3, Part I, American Physiological Society, Bethesda, MD.
Hawgood, S., Benson, B. J., & Hamilton, R. L., Jr. (1985) *Biochemistry 24*, 184–190.
Hook, G., Spalding, J. W., Ortner, M. J., Tombropoulos, E. G., & Chignell, C. F. (1984) *Biochem. J. 223*, 533–542.
Jaworsky, M., & Mendelsohn, R. (1985) *Biochemistry 24*, 3422–3428.
Keough, K. M. W., Farrell, E., Cox, M., Harrell, G., & Teusch, H. W., Jr. (1985) *Can. J. Physiol. Pharmacol. 63*, 1043–1051.
King, R. J. (1982) *J. Appl. Physiol.: Respir., Environ. Exercise Physiol. 53*, 1–8.
King, R. J., & MacBeth, M. C. (1979) *Biochim. Biophys. Acta 557*, 86–101.
King, R. J., Carmichael, M. C., & Horowitz, P. M. (1983) *J. Biol. Chem. 258*, 10672–10680.
Kouaouci, R., Silvius, J. R., Graham, I., & Pezolet, M. (1985) *Biochemistry 24*, 7132–7140.
Laroche, G., Carrier, D., & Pezolet, M. (1988) *Biochemistry 27*, 6220–6228.
MacDonald, R. I., & MacDonald, R. C., (1975) *J. Biol. Chem. 250*, 9206–9213.
Mautone, A. J., Reilly, K. E., & Mendelsohn, R. (1987) *Biochim. Biophys. Acta 896*, 1–10.
Mendelsohn, R., & Mantsch, H. H. M. (1986) in *Progress in Protein–Lipid Interactions 2* (Watts, A., & De Pont, J. J. H. H. M., Eds.) Chapter 4, Elsevier Science Publishers, Amsterdam, The Netherlands.
Mendelsohn, R., Dluhy, R., Taraschi, T., Cameron, D. G., & Mantsch, H. H. (1981) *Biochemistry 20*, 6699–6706.
Mendelsohn, R., Anderle, G., Jaworsky, M., Mantsch, H. H., & Dluhy, R. A. (1984) *Biochim. Biophys. Acta 775*, 215–224.
Ng, V. L., Herndon, V. L., Mendelson, C. R., & Snyder, J. M. (1983) *Biochim. Biophys. Acta 754*, 218–236.
Notter, R. H. (1983) *Chem. Phys. Lipids 33*, 67–80.
Notter, R. H. (1984) in *Pulmonary Surfactant*, pp 17–65, Elsevier, The Netherlands.
Ross, G. F., Notter, R. H., Meuth, J., & Whitsett, J. A. (1986) *J. Biol. Chem. 261*, 14283–14291.
Scarpelli, E. (1988) *Surfactants and the Lining of the Lung*, Johns Hopkins University Press, Baltimore, MD.
Schiffer, K., Hawgood, S., Duzgunes, N., & Goerke, J. (1988) *Biochemistry 27*, 2689–2695.
Snyder, R. G., Strauss, H. L., & Elliger, C. A. (1982) *J. Phys. Chem. 86*, 5145–5150.
Sueishi, K., & Benson, B. J. (1981) *Biochim. Biophys. Acta 665*, 442–453.
Taylor, M. O., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 599*, 140–151.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
White, C. A., & Kennedy, J. F. (1981) *Techniques in Carbohydrate Metabolism*, Vol. B312, p12, Elsevier/North-Holland Scientific Publishers Ltd., Amsterdam, The Netherlands.
White, R. T., Damm, D., Miller, J., Spratt, K., Schilling, J., Hawgood, S., Benson, B., & Cordell, B. (1985) *Nature 317*, 361–363.
Wright, J. R., Benson, B., Williams, M. C., George, J., & Clements, J. A. (1984) *Biochim. Biophys. Acta 791*, 320–332.
Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L., & Clements, J. A. (1987) *J. Biol. Chem. 262*, 2888–2894.
Yu, S., & Possmayer, F. (1986) *Biochem. J. 236*, 85–89.

Differential Sensitivity of Chicken Progesterone Receptor Forms to Sulfhydryl Reactive Reagents†

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ABSTRACT: DNA binding of chick progesterone receptor B form (PRB) has been examined and compared to that of the A form (PRA). We found that the elution profiles of the two receptors overlap on DNA–cellulose columns. Both PRA or PRB could bind to plasmid DNA equivalently as assayed by sedimentation velocity studies. However, DNA-binding activity of the two receptor forms showed differential sensitivity to reducing agents and to sulfhydryl (SH) reactive reagents. Reducing agents stabilized DNA-binding activity of PRA more efficiently than they stabilized PRB. Moreover, removal of reducing agents from receptor preparations caused preferential loss of DNA binding by PRB compared to the PRA. DNA-binding activity of PRA was readily destroyed by sulfhydryl modifying reagents such as *N*-ethylmaleimide and iodoacetamide while PRB was 3–4 times less sensitive to these reagents. We conclude the DNA-binding activity of PRB is less stable due to altered accessibility of SH groups despite the amino acid sequence identity of the DNA-binding domains of PRA and PRB.

The chicken progesterone receptor (PR)[1] exists in two forms: receptor A (PRA) of 78 kDa and receptor B (PRB) of 108 kDa (Birnbaumer et al., 1983). These receptor forms are products of two translation initiation sites of a single mRNA (Conneely et al., 1987a). In earlier studies, we found that PRA could bind tightly to DNA–cellulose (Schrader et al., 1972) and to soluble DNA (Compton et al., 1984). By contrast, PRB showed only weak binding to DNA–cellulose (Vedeckis et al.,

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[1] Abbreviations: PRB, progesterone receptor B form (108 kDa); PRA, progesterone receptor A form (78 kDa); SH, sulfhydryl; NEM, *N*-ethylmaleimide; DES, diethylstilbestrol; TAA, triamcinolone acetonide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; βME, β-mercaptoethanol; DTT, dithiothreitol.

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1980) and no binding to DNA in solution (Schrader et al., 1972). Gronemeyer et al. (1985) demonstrated that a significant proportion of PRB from hen oviducts could bind tightly to DNA–cellulose in large-scale preparations, although PRB from DES-treated immature chicks did not possess this activity. We have confirmed this observation (Peleg et al., 1988) but showed that even in immature chicks a small proportion of PRB can bind tightly to DNA. In addition, we showed that DNA binding of PRB was more sensitive than that of PRA to oxidation, and loss of DNA binding of both receptor forms was irreversible in vitro possibly due to inaccessibility of reducing agents to the oxidized residues. In that study, we speculated that higher sensitivity of PRB to oxidation was not likely to be due to a higher number of cysteines on this molecule, since the two receptor forms have a similar number of cysteines.

In the present study, we further investigated DNA-binding activity of the two receptor forms. We compared their binding characteristics to insoluble and soluble DNA and the sensitivity of DNA binding to sulfhydryl reactive reagents. We report here that binding characteristics of PRB to nonspecific DNA are similar to those of PRA. However, stabilization of this activity by reducing agents is more efficient for PRA than for PRB possibly because of limited accessibility of SH groups on the DNA-binding form of PRB to these reagents.

Experimental Procedures

Chemicals. [^{3}H]Progesterone (66 Ci/mmol) and [^{3}H]-R5020 [promegestone (17α-methyl ^{3}H); 86 Ci/mmol] were obtained from New England Nuclear. Progesterone and triamcinolone acetonide (TAA)[1] were from Steraloids. Molecular weight standards for electrophoresis were from Bio-Rad; *N*-ethylmaleimide, iodoacetamide, (*p*-chloromercuri)-benzoate, and mersalylic acid were from Sigma. *Buffer TEG* contained 10 mM Tris-HCl, pH 7.4, 1 mM Na_2EDTA,[1] 10% (w/v) glycerol, and 50 mM NaCl. This buffer was supplemented with thiols when indicated in the text.

Preparation of Cytosol and Partial Purification of Progesterone Receptor. Oviducts were removed from diethylstilbestrol-treated 5-week-old chicks and rinsed in ice-cold saline. Homogenization was done at 0 °C in TEG buffer with or without thiols as indicated in the text, at a ratio of 5 mL of buffer per gram of wet tissue, using a Polytron PT-10 at a setting of 5.5, with three 10-s bursts separated by 1-min intervals. The homogenate was then centrifuged at 27000*g* for 20 min. A floating lipid layer was removed, and the 27000*g* supernatant fraction was centrifuged for 1 h at 105000*g* to yield the high-speed cytosol.

High-speed cytosol prepared in TEG buffer with or without thiols was applied to a 30-mL phosphocellulose column, and the flow-through material was labeled for 2 h with [^{3}H]-progesterone. The receptor was precipitated at 40% saturation of ammonium sulfate. The pellet was collected after 30 min by centrifugation and redissolved in TEG buffer containing 100 mM NaCl with or without thiols.

To separate receptor able to bind to DNA from receptor unable to bind to DNA, we prepared cytosol and ammonium sulfate pellets as described above and applied the redissolved ammonium sulfate pellets onto a DNA–cellulose column. Receptor unable to bind to DNA was collected in the DNA–cellulose flow-through and in a 0.1 M NaCl wash of that column. The DNA-binding receptor fraction was eluted from the DNA–cellulose column either by stepwise elution with 0.3 M NaCl in TEG buffer or by a NaCl gradient (0.1–0.5 M).

Photoaffinity Labeling of Receptors. Photolabeling of receptor was performed using receptor samples (see text) prepared from cytosol incubated with [^{3}H]R5020 instead of [^{3}H]progesterone (Birnbaumer et al., 1983). For UV irradiation, receptor solutions (10 mL) were placed in plastic petri dishes (Fisher). The dishes were placed over a transillumination UV light-box with wavelength cutoff of 290 nm and irradiated for a period of 5 min. After irradiation, the pools of receptor proteins were dialyzed at 4 °C against several changes of 50 mM NH_4HCO_3 (pH 8.5) and lyophilized.

Polyacrylamide Gel Electrophoresis. Receptor-containing samples were heated in Laemmli sample buffer (Laemmli, 1970). The electrophoresis separation was carried out in slabs containing 1% (w/v) SDS[1] and formed of 7.5% acrylamide/0.2% bis(acrylamide). Electrophoresis, gel staining by Coomassie Blue, and fluorography were carried out as described by Birnbaumer et al. (1983).

Preparation of Plasmid DNA. All experiments were performed using DNA from bacterial plasmid pOV1.7 containing 1.7-kb sequences of the ovalbumin gene (Dugaiczyk et al., 1979). Supercoiled plasmid was isolated by equilibrium sedimentation in an ethidium bromide/cesium chloride gradient (Maniatis et al., 1982). Tubes were pierced from the side, and the fluorescent band of supercoiled DNA was removed. Dye was removed by precipitation at −20 °C overnight in absolute ethanol. Linear, double-stranded DNA was prepared by digesting the DNA to completion at a unique *Pvu*II site or *Eco*RI site. DNA concentrations were measured spectrophotometrically, assuming that 50 μg/mL DNA has an absorbance of 1.0 at 260 nm.

Double-stranded DNA, linearized by *Eco*RI, was labeled to a specific activity of 1–5 μCi/μg with ^{32}P at the 5′ ends of each strand using Klenow fragment of DNA polymerase I (Maniatis et al., 1982). Samples [(5–10) × 10^3 cpm] of [^{32}P]DNA were added to some of the receptor–DNA mixtures as molecular weight markers.

Interaction of Receptor with DNA in Solution. Receptor samples were prepared as described in the text. Aliquots were incubated at a final NaCl concentration of 100 mM with plasmid DNA at 0 °C for 1 h. For sedimentation velocity analysis, linear gradients of 5–20% sucrose in buffer TEB (10 mM Tris, pH 7.4, 1 mM EDTA, and 12 mM 1-thioglycerol) containing 100 mM NaCl were prepared by using a Beckman gradient former. The volume of the gradients was 5 mL. The receptor–DNA samples (200 μL) were then applied to the top of the sucrose gradients, the tubes were sealed, and centrifugation was performed in the VTi 65 rotor for 35 min at 65000 rpm. Each 200-μL aliquot contained receptor and DNA at final concentrations of 1.7 and 1–5 nM, respectively. Fractions (0.2 mL) collected from the bottom of the tubes were assayed for ^{3}H and ^{32}P by scintillation counting. Sedimentation coefficients of [^{3}H]progesterone receptor–DNA complexes were compared with that of *Eco*RI-cut linear [^{32}P]DNA (18 S) or with that of unlabeled supercoiled DNA.

Assay of Receptor Binding to DNA–Cellulose. [^{3}H]-Progesterone receptor complexes treated with reducing agents or with SH reactive reagents were applied to small (2-mL) DNA–cellulose columns. The columns were washed with 10 column volumes of TEG buffer containing 0.1 M NaCl. Receptors were eluted by 0.3 M NaCl, and aliquots were analyzed by counting in a liquid scintillation spectrometer. Receptor size was analyzed by Western immunoblots using polyclonal antibody against the chicken progesterone receptor (Birnbaumer et al., 1987).

Immunoblotting (Western) Analysis. For immunoanalysis, receptor-containing aliquots (40 μL) were boiled in Laemmli sample buffer immediately after elution from the columns and

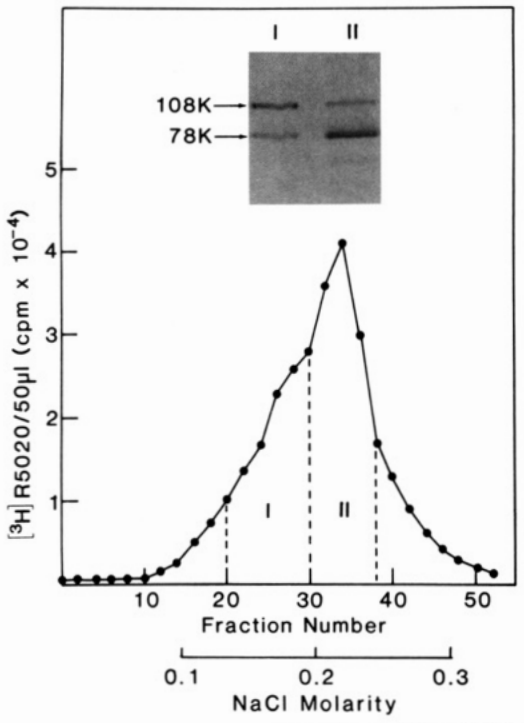

FIGURE 1: Salt gradient elution of ^3H-labeled receptor proteins from DNA–cellulose. [^3H]R5020–receptor complexes were partially purified and chromatographed on DNA–cellulose, as described under Experimental Procedures. Pooled samples from fractions 20–30 (pool I) and fractions 30–40 (pool II) were UV-irradiated, lyophilized, and analyzed by gel electrophoresis as described under Experimental Procedures. Insert at top shows fluorograms of pools I and II. Receptor size was determined by comparison to molecular weight standards migrating in the adjacent lane (not shown). An NaCl gradient elution (0.1–0.5 M) of ^3H from the DNA–cellulose column is shown at the bottom.

kept frozen (−20 °C) until analyzed. Gel electrophoresis of these samples was performed as described above. Proteins were electroeluted from the slab gels onto nitrocellulose paper in Tris–glycine buffer, as described previously (Towbin et al., 1973). After transfer, additional protein-binding sites on the paper were blocked by incubating the filter for 2 h at room temperature in Tris-HCl (pH 7.4), containing 0.2 M NaCl, and 2% (w/v) nonfat dry milk (Carnation). Incubation with polyclonal antibodies (1 μg of DEAE-purified IgG/mL of buffer) was done in the same buffer at room temperature for 18 h. Unreacted antibodies were removed by three washes (15 min each) of the filter in the same buffer. The receptor–antibody complexes were detected by incubating the filter with ^{125}I protein A (Amersham) for 1 h at room temperature. Unbound protein A was removed by three washes in the same buffer. The nitrocellulose paper was then air-dried and exposed for 24–72 h at −72 °C to Kodak X-OMAT XAR5 film in a cassette with a DuPont Cronex Quanta III intensifying screen.

Results

Binding Characteristics of Receptor A and B Forms to Nonspecific DNA. In a recent study (Peleg et al., 1988) we reported that the two chicken PR forms existed in vitro in a reduced (DNA-binding) state and an oxidized (non-DNA-binding) state. Since the DNA-binding activity of the PRB form showed higher sensitivity to oxidation than PRA, we wished to find if this characteristic of PRB was correlated with lower affinity of this receptor form to DNA, or merely reflected lower accessibility of reducing agents to sulfhydryl groups which are essential for the maintenance of DNA-binding activity.

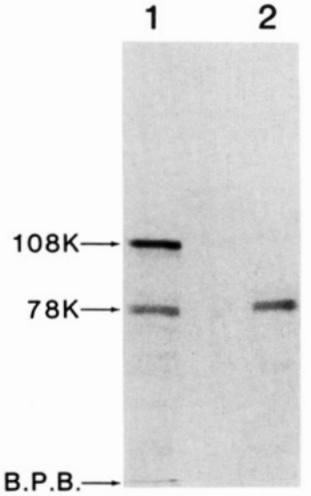

FIGURE 2: Fluorography of receptor forms eluted from DNA–cellulose and separated by DEAE-cellulose chromatography. [^3H]PR complexes were applied on a DNA–cellulose column at 0.1 M NaCl, and step-eluted by 0.3 M NaCl. The eluate was diluted to 50 mM by NaCl and applied to a 2-mL DEAE-cellulose column. PRA was prepared by eluting that column with buffer containing 0.15 M NaCl. PRB was prepared by subsequent elution of that column with buffer containing 0.3 M NaCl. Samples of the ^3H receptor complexes were UV-irradiated, lyophilized, and analyzed by gel electrophoresis and fluorography as described under Experimental Procedures. Shown are the fluorograms of samples eluted from the DEAE-cellulose column: lane 1, [^3H]PRB, obtained by 0.3 M NaCl elution from DEAE-cellulose; lane 2, [^3H]PRA, obtained by 0.15 M NaCl elution from DEAE-cellulose. Receptor size (kilodaltons) was determined by comparison with molecular weight standards, migrating in adjacent lanes (not shown).

We used salt elution profiles of receptor from calf thymus DNA–cellulose as an assay for the affinity of these proteins to nonspecific DNA. For that purpose, we prepared [^3H]-R5020–receptor complexes, as described by Birnbaumer et al. (1983). The [^3H]R5020–PR complexes were applied to a DNA–cellulose column at 0.1 M NaCl and eluted by an NaCl gradient as shown in Figure 1. The radioactive fractions eluted from the column were collected as two pools (I and II), irradiated, and analyzed for receptor size by gel electrophoresis, followed by fluorography. The fluorograph of the two pools is shown in the inset of Figure 1. Each pool contained PRA and PRB. However, the early eluting material contained a higher proportion of PRB than did pool II. Conversely, pool II was enriched in PRA. This apparent difference in elution of PRA and PRB could be due to a slightly lower affinity of PRB for DNA–cellulose.

Salt gradient chromatography using DNA–cellulose is not a very sensitive method for analyzing relative affinity to DNA. As an alternative to this method, we used a sedimentation velocity ultracentrifugation assay which had been used previously to study steroid receptor binding to DNA in solution (Compton et al., 1984). We tested three receptor fractions. The first and the second receptor fractions were prepared from the DNA–cellulose eluate: A pool of DNA-positive PRA and PRB was eluted from DNA–cellulose by 0.3 M NaCl. The two receptor forms were then separated by DEAE-cellulose chromatography, as shown in Figure 2. Lane 2 shows that the fraction eluted from that column by 0.15 M NaCl contained PRA only. Lane 1 shows that the fraction eluted subsequently from the DEAE-cellulose by 0.3 M NaCl contained mainly PRB. The third receptor fraction of PRB lacking binding activity to DNA–cellulose was prepared by collecting the DNA flow-through fraction on the DEAE-cellulose column. Then this column was washed with 0.15 M NaCl, followed by a step eluting PRB, using 0.3 M NaCl

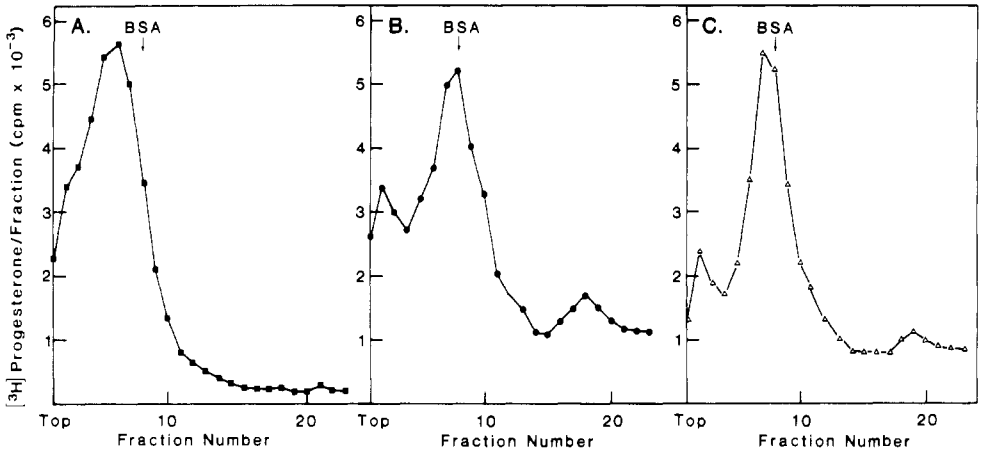

FIGURE 3: Sedimentation velocity analysis of PRA and PRB forms. Ammonium sulfate precipitates containing [^{3}H]progesterone receptor were prepared as described under Experimental Procedures. The resuspended pellets were applied to a DNA–cellulose column, and the flow-through of that column was adsorbed to DEAE-cellulose. DNA-negative PRB was step-eluted from that DEAE-cellulose column. DNA-positive PRA and PRB were step-eluted from the DNA–cellulose column by 0.3 M NaCl, diluted to 50 mM NaCl, and separated by DEAE-cellulose chromatography as described in Figure 2. Receptor samples were diluted to a final NaCl concentration of 0.1 M. Samples were applied onto 5–20% sucrose gradients, and ultracentrifugation was carried out in a VTi-65 rotor at a speed of 65 000 rpm for 2 h. BSA (4.2 S) was run in a separate tube. Panel A, receptor A able to bind DNA; panel B, receptor B able to bind to DNA; panel C, receptor B unable to bind to DNA.

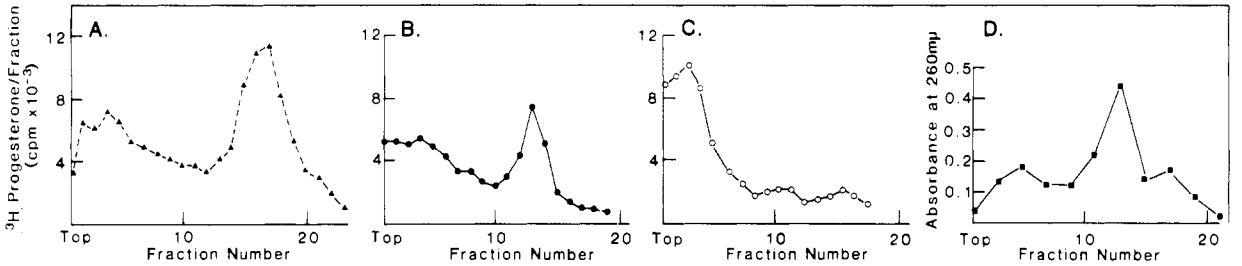

FIGURE 4: Sedimentation velocity analysis of progesterone receptor binding to DNA. [^{3}H]Progesterone receptor (A or B) complexes were prepared exactly as described in Figure 3. Receptor samples (1.7 nM) were incubated 1 h at 4 °C with plasmid DNA pOV1.7 (1–5 nM), at a final NaCl concentration of 0.1 M. Ultracentrifugation of the sucrose gradients was carried out in a VTi-65 rotor at a speed of 65 000 rpm for 35 min. Panel A, DNA-positive PRB; panel B, DNA-positive PRA; panel C, DNA-negative PRB; panel D, UV absorbance profile of plasmid DNA alone.

wash. Photoaffinity labeling of PRB obtained from the DEAE-cellulose column showed exclusively the 108-kDa band upon fluorography (data not shown). All three receptor pools to be tested were prepared simultaneously and were then incubated with plasmid pOV1.7 DNA for 1 h in TEG buffer containing 0.1 M NaCl. DNA concentrations ranged from 1 to 5 nM. Receptor concentration was 1.7 nM.

The results of these experiments are shown in Figures 3 and 4. All three receptor pools tested had a sedimentation coefficient of about 4 S in the absence of DNA (Figure 3). Figure 3C shows that the sedimentation coefficient of the PRB pool which lacked DNA-binding activity was also 4 S; thus, the reason for its inability to bind DNA was not due to self-aggregation or association with a large protein such as hsp90 (Puri et al., 1982).

Plasmid DNA, sedimented in fraction 12 (Figure 4, panel D), was clearly resolved from the 4S receptor region of the gradients. Panels A–C of Figure 4 show results obtained when the three receptor pools were incubated with plasmid DNA. The DNA shifted both PRB (Figure 4, panel A) and PRA (Figure 4, panel B) obtained from DNA–cellulose. However, PRB, unable to bind DNA–cellulose, also failed to bind to DNA in solution (Figure 4, panel C). These results indicate that both PRA and PRB have similar affinity for plasmid DNA since significant binding of both receptor forms to this DNA occurred at the same receptor:DNA molar ratio of about 1.0. Increased salt concentrations of 0.15 or 0.2 M NaCl disrupted DNA–receptor complexes in solution for both PRA and PRB (data not shown).

We cannot exclude the possibility that receptor B, unable to bind to DNA, was associated with a small molecule which altered its DNA-binding activity and caused an undetectable shift in sedimentation. However, we also eluted a sample of the same receptor preparation from a Sephadex G-100 column run in 0.3 M NaCl. The receptor pool, when diluted to 0.1 M NaCl, still lacked DNA-binding activity (data not shown). Since the gel filtration would have removed loosely bound cofactors from the receptor, such factors (if they exist) are not dissociable in 0.3 M NaCl.

Differential Sensitivity of PRA and PRB to Reducing Agents. The experiments shown above indicate that the affinity of PRB for nonspecific DNA is not significantly different from that of PRA. These results agree with the nucleic acid sequence data which show that the DNA-binding domains of both receptor forms are identical (Conneely et al., 1987a,b). We speculated, therefore, that differences in the sensitivity of DNA-binding activity to oxidation between both receptor forms is a result of structural difference outside the DNA-binding domain. This structural difference might cause an altered accessibility of reducing agents to cysteines on PRB. To test this hypothesis, we titrated the concentrations of reducing agent required for maintenance of DNA binding of the two receptor forms as shown in Figure 5.

The experiment shown in Figure 5B indicates that the concentrations of β-mercaptoethanol (βME)[1] required to achieve 50% of maximal retention of PRA and PRB on DNA–cellulose columns were 5 and 20 mM, respectively. The concentration of DTT[1] required to achieve the same effect was

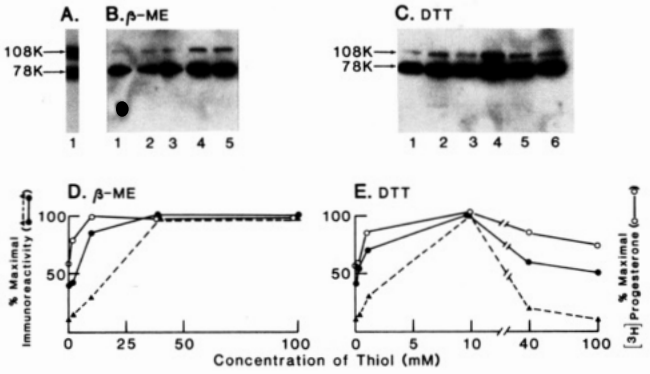

FIGURE 5: Effect of thiol concentration on progesterone receptor binding to DNA–cellulose. [^{3}H]Progesterone receptor complexes were prepared in buffer TEG supplemented with 5 mM β-mercaptoethanol as described under Experimental Procedures. The ammonium sulfate pellets were then resuspended in the same buffer containing 100 mM NaCl, and thiols were added. After 3 h at 4 °C, samples were tested for DNA-binding activity using DNA–cellulose adsorption and elution. Panels A, B, and C show the autoradiograms of samples analyzed after gel electrophoresis, transfer, and immunoblotting with rabbit anti-receptor antibodies as described under Experimental Procedures. Panel A, immunoreactivity in start material. Panel B, samples treated with 0, 1, 10, 40, and 100 mM βME (lanes 1–5, respectively). Panel C, samples treated with 0, 1, 0.1, 10, 40, and 100 mM DTT (lanes 1–6, respectively). Panels D (βME treatment) and E (DTT treatment) express quantitatively the results obtained by densitometric scanning of the autoradiograms: (●—●) Immunoreactivity of the PRA band; (▲---▲) immunoreactivity of the PRB band. Also shown are the results obtained by counting of the tritium content of each sample (○—○).

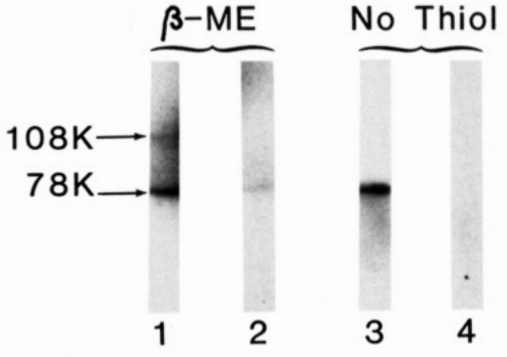

FIGURE 6: Effect of time and reducing environment on progesterone receptor retention on DNA–cellulose. [^{3}H]Progesterone receptor complexes were prepared as described under Experimental Procedures, in TEG buffer with or without 5 mM β-mercaptoethanol. The ammonium sulfate pellets were dissolved in the same buffer containing 100 mM NaCl and applied to a DNA–cellulose column immediately, or after 3 h at 4 °C. Samples of receptor eluted from the DNA–cellulose column at 0.3 M NaCl were electrophoresed, transferred to nitrocellulose filters, and incubated with rabbit anti-receptor antibodies as described under Experimental Procedures. Lane 1, material prepared with β-mercaptoethanol and applied immediately on DNA–cellulose. Lane 2, material prepared with βME and applied on DNA–cellulose after 3 h at 4 °C. Lane 3, material prepared without thiols and applied immediately on DNA–cellulose. Lane 4, material prepared and incubated 3 h at 4 °C without thiols and then applied on DNA–cellulose.

1 mM for PRA and about 3 mM for PRB. The fraction of PRA detected by Western immunoblot was significantly larger than that of PRB. We thus expected to observe that the elution profile of [^{3}H]progesterone from the DNA–cellulose column in this experiment would follow the immunoreactive profile of PRA rather than that of PRB. Figure 5 shows that this is the case; the ^{3}H elution profile indeed resembled that of immunoreactive PRA. We conclude that PRA and PRB forms show different responses to the two reducing agents tested. The experiment also shows that DTT is more efficient in maintenance of receptor binding to DNA since 3–5 times higher concentration of βME was required to achieve 50% of maximal response.

Since PRA showed 30–40% retention of DNA-binding activity even in the absence of reducing agent during 3-h incubation, we speculated that the residual amount of βME in the buffer remaining after preparation of cytosol and ammonium sulfate precipitation was sufficient to maintain this activity. To test this possibility, we prepared cytosol and ammonium sulfate pellets with and without βME, and redissolved the pellets in the same buffers. This material was tested for DNA-binding activity immediately or after 3-h incubation at 4 °C. The results shown in Figure 6 indicate that PRA prepared in βME maintained part of this activity during 3-h incubation in the presence of that thiol but completely lost DNA-binding activity if it was prepared and maintained without thiol. This experiment shows the absolute requirement of reducing agents for DNA-binding activity of both receptor forms and confirms their differential sensitivity to the reducing environment.

Differential Accessibility of SH on PRA and PRB to SH Reactive Reagents. The previous experiments indicated that PRB is more sensitive to oxidation than PRA. Since the PRB responded to thiols less efficiently than PRA, it was possible that some SH groups on this receptor form are less accessible to thiols than on PRA. To test this idea, we examined the accessibility of SH groups important for maintenance of DNA-binding activity by treating receptor with SH reactive reagents.

The reagents *N*-ethylmaleimide and iodoacetamide have been described previously as poor inhibitors of DNA binding of chicken PR (Coty et al., 1983). We modified the conditions previously described and prolonged the incubation period of the reagents with receptor. Since receptor binding to DNA requires the continuous presence of thiols, we compared the effect of these reagents in the absence or presence of low concentrations of DTT. The results of this experiment are shown in Figure 7. Panels A and B show autoradiograms of Western immunoblots using samples eluted from the DNA-cellulose after treatment with the reagents. The bottom panels (C–F) describe the results quantitatively after densitometric scanning of the autoradiograms.

The results of this experiment show that iodoacetamide is more effective than *N*-ethylmaleimide. However, it is clear that PRA was more sensitive to either reagent than was PRB. This difference is particularly apparent when receptor samples were incubated with *N*-ethylmaleimide in the absence of reducing agents (compare panel F to panels C–E). In this experiment, DNA binding of receptor B is completely unaffected by the maleimide while PRA lost 80% of its DNA-binding activity. In the presence of DTT, however, PRB showed a limited response to the maleimide (25% loss of DNA binding) while PRA lost 70% of this activity, in the same incubation conditions. Similarly, PRA maintained only 5% of its DNA-binding activity with iodoacetamide in the absence of DTT, while PRB retained 30% of this activity under the same conditions.

We conclude that iodoacetamide and *N*-ethylmaleimide are significantly less effective inhibitors of the DNA-binding activity of PRB than that of PRA.

Mercurial reagents also react specifically with SH groups and have been reported to have better accessibility to SH groups on proteins in general (Means & Feeney, 1971). Mercurials are efficient blockers of DNA binding of steroid receptors in particular (Coty et al., 1983). In Figure 8, we

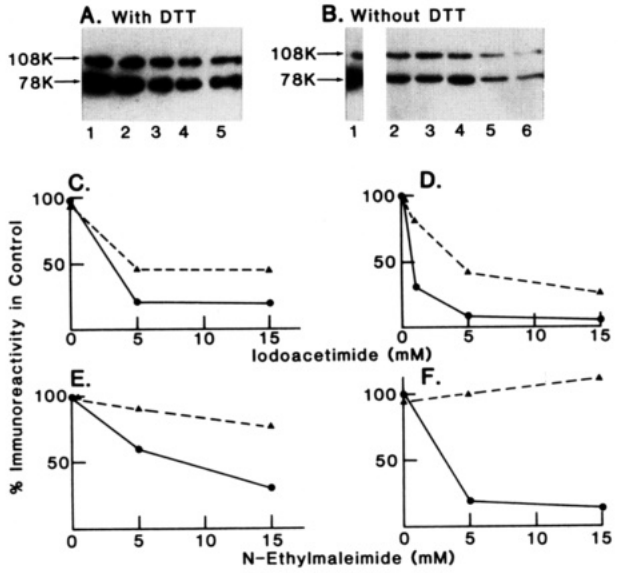

FIGURE 7: Effect of SH blocking reagents on DNA binding of PRA and PRB. [^{3}H]Progesterone receptor complexes were prepared in TEG buffer supplemented with 5 mM β-mercaptoethanol and precipitated by ammonium sulfate as described under Experimental Procedures. The pellets were resuspended in the same buffer adjusted to pH 7, at a final NaCl concentration of 0.1 M. *N*-Ethylmaleimide or iodoacetamide were added with (panels A, C, E) or without (panels B, D, F) 1 mM DTT. After 30-min incubation at 4 °C, the samples were applied to DNA–cellulose columns. The material retained by the columns was step-eluted by 0.3 M NaCl and analyzed after gel electrophoresis, transfer, and immunoblot with rabbit anti-receptor antibodies. Panel A, autoradiograms of samples treated with SH blocking reagent and DTT: lane 1, DTT only; lane 2, 1 mM DTT, 5 mM NEM; lane 3, 1 mM DTT, 15 mM NEM; lane 4, 1 mM DTT, 5 mM iodoacetamide; lane 5, 1 mM DTT, 15 mM iodoacetamide. Panel B, autoradiograms of samples treated with SH blocking reagents only: lane 1, no reagent; lanes 2 and 3, 5 and 15 mM NEM, respectively; lanes 4, 5, and 6, 1, 5, and 15 mM iodoacetamide, respectively. Panels C–F describe quantitatively the results obtained by densitometric scanning of the autoradiograms: (●—●) immunoreactivity of the PRA band; (▲---▲) immunoreactivity of the PRB band.

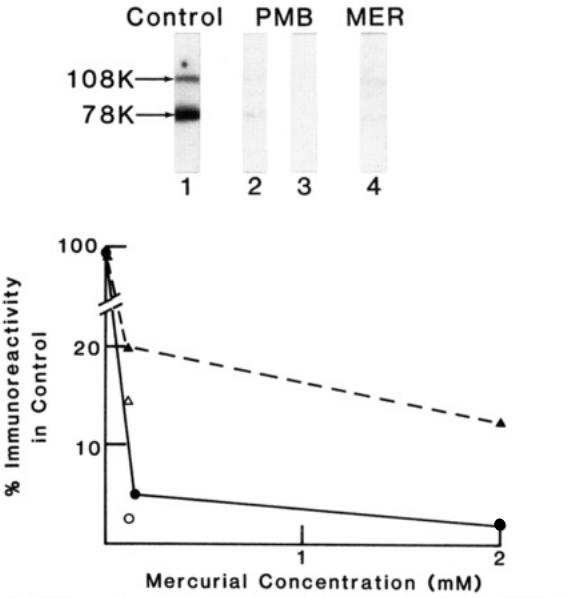

FIGURE 8: Effect of mercurials on DNA binding of PRA and PRB. [^{3}H]Progesterone receptor complexes were prepared and precipitated by ammonium sulfate as described in Figure 7 and under Experimental Procedures. The receptor-containing pellets were resuspended in TEG buffer containing 0.1 M NaCl and (*p*-chloromercuri)benzoate (PMB) or mersalyl acid (MER) and applied immediately to DNA–cellulose columns. The material retained on the columns was eluted by 0.3 M NaCl and analyzed by Western immunoblots as described in Figure 7. The upper panel shows the autoradiograms: lane 1, control, without mercurials, lanes 2 and 3, 0.1 and 2 mM PMB, respectively; lane 4, 0.1 mM mersalyl. The lower panel describes quantitatively the results obtained by densitometric scanning of the autoradiograms: (●—●) immunoreactivity of the PRA band after PMB treatment; (▲---▲) immunoreactivity of PRB band after PMB treatment; (○) PRA after mersalyl; (△) PRB after mersalyl.

show that two mercurials [(*p*-chloromercuri)benzoate and mersalyl] abolish DNA-binding activity of chicken PR at a concentration 150 times lower than *N*-ethylmaleimide or iodoacetamide (0.1 mM). However, the fraction of PRB which remains unaffected is 4–5 times higher (15–20% of control) than the unaffected fraction of receptor A (<5%).

We conclude that SH groups involved in DNA-binding activity of chicken PRB show a general lower sensitivity than SH groups on PRA to a wide variety of SH reactive reagents. Therefore, it is possible that these SH groups are partially masked on the B form of the chicken progesterone receptor.

Discussion

In a recent study (Peleg et al., 1988), we reported that the chicken PR exists in vitro in an oxidized (non-DNA-binding) and a reduced (DNA-binding) state. In that study, we demonstrated that the chicken PRB form (108 kDa) oxidized more easily than the PRA form and speculated that a conformational difference between the two molecules might cause this differential sensitivity to oxidation. In the present study, we examined the possibility that the DNA-binding site of PRB is partially masked, thus rendering the cysteines in that region inaccessible to reducing agents. We compared DNA-binding characteristics of both PRA and PRB and measured the sensitivity of both to a variety of SH reactive reagents.

Our findings indicate a clear difference between the two receptor forms in the responsiveness of cysteines associated with DNA-binding activity to either reducing agents, alkylating reagents, or mercurials. Maintenance of DNA-binding activity of PRB required 3–4-fold higher concentration of thiols than PRA. Alkylating reagents reacted poorly with PRB but very efficiently with PRA. Mercurials altered rapidly DNA binding of both receptor forms but still showed preference for PRA.

Although the two receptor forms in their DNA-binding state showed a clear difference in the accessibility of their cysteines to SH reactive reagents, their binding characteristics to nonspecific DNA are almost identical. Proteins PRA and PRB have overlapping elution profile from DNA–cellulose columns, and they interact similarly with plasmid DNA in solution. This observation is further supported by a recent study by Tsai et al. (1988), who performed DNA-binding studies of chicken PRA and PRB to the GRE/PRE and demonstrated that the interaction sites of the two receptor forms with the GRE/PRE element are identical in vitro. These results suggest that cysteines at the DNA-binding domain are not directly involved in the receptor–DNA interaction, although they are important for maintenance of DNA-binding activity. An explanation for this phenomenon could come from the current model proposed for the structure of the DNA-binding site of steroid receptors in general: it has been suggested that the cysteines in that region interact to form two fingerlike structures (Weinberger et al., 1985; Miesfeld et al., 1986). This interaction is coordinated by zinc (Freedman et al., 1988). If the actual interaction of receptor proteins with DNA is via the "fingers", then masking the cysteines which are located at the base of the fingers will not alter the DNA-binding site per se. However, oxidation of these cysteines due to prolonged alteration of the reducing environment will eventually destroy

the finger structure and DNA-binding activity as well.

A structural feature on chicken PRB which could contribute to the masking of cysteines at the DNA-binding domain could be the long stretch of glutamic acid near the N-terminus of this receptor form (Conneely et al., 1987b). This tract is absent on the PRA, and due to its negative charge could have some affinity to the basic amino acids surrounding the "fingers" at the DNA-binding domain, thus masking the cysteines at that region. Another possibility is that modification of cysteines which alter DNA-binding activity occurred outside the DNA-binding domain as has been suggested for the glucocorticoid receptor (Bodwell et al., 1984). The amino acid sequence of the PRB shows that it contains two cysteines, close to the amino terminus, which do not appear in PRA. Thus, modification of these cysteines by formation of disulfide bonds with cysteines located near the DNA-binding domain could also alter preferentially DNA-binding activity of PRB.

Apparently, the conformational difference between PRA and PRB involves masking of cysteines affecting DNA-binding activity. We speculate that this difference does not affect cysteines associated with the steroid-binding activity which is located closer to the carboxyl end of steroid receptor molecules. This assumption is supported by earlier studies by Coty et al. (1983) which show that steroid-binding activity of chicken PR in cytosol preparations (which contain equivalent amounts of PRA and PRB) is completely (and rapidly) abolished by alkylating reagents or mercurials. Therefore, it appears that steroid-binding activity, but not DNA-binding activity of both progesterone receptor forms, is equally sensitive to SH reactive reagents.

References

Birnbaumer, M., Schrader, W. T., & O'Malley, B. W. (1983) *J. Biol. Chem. 255*, 1637–1644.

Birnbaumer, M., Hinrichs-Rosells, M. V., Cook, R. G., Schrader, W. T., & O'Malley, B. W. (1988) *Mol. Endocrinol. 1*, 249–259.

Bodwell, T. E., Holbrook, N. J., & Munck, A. (1984) *Biochemistry 23*, 4237–4242.

Compton, J. G., Schrader, W. T., & O'Malley, B. W. (1984) *J. Steroid Biochem. 20*, 89–94.

Conneely, O. M., Maxwell, B. L., Toft, D. O., Schrader, W. T., & O'Malley, B. W. (1987a) *Biochem. Biophys. Res. Commun. 149*, 493–501.

Conneely, O. M., Dobson, A. D., Tsai, M. J., Beattie, W., Toft, D. O., Huckaby, C. S., Zarucki, T., Schrader, W. T., & O'Malley, B. W. (1987b) *J. Mol. Endocrinol. 1*, 517–525.

Coty, W. A., Klooster, T. A., Griest, R. E. & Profita, J. A. (1983) *Arch. Biochem. Biophys. 225*, 748–757.

Dugaiczyk, A., Woo, S. L. C., Colbert, D. A., Lai, E. C., Mace, M. D., & O'Malley, B. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2253–2257.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavppa, R., Sigler, P. B., & Yamamoto, K. P. (1988) *Nature 334*, 543–546.

Gronemeyer, H., Govindan, M. V., & Chambon, P. (1985) *J. Biol. Chem. 260*, 6916–6925.

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

Maniatis, T., Fritsch, E. F., & Sambrook, T. (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Spring Harbor, NY.

Means, G. E., & Feeney, R. E. (1971) in *Chemical Modification of Proteins*, Holden-Day, San Francisco, CA.

Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikström, A. C., Gustaffson, J. A., & Yamamoto, K. R. (1986) *Cell 46*, 389–399.

Peleg, S., Schrader, W. T., & O'Malley, B. W. (1988) *Biochemistry 27*, 358–367.

Puri, R. K., Grandics, P., Daugherty, J. J., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 10831–10837.

Schrader, W. T., Toft, D. O., & O'Malley, B. W. (1972) *J. Biol. Chem. 247*, 2401–2407.

Towbin, H., Staehelin, T., & Gordon, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, T. A., Tsai, M.-J., & O'Malley, B. W. (1988) *Cell 55*, 361–363.

Vedeckis, W. V., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry 19*, 343–349.

Weinberger, C., Hollenberg, S. M., Rosenfeld, M. G., & Evans, R. M. (1985) *Nature 318*, 670–672.