å. (1987) *J. Biol. Chem. 262*, 11803–11806.

Denis, M., & Gustafsson, J.-Å. (1989) *J. Biol. Chem. 264*, 6005–6008.

Denis, M., Poellinger, L., Wikström, A.-C., & Gustafsson, J.-Å. (1988) *Nature (London) 333*, 686–688.

Denis, M., Gustafsson, J.-Å., & Wikström, A.-C. (1988) *J. Biol. Chem. 263*, 18520–18523.

Groyer, A., Schweizer-Groyer, G., Cadepond, F., Mariller, M., & Baulieu, E. E. (1987) *Nature (London) 328*, 624–626.

Gustafsson, J.-Å., Carlstedt-Duke, J., Poellinger, L., Okret, S., Wikström, A.-C., Brönnegård, M., Gillner, M., Dong, Y., Fuxe, K., Cintra, A., Härfstrand, A., & Agnati, L. (1987) *Endocr. Rev. 8*, 185–234.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., & Evans, R. M. (1985) *Nature (London) 318*, 635–641.

Hollenberg, S. M., Giguere, V., Segui, P., & Evans, R. M. (1987) *Cell 49*, 39–46.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

McBlain, W. A., & Shyamala, G. (1980) *J. Biol. Chem. 255*, 3884–3891.

McBlain, W. A., Toft, D. O., & Shyamala, G. (1981) *Biochemistry 20*, 6790–6798.

Mendel, D. B., Bodwell, J. E., Gametchu, B., Harrison, R. W., & Munck, A. (1986) *J. Biol. Chem. 261*, 3758–3763.

Moudgil, V. K., Lombardo, G., Eessalu, T., & Eliezer, N. (1986) *J. Biochem. 99*, 1005–1016.

Munck, A., & Brinck-Johnsen, T. (1968) *J. Biol. Chem. 243*, 5556–5565.

Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752–754.

Nemoto, T., Ohara-Nemoto, Y., & Ota, M. (1987) *J. Biochem. 102*, 513–523.

Nemoto, T., Ohara-Nemoto, Y., & Ota, M. (1988) *J. Biochem. 103*, 920-927.
Nielsen, C. J., Vogel, W. M., & Pratt, W. B. (1977) *Cancer Res. 37*, 3420-3426.
Ohara-Nemoto, Y., Nemoto, T., Sato, N., & Ota, M. (1988) *J. Steroid Biochem. 31*, 295-304.
Okret, S., Wilkström, A.-C., & Gustafsson, J.-Å. (1985) *Biochemistry 24*, 6581-6586.
Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M.-G., Evans, R. M., & Baulieu, E.-E. (1988) *J. Biol. Chem. 263*, 267-273.
Ringold, G. M. (1985) *Annu. Rev. Pharmacol. Toxicol. 25*, 529-566.
Rusconi, S., & Yamamoto, K. R. (1987) *EMBO J. 6*, 1309-1315.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660-672.
Schmidt, T. J., & Litwack, G. (1982) *Physiol. Rev. 62*, 1131-1192.
Sherman, M. R., Moran, M. C., Tuazon, F. B., & Stevens, Y.-W. (1983) *J. Biol. Chem. 258*, 10366-10377.
Wrange, Ö., Okret, S., Radojcic, M., Carlstedt-Duke, J., & Gustafsson, J.-Å. (1984) *J. Biol. Chem. 259*, 4534-4541.

Lentiginosine, a Dihydroxyindolizidine Alkaloid That Inhibits Amyloglucosidase†

Irena Pastuszak,‡ Russell J. Molyneux,§ Lynn F. James,∥ and Alan D. Elbein*,‡

Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710, and Poisonous Plant Research Laboratory, U.S. Department of Agriculture, Logan, Utah 84321

Received July 26, 1989; Revised Manuscript Received October 17, 1989

ABSTRACT: Lentiginosine, a dihydroxyindolizidine alkaloid, was extracted from the leaves of *Astragalus lentiginosus* with hot methanol and was purified to homogeneity by ion-exchange, thin-layer, and radial chromatography. A second dihydroxyindolizidine, the 2-epimer of lentiginosine, was also purified to apparent homogeneity from these extracts. Gas chromatography of the two isomers (as the TMS derivatives) showed that they were better than 95% pure; lentiginosine eluted at 8.65 min and the 2-epimer at 9.00 min. Both compounds had a molecular ion in their mass spectra of 157, and the NMR spectra demonstrated that both were dihydroxyindolizidines differing in the configuration of the hydroxyl group at carbon 2. Lentiginosine was found to be a reasonably good inhibitor of the fungal α-glucosidase, amyloglucosidase ($K_i = 1 \times 10^{-5}$ M), but it did not inhibit other α-glucosidases (i.e., sucrase, maltase, yeast α-glucosidase, glucosidase I) nor any other glycosidases. The 2-epimer had no activity against any of the glycosidases tested.

Plants produce many different types of alkaloids, but the biochemical mode of action of most of these has not been elucidated. On the other hand, a number of plant indolizidine and pyrrolizidine alkaloids have been shown to be potent inhibitors of various glycosidases (Elbein & Molyneux, 1987; Elbein, 1987). For example, swainsonine (1,7,8-trihydroxyindolizidine) is a toxic alkaloid that is found in the leaves and stems of *Swainsona* species that grow in western Australia (Colegate et al., 1979), and in *Astragalus* species that grow in the western U.S. (Molyneux & James, 1982; Davis et al., 1984; Molyneux et al., 1985). Members of both of these genera are considered to be poisonous to animals (Hartley, 1978). Swainsonine was shown to be a potent inhibitor of lysosomal α-mannosidase (Dorling et al., 1980), as well as other arylmannosidases (Kang & Elbein, 1983a). This alkaloid also inhibited the glycoprotein processing mannosidase II (Tulsiani et al., 1982) and in cultured cells caused the formation of N-linked glycoproteins having hybrid types of oligosaccharides (Elbein et al., 1981; Tulsiani et al., 1984; Arumughan & Tanzer, 1983; Kang & Elbein, 1983b).

Another indolizidine alkaloid (1,6,7,8-tetrahydroxyindolizidine), castanospermine, was crystallized from the seeds of the Australian tree *Castanospermum australe* (Hohenschutz et al., 1980) and found to be a potent inhibitor of α- and β-glucosidases (Saul et al., 1983). This alkaloid also inhibited the processing of N-linked glycoproteins. In this case, the inhibition was at the glucosidase I stage (Pan et al., 1983), resulting in the accumulation of glycoproteins with $Glc_3Man_9(GlcNAc)_2$ structures in cultured cells incubated in the presence of castanospermine (Repp et al., 1985; Arakaki et al., 1987; Pan et al., 1983).

After removal of the castanospermine from the seed extract by crystallization, several other new alkaloids were identified in these extracts. One of these was purified to homogeneity and identified as 6-epicastanospermine. Although this tetrahydroxyindolizidine alkaloid still had good activity against the amyloglucosidase, it had lost the inhibitory activity toward β-glucosidase (Molyneux et al., 1986). A second alkaloid was purified from these extracts and shown to be a tetrahydroxypyrrolizidine alkaloid. This compound, australine, had a unique substitution pattern and was also found to inhibit α-glucosidases (Molyneux et al., 1988). In addition, it inhibited glycoprotein processing at the glucosidase I stage, although not as effectively as castanospermine, and caused the accumulation of $Glc_3Man_{7-9}(GlcNAc)_2$ structures in cultured cells (Tropea et al., 1989). The fact that a pyrrolizidine alkaloid can also inhibit these glycosidases indicates that a six-membered ring is not essential for inhibitory activity. Previously, a polyhydroxypyrrolidine called DMDP had also been shown to inhibit α- and β-glucosidases, as well as the

† This study was supported by grants from the National Institutes of Health (HL-17783) and the Robert A. Welch Foundation.
‡ University of Texas Health Science Center.
§ Western Regional Research Center.
∥ Poisonous Plant Research Laboratory.

0006-2960/90/0429-1886$02.50/0