Evidence for Distinct Sulfhydryl Groups Associated with Steroid- and DNA-Binding Domains of Rat Thymus Glucocorticoid Receptors†

Jack E. Bodwell,* Nikki J. Holbrook,‡ and Allan Munck

ABSTRACT: We have found that nonactivated and activated forms of the rat thymus glucocorticoid–receptor complex (GRC) will react with reactive sulfhydryl matrices to form covalently immobilized complexes that can subsequently be eluted with reducing agents. The interaction of GRCs with these matrices depends on the nature of both the immobilized sulfhydryl group and the type of leaving group attached. One matrix, agarose CL-4B–diaminoethyl–succinyl–thioethylamine–2-thiopyridyl (DSTT), binds total receptor-bound steroid. A second matrix, agarose CL-4B–diaminoethyl–succinyl–cysteinyl–2-thiobenzoic acid (DSCT), binds activated but not nonactivated complexes. The reaction of activated complexes with the DSCT matrix is apparently through a sulfhydryl group located near the DNA binding domain, as soluble DNA interferes with the reaction. This sulfhydryl group(s) appears to be located in a portion of the GRC that is resistant to degradation, since proteolytic digestion of activated GRC to a point where DNA binding is lost results in only a moderate decrease in binding with the DSCT matrix. Purified receptor, covalently labeled with [^{3}H]dexamethasone to the sulfhydryl associated with the steroid binding domain, was able to bind to DSCT matrix, providing evidence for distinct sulfhydryl groups associated with the steroid and DNA binding domains.

The physiological effects of glucocorticoids on target cells are mediated by specific receptor molecules through a mechanism that consists of several stages. The initial stage involves the formation of a cytosolic glucocorticoid–receptor complex (GRC),[1] a form designated as nonactivated. The second stage is characterized by the transformation of the nonactivated complex to an activated form characterized by the ability to bind to nuclei and DNA (Munck & Foley, 1979). The transformation process, or "activation", is usually initiated by the application of heat (25–37 °C) for a specified period of time (15–30 min), although in cell-free systems high ionic strength (Higgins et al., 1975), gel filtration, and dilution (Goidl et al., 1977) are also used. The activated complex is distinguishable from the nonactivated form by increased affinity not only for nuclei and DNA but also for ATP–Sepharose (Moudgil & John, 1980), phosphocellulose (Kalimi et al., 1975), and carboxymethylcellulose (Milgrom et al., 1973). The activated complex also has a smaller molecular weight (Holbrook et al., 1983) and a weaker interaction with DEAE-cellulose (Sakaue & Thompson, 1977) than the nonactivated form. An even smaller GRC is the meroreceptor which does not bind to DEAE-cellulose or DNA–cellulose and is thought to be a degradation product.

Glucocorticoid receptors in rat thymus and liver cytosols are sensitive to sulfhydryl-modifying reagents. Treatment of unbound receptors with such reagents prevents binding of glucocorticoid to form GRC complexes (Schaumburg, 1972; Kobolinski et al., 1972; Rees & Bell, 1975). However, once the glucocorticoid has bound to the receptor, these reagents have little effect on the integrity of the complex (Young et al., 1975; Kalimi & Love, 1980; Bodwell et al., 1984). One class of sulfhydryl-modifying reagents, the organomercurials, are exceptions and completely dissociate the glucocorticoid–receptor complex (Banerji & Kalimi, 1981). In addition to the sulfhydryl group presumed from these results to be associated with the steroid binding domain, there is also evidence for a sulfhydryl group associated with the receptor's DNA binding domain as treatment of activated complexes from rat thymus with sulfhydryl-modifying reagents will significantly decrease binding to DNA–cellulose (Bodwell et al., 1984).

Sulfhydryl-modifying reagents could be inhibiting glucocorticoid and DNA binding by reacting either with a single sulfhydryl that has access to both binding domains or with separate sulfhydryl groups associated with each domain. We have used covalent chromatography and affinity labeling to study this problem and present evidence that there are separate sulfhydryl groups associated with each binding domain.

Experimental Procedures

Materials

[6,7-^{3}H]Dexamethasone (38–50 Ci/mmol), [^{3}H]dexamethasone 21-mesylate, and [^{3}H]TA were purchased from New England Nuclear, Boston, MA. Unlabeled dexamethasone and unlabeled TA were purchased from Steraloids, Wilton, NH. Cellulose CF11 was obtained from Whatman, Clifton, NJ. P60 (100–200 mesh) gel filtration matrices and hydroxylapatite (Bio-Gel HT) were obtained from Bio-Rad Laboratories, Richmond, CA. Calf thymus DNA was purchased from P-L Biochemicals, Milwaukee, WI. All other reagents and enzymes were obtained from Sigma Chemicals, St. Louis, MO.

Methods

Buffers. The buffers used in this study were the following: buffer A, 10 mM HEPES, pH 7.6; buffer B, 50 mM HEPES, pH 7.6; buffer C, 50 mM HEPES, pH 8.0; buffer D, buffer

† From the Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire 03756. *Received January 4, 1984.* This research was supported by Research Grants AM 03535 and CA 17323 from the National Institutes of Health and by Norris Cotton Cancer Center Core Grant CA 23108 from the U.S. Public Health Service. N.J.H. was supported, during part of these studies, by National Research Service Award CA09367 and currently by a Fellowship Award from the Leukemia Society of America.

‡ Present address: Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20014.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HAP, hydroxylapatite; DSS, agarose CL-4B–diaminoethyl–succinyl–*N*-hydroxysuccinimide ester; DSCT, agarose CL-4B–diaminoethyl–succinyl–cysteinyl–2-thiobenzoic acid; DSTT, agarose CL-4B–diaminoethyl–succinyl–thioethylamine–2-thiopyridyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GRC, glucocorticoid–receptor complex; TA, triamcinolone acetonide; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-4237$01.50/0

C plus 0.3 M NaCl and 1 mM EDTA. Buffers were adjusted to the appropriate pH at 23 °C.

Preparation and Activation of ^{3}H-Labeled Glucocorticoid–Receptor Complexes. As described in detail elsewhere (Bodwell et al., 1984), suspensions of adrenalectomized rat thymus cells (0.3–0.4 mL of packed cells/mL of suspension) were incubated in Krebs–Ringer bicarbonate buffer supplemented with 10 mM glucose and 10 mM HEPES,[1] with 10–30 nM [^{3}H]dexamethasone or 10–30 nM [^{3}H]TA for 2 h at 0 °C. Cytosols were prepared by adding the cell suspension to 5 volumes of lysing solution (3 °C). This solution contained 80% (v/v) 1.5 mM $MgCl_2$ with dextran-coated charcoal (Munck & Wira, 1975) and 20% WEHI-7 stabilizing factor. This factor greatly stabilizes thymus cytosols against degradation and was prepared as described by Holbrook et al. (1984). After 15 min the preparation was centrifuged for 2.5 min at 11000*g* to obtain cytosols containing the ^{3}H-labeled GRC.

The same general procedure was used to obtain complexes covalently labeled with [^{3}H]dexamethasone. [^{3}H]Dexamethasone 21-mesylate (10–30 nM) was used during the incubation, the lysing solution was supplemented with 15 mM HEPES (pH 8.5, 0 °C), and the cytosol was left at 0 °C for 20 min after centrifugation to ensure good covalent attachment to the receptor.

Cytosols were activated by warming at 25 °C for 15 min.

Measurement of Radioactivity and Specific Binding. Radioactivity was assayed with a Packard 3390 liquid scintillation counter using Hydrofluor scintillation fluid (National Diagnostics) at about 34% efficiency for tritium. All samples were corrected for quenching by the external standard method. Samples containing chromatography matrices that had GRC covalently labeled with [^{3}H]dexamethasone bound to them had poor counting efficiencies. This was overcome by eluting the complexes from DEAE-cellulose, DNA–cellulose, and HAP by pretreating with 1 mL of 3 N KCl for 60 min (25 °C) and from DSCT and DSTT with 1 mL of 1 M mercaptoethanol (60 min, 25 °C) before adding 4 mL of Hydrofluor.

Initial experiments indicated that nonsaturable binding, measured in the presence of 2.6 μM unlabeled and 10–30 nM [^{3}H]dexamethasone or [^{3}H]TA, was about 5% of total receptor-bound steroid. This amount was considered negligible, so no correction has been applied for nonsaturable binding. Crude cytosols labeled with [^{3}H]dexamethasone 21-mesylate have substantial nonsaturable binding (measured in the presence of 2.6 mM cold TA); this value was always subtracted from total binding to give saturable binding.

Assays. Binding to DNA–cellulose and HAP was measured as described elsewhere (Bodwell et al., 1984). Minicolumn analysis, a rapid chromatographic method for the simultaneous determination of nonactivated, activated, and mero (degraded) forms of the receptor, was used as described previously (Holbrook et al., 1983) except that buffer A was used to equilibrate and wash the columns.

To determine the fraction of GRC covalently labeled with [^{3}H]dexamethasone, samples were made 1% in Triton X-100, boiled for 2 min, and cooled to 25 °C, and a 100-μL aliquot was applied to a 0.25-mL column of HAP packed in a 1.0 mL syringe (25 °C). After the sample was allowed to enter the bed, the column was washed with 4 mL of buffer A. The column and a sample of the eluate were counted for radioactivity; the amount of ^{3}H bound to HAP was used as a measure of covalently bound dexamethasone.

Binding to DSCT and DSTT matrices was performed at 3 °C by applying a 50-μL sample to a 0.45 × 1.15 cm (0.2 mL) column (equilibrated in buffer B) and allowing the sample to enter the bed. After 5 min the column was washed with 4 mL of equilibrating buffer. Both the matrix and a sample of the eluant were assayed separately for radioactivity.

Gel Filtration. Cytosols were desalted on small columns as described previously (Bodwell et al., 1984) except that P6DG was used instead of the P60 matrix.

Preparation of Reactive Sulfhydryl Matrices. Agarose CL-4B was activated with 2,2′-carbonyldiimidazole (0.12 g/3 mL of acetone-washed agarose) by the method of Bethel et al. (1979) except that dry acetone was used as the solvent. After 15 min at 23 °C the activated agarose was washed on a sintered glass funnel with dry acetone to remove reaction products. One milliliter of activated agarose was added to 2 mL of cold 0.9 M ethylenediamine (pH 10) and was gently agitated overnight at 3 °C. The coupled agarose was then washed with water, 1 M NaCl, and water, followed by succinylation as described by Parikh et al. (1974). This procedure usually had to be repeated in order to couple all of the amino groups as judged by reaction with fluorescamine (Udenfriend et al., 1972) and visual observation with a hand-held UV lamp. The agarose–diaminoethyl–succinic acid was converted to the *N*-hydroxysuccinimide ester as described by Parikh et al. (1974) except that dry acetone was used as the solvent. The resultant agarose–diaminoethyl–succinyl–*N*-hydroxysuccinimide ester (DSS) was then used to synthesize the various reactive sulfhydryl- and protease-containing matrices.

Agarose–diaminoethyl–succinyl–cysteinyl-2-thiobenzoic acid (DSCT) was prepared by reacting 1 mL of DSS with 2 mL of 0.3 M cysteine in buffer B overnight at room temperature. The gel was washed with at least 10 volumes of water, buffer A, 1 N NaCl, and buffer D. The washed gel was shaken (60 cpm) for 60 min at 25 °C with 3–5 volumes of 50 mM dithioerythritol in buffer D. The gel was then washed with buffer C until no sulfhydryl groups were detected in the washes (Elman, 1958). The leaving group was attached by placing the gel into 5 volumes of 3.2 mM bis(2,2′-carboxyphenyl) disulfide in buffer C. After 24 h at 25 °C the DSCT was stored in the same solution at 3 °C, and when needed the appropriate volume of matrix was removed and washed before each experiment.

Agarose–diaminoethyl–succinyl–thioethylamine–thiopyridyl (DSTT) was prepared as described for DSCT except that DSS was incubated with 0.3 M cystamine in buffer D and the leaving group was attached with (and stored in) a solution containing 40% ethanol–4.5 mM, 2,2′-dipyridyl disulfide–10% buffer D.

There were no detectable sulfhydryl groups on either of the matrices as judged by reaction (Elman, 1958) with 5,5′-dithiobis(2,2′-nitrobenzoic acid). The content of reactive disulfide groups was determined by reducing the matrices with 50 mM dithioerythritol in buffer C for 30 min at 25 °C, washing out the dithioerythritol (buffer C), reacting the sulfhydryl groups on the matrix with 1 mM 5,5′-dithiobis-(2,2′-nitrobenzoic acid) for 5 min, washing with buffer C, eluting the bound thionitrobenzoate with 5 mM dithioerythritol in buffer C, and determining the absorbance at 412 nM (E_{412} = 13 600 m^{-1} cm^{-1}; Elman, 1958). Disulfide content for the matrices ranged between 1.5 and 2.5 μmol/mL of packed matrix.

Preparation of Proteolytic Matrices. DSS matrix was reacted with 1.5 volumes of a 1.0–1.2 mg/mL solution of trypsin, α-chymotrypsin, thermolysin, subtilisin BPN, or *Staphylococcus aureus* strain V8 protease in buffer B containing 20 mM $CaCl_2$ overnight at 0 °C. Nonreacted *N*-hydroxy-

Table I: Binding of Activated and Nonactivated GRC to DNA-Cellulose, DSTT, and DSCT[a]

treatment of cytosol	receptor-bound [^{3}H]TA (cpm) applied to columns	[^{3}H]TA (cpm) retained on columns: DNA-cellulose	DSCT	DSTT
		DSCT		
unwarmed	4796 ± 54	162 ± 27	133 ± 5	
warmed before DNA-cellulose	3698 ± 18	2359 ± 36	2334 ± 134	
warmed after DNA-cellulose	764 ± 34	159 ± 13	200 ± 10	
		DSTT		
unwarmed	4941 ± 98	327 ± 51		4938
warmed	4807 ± 101	2717 ± 17		4940

[a] Warmed or unwarmed cytosol, labeled with [^{3}H]TA, was assayed for binding to DNA-cellulose and the DSCT matrix as described under Experimental Procedures. The activated cytosol (1 mL) was then put over a DNA-cellulose column (0.9 × 5.1 cm), the column was washed with equilibrating buffer (10 mM HEPES, pH 7.6), and each fraction (0.5 mL) was assayed for HAP binding. The fraction containing the most receptor-bound tritium was assayed for binding to DNA-cellulose and DSCT. Values are the means (± half of the range) of duplicate samples, except for DSTT data which were determined from single columns.

succinimide esters were eliminated by incubating the matrices for 60 min at 25 °C after adding ethanolamine to a final concentration of 100 mM from a 10-fold concentrated stock solution that had been adjusted to pH 7.6. The degree of substitution for each gel was approximately 1 mg/mL as judged from protein determinations (Bradford, 1976) of the supernatants after the coupling procedure was complete.

SDS-PAGE. Electrophoresis was performed as described previously (Bodwell & Meyer, 1981) except that a 5–15% acrylamide gradient separating gel was used. Samples were prepared for SDS-PAGE by adding 45% trichloroacetic acid to obtain a 10% final concentration. After 16 h at 0 °C, samples were centrifuged for 10 min at 10000*g*, and the supernates were removed. The pellets were redissolved in 2–10 μL of 1 N NaOH, and boiling sample buffer (50 μL) was added immediately. The tubes were quickly placed in a boiling water bath for 2 min, cooled, and electrophoresed.

After staining/destaining (Bodwell & Meyer, 1981), the gels were sliced into 2-mm segments and the segments placed into glass vials with 10 mL of Econofluor with 3% protosol (New England Nuclear). Vials were counted after 24 h at 37 °C. Molecular weights of various peaks were estimated from a plot of the migration distance (slice number) vs. the logarithm of molecular weights for the following standards: rabbit muscle phosphorylase B (M_r 97 400), bovine serum albumin (M_r 68 000), bovine liver catalase (M_r 58 000), hen egg albumin (M_r 45 000), rabbit muscle aldolase (M_r 40 000), rabbit muscle lactate dehydrogenase (M_r 36 000), and bovine erythrocyte carbonic anhydrase (M_r 29 000).

Results

Binding of GRC to DSCT and DSTT. The reactivity of sulfhydryl groups on model peptides is sensitive to changes in the local environment. Peptides with different neighboring amino acids can have reaction rates with sulfhydryl-modifying reagents that differ by many orders of magnitude (Snyder et al., 1981). Since the glucocorticoid–receptor complex undergoes marked changes in conformation and DEAE binding properties during activation, a sulfhydryl group located in the area on the receptor where the DNA binding site is formed or unmasked might have sufficiently different reactivity between the nonactivated and activated forms that it could be distinguished by chromatography on reactive sulfhydryl matrices.

Reactive sulfhydryl columns (Brocklehurst, 1979) function as shown in Figure 1A. A thio anion on the glucocorticoid–receptor complex undergoes a thio–disulfide interchange reaction with the disulfide of the matrix, thereby covalently attaching the complex to the matrix (I). This process can be reversed and the complex eluted from the column by the addition of compounds containing a sulfhydryl group (II).

We have synthesized over 20 reactive sulfhydryl matrices with different spacer arms and leaving groups. Of these, the matrices designated as DSCT and DSTT (Figure 1B) have proved to be the most useful. The DSTT matrix binds nonactivated and activated GRC (Tables I and II), while the DSCT matrix binds mainly activated complexes. Many of the columns synthesized gave results intermediate between DSCT and DSTT except for a diamino-succinyl-cysteinyl-thioaminophenol column which bound receptor poorly (data not shown).

To establish that the DSCT matrix binds activated complexes while binding little if any nonactivated complex, no-

Table II: Binding of GRC Covalently Labeled with Dexamethasone to DSCT, DSTT, and Minicolumns[a]

treatment	GRC (cpm) bound to minicolumns: activated (DNA)	nonactivated (DEAE)	mero (HAP)	total	reactive sulfhydryl columns: DSTT	DSCT
	Experiment I					
original cytosol	57	1289	76	1422	1166	0
after first DNA column and warming	654	17	85	756	632	670
after elution from second DNA column and desalting	1391	60	34	1485	1634	1375
	Experiment II					
original cytosol	76	1193	143	1412	1334	0
after first DNA column and warming	792	176	96	1064	1102	538
after elution from second DNA column and desalting	3611	177	110	3898	3880	3307

[a] Unwarmed cytosol (8 mL), covalently labeled with dexamethasone 21-mesylate (with and without 2.6 × 10^{-6} M unlabeled TA), was applied to a DNA-cellulose column (0.9 × 3.4 cm) equilibrated in 10 mM HEPES (pH 7.6) buffer and the column washed with 3 mL of the same buffer. The eluate was warmed to 25 °C for 15 min, cooled to 3 °C, and applied to a second DNA-cellulose column (0.9 × 2.5 cm). After washing with 50 mM HEPES buffer (pH 7.6), the GRC was eluted from the column with 50 mM HEPES–0.4 M NaCl buffer (pH 7.6) containing 0.1 mg/mL ovalbumin. The NaCl was removed by gel filtration as described under Experimental Procedures. Binding of GRC to minicolumns, DSCT, and DSTT was determined on aliquots obtained from the original cytosol, after it had been warmed and after gel filtration. cpm represent saturable binding. Minicolumn analysis determines the amount of activated (DNA-cellulose binding), nonactivated (DEAE-cellulose binding), and meroreceptor (HAP binding) forms of the GRC in a particular sample. Total receptor is the sum of activated, nonactivated, and meroreceptor forms of the GRC. The increase in cpm observed after the gel filtration step is due to the concentrating effect of the second DNA-cellulose column.

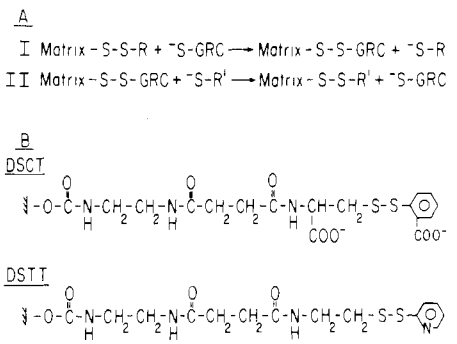

FIGURE 1: See text for details.

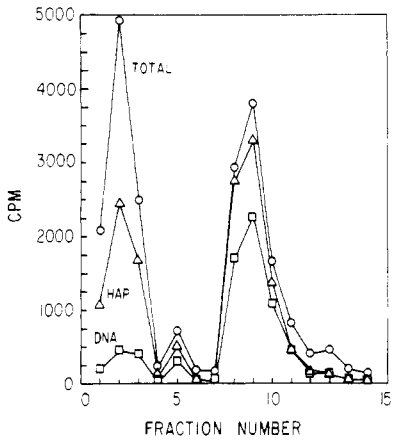

FIGURE 2: DSCT chromatography of warmed cytosol labeled with [^{3}H]dexamethasone. Warmed cytosol (1 mL) was applied to a 0.45 × 1.15 cm column (0.2 mL) of DSCT equilibrated in 50 mM HEPES (pH 7.6) buffer, and the column was then washed in the same buffer. At fraction 4 the eluting buffer was changed to 50 mM HEPES (pH 8.)–0.3 M NaCl and at fraction 7 to 50 mM HEPES (pH 8.)–75 mM mercaptoethylamine. Fraction size was 0.5 mL. Each fraction was assayed for total radioactivity (○), for binding to HAP, which gives total receptor bound tritium (△), and for binding to DNA–cellulose, which gives activated GRC (□).

nactivated complexes were assayed for binding to DNA–cellulose and the DSCT matrix. As shown in Table I, nonactivated complexes bind poorly to both DNA–cellulose and the DSCT matrix. In contrast activated complexes bind equally well to both DNA–cellulose and DSCT columns. After removal of activated complexes from the cytosol by passage over a 2-mL DNA–cellulose column, binding to DSCT matrix and DNA–cellulose were at low levels. Additional evidence that the DSCT matrix does not bind non-activated complexes is presented in Figure 2. Here a warmed cytosol was applied to a column of DSCT. Less than 20% of the GRC that initially passed through the column (fractions 1–4) bound to DNA–cellulose and probably represents nonactivated complexes. Washing the column with high salt buffer (0.3 N NaCl) did not remove a significant amount of GRC from the matrix (fractions 5–7), but 50 mM mercaptoethylamine (in buffer C) effectively eluted the bound complexes. Other reducing agents such as dithioerythritol, cysteine, and mercaptoethanol will elute the bound GRC but less effectively (data not shown). Elution by mercaptoethylamine caused little dissociation of the steroid from the complex, and over 70% of the eluted complexes bound to DNA–cellulose.

From the data presented above it appears that the GRC is probably bound to the DSCT column through a disulfide bond between the complex and the matrix. In fact, among many compounds we have tested, only those with sulfhydryl groups will elute the GRC from the matrix. Activated GRC, covalently labeled with [^{3}H]dexamethasone 21-mesylate (see Dexamethasone 21-Mesylate Studies), were not significantly eluted from the DSCT column with 1% sodium dodecyl sulfate (89% of the complexes retained) which would disrupt hydrophobic interactions or with 1% Triton X-100 in 2 M KCl (95% of the complexes retained) which would disrupt ionic interaction. Furthermore, when activated complexes labeled with [^{3}H]dexamethasone 21-mesylate were treated with the sulfhydryl-modifying reagent *p*-(hydroxymercuri)phenylsulfonic acid (1 mM final concentration, 25 °C, 30 min), binding to the DSCT matrix was reduced by 52% from controls treated with buffer alone, and binding to DNA–cellulose was reduced by 52%. Thus, all our data are consistent with the idea that the GRC undergoes a thio–disulfide interchange with the DSCT matrix and becomes covalently attached to the matrix through a disulfide bond.

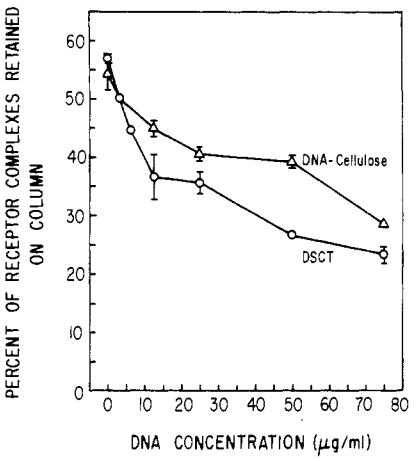

FIGURE 3: Inhibition of the binding of GRC to DNA–cellulose and DSCT by soluble DNA. Activated cytosol was made up to the appropriate DNA concentration by addition from a 10-fold concentrated stock solution. Cytosols were then applied to DNA–cellulose or DSCT columns preequilibrated with the appropriate concentration of DNA. The columns were run as described under Experimental Procedures except that eluting buffer contained the same concentration of DNA as it was equilibrated with. Values are the means of duplicate determinations. Error bars when larger than the symbol show the range of values.

Inhibition of DSCT and DNA–Cellulose Binding by Soluble DNA. The data from experiments of Figure 1 and Table I plus previously reported data (Bodwell et al., 1984) suggested that the activated complex might be interacting with the DSCT matrix through a sulfhydryl group located within the DNA-binding domain. If this hypothesis is correct, then DNA might block binding of the complex to the DSCT matrix. As shown in Figure 3, the addition of different concentrations of calf thymus DNA to cytosols before applying them to DNA–cellulose or DSCT columns (equilibrated in the same concentrations of DNA) reduced complex binding to both matrices. Binding to DSCT matrix was inhibited slightly more than to DNA–cellulose, but both columns had similar DNA concentration-dependent inhibition. This supports the hypothesis that the DSCT matrix is interacting with a sulfhydryl(s) located near the DNA binding domain.

Dexamethasone 21-Mesylate Studies. Assuming that GRC interact with the DSCT matrix via a sulfhydryl group located near the DNA binding site, we determined if this sulfhydryl group was also associated with the glucocorticoid binding domain by blocking the sulfhydryl group in the steroid binding site by covalently labeling the receptor with dexamethasone 21-mesylate, a glucocorticoid that selectively reacts with sulfhydryl groups to form a stable thioether bond (Simons et al., 1980). Failure of the covalently labeled GRC to bind to

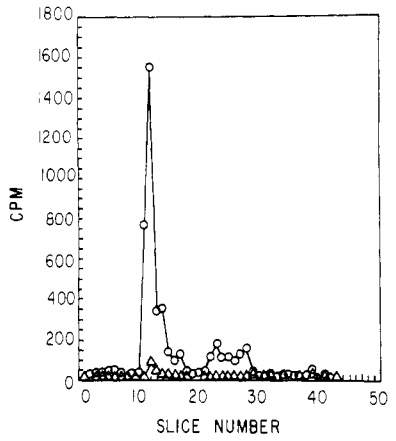

FIGURE 4: SDS–PAGE of purified GRC covalently labeled with [^{3}H]dexamethasone. A sample of the purified GRC from the experiment described in Table II was subjected to SDS–PAGE as described under Experimental Procedures. The symbol (○) represents saturable binding and (△) nonsaturable binding (determined in the presence of 2.6×10^{-6} M unlabeled TA).

the DSCT matrix would suggest that a single sulfhydryl group was associated with both the glucocorticoid and DNA binding domains. Alternatively, binding to the matrix would suggest that different sulfhydryl groups are involved.

GRC, covalently labeled with dexamethasone 21-mesylate, were purified by using a two-stage DNA–cellulose procedure (Simons et al., 1983) in order to determine which of the two possiblies was correct. Cells were incubated at 0 °C with [^{3}H]dexamethasone 21-mesylate, and the cytosols obtained were assayed for binding to DSCT, DSTT, and minicolumns. Minicolumns consist of three 0.2-mL columns (DNA–cellulose, DEAE-cellulose, and HAP) connected in series. As a sample containing GRC moves through the columns, the activated complexes bind to the DNA–cellulose, the nonactivated complexes bind to the DEAE-cellulose, and the meroreceptor binds to HAP (Holbrook et al., 1983). Assay of total radioactivity bound to each column thus permits rapid determination of the different forms of the GRC and also measures total receptors as the sum of the three columns.

Minicolumn analysis of the unwarmed cytosol showed that most GRC were in the nonactivated form (Table II, column labeled DEAE) with only small amounts of activated (column labeled DNA), and meroreceptor forms (column labeled HAP). There was negligible binding to the DSCT matrix. The cytosol was passed through a DNA–cellulose column to remove DNA binding proteins and was then warmed to 25 °C for 15 min. Minicolumn analysis at this stage revealed that most of the complexes were in the activated form. There was also substantial binding to the DSCT matrix. The activated complexes were then bound to a second DNA–cellulose column and eluted from that column with 0.4 M salt. The eluate was desalted by gel filtration and analyzed again by minicolumn. There is a good correlation in Table II between the amount of activated receptor complexes (that which is bound to DNA–cellulose on the minicolumns) and the amount bound to the DSCT matrix. This correlation suggests that dexamethasone 21-mesylate and the DSCT matrix do not interact with the same sulfhydryl group on the receptor. Apparently the GRC binds to the matrix through a sulfhydryl group(s) associated with the DNA binding domain.

The GRC purified by the above procedure show a large peak with a molecular weight of 87 000 ± 2000 (N = 4) and is contaminated only slightly by degradative forms of the GRC (Figure 4). In four such experiments where the amount of [^{3}H]dexamethasone covalently bound to the receptor was

Table III: Binding of GRC to DSCT and Minicolumns after Protease Treatment[a]

	% of total receptor-bound [^{3}H]TA applied to				
	minicolumns				reactive sulfhydryl column, DSCT (bound)
treatment	activated (DNA)	nonactivated (DEAE)	mero (HAP)	free	
Immobilized Proteases					
matrix (CL-4B)	52	14	21	13	58
trypsin	3	4	79	13	46
chymotrypsin	34	6	44	15	58
thermolysin	2	2	78	18	48
subtilisin BPN	7	4	73	15	45
S. aureus V8	51	12	25	12	62
Endogenous Thymic Enzyme(s)					
0 h	45	20	27	8	47
24 h	4	9	69	17	23

[a] Warmed cytosol prepared without stabilizing factor and labeled with [^{3}H]TA was made 50 mM in HEPES (pH 7.6) and 10 mM in $CaCl_2$ by the addition of 20-fold concentrated stock solutions; 50 μL of the appropriate immobilized protease was added to 1 mL of cytosol and incubated for 60 min at 3 °C with slight agitation. The supernatant was removed following centrifugation (10000g for 30 s) and assayed for binding to minicolumn, DSCT, and DSTT (see Experimental Procedures). Thymic digestion was performed by allowing the cytosol to sit for 24 h at 3 °C. Assays were performed as above at 0 and 24 h. The values presented are the means of duplicate samples, and the range was less than ±2%.

monitored, at least 95% of the ^{3}H was covalently attached (see Experimental Procedures).

There may in fact be more sulfhydryl groups on the GRC as the DSTT matrix binds total receptor-bound steroid throughout the purification procedure, indicating the presence of a reactive sulfhydryl group(s) even on the nonactivated GRC. The DSTT matrix may have different reactivity for the same sulfhydryl(s) that reacts with the DSCT matrix or may react with different sulfhydryls on the receptor.

Removal of DNA Binding Function with Proteolytic Enzymes. Since the DSCT matrix seems to react with a sulfhydryl group in the DNA binding domain, we attempted to digest the GRC to a point where binding to DNA–cellulose was lost to see what effect this had on DSCT binding. Two protocols were used. The first consisted of incubating a warmed cytosol overnight at 3 °C, allowing the native enzymes to digest the complex (Holbrook et al., 1983). The second involved incubating warmed cytosols with immobilized proteases or Sepharose CL-4B for 1 h at 0 °C and then assaying the supernatant for binding to DSCT and minicolumns.

As shown in Table III, treatments of GRC with immobilized trypsin, thermolysin, subtilisin, and the thymus cell enzymes all gave similar results. There was a drastic loss of DNA–cellulose binding with a corresponding large increase in HAP binding, indicating that proteolysis has occurred. However, there was only a moderate decrease in DSCT binding. This result may mean that the DNA binding site is not totally excised by protease treatment, so that the sulfhydryl group that reacts with the DSCT matrix remains with the partially digested complex. The decrease in DSCT binding might be accounted for by a reduction in that sulfhydryl group's reactivity as a result of the protease treatment. Alternatively, there could also be more than one cleavage site in the DNA binding domain so that multiple cleavage products are obtained, some forms retaining the sulfhydryl while others do not.

If the sulfhydryl group associated with DNA binding is located within the binding domain, then meroreceptor forms could be produced from cleavage within the DNA-binding

domain without total removal of the domain.

Discussion

The usual purpose of chromatography on reactive sulfhydryl matrices is to separate sulfhydryl-containing macromolecules from molecules without a sulfhydryl group. However, by altering the composition of the matrix, we have been able to use this techique to selectively bind to a particular sulfhydryl in the presence of at least one other sulfhydryl group and probably others. The DSCT matrix has allowed us to detect a sulfhydryl group that is apparently located near the DNA binding domain yet is distinct from the sulfhydryl group associated with steroid binding.

While this paper was in preparation, a report appeared by Harrison et al. (1983) demonstrating that mouse AtT-20 GRC labeled with dexamethasone 21-mesylate, after denaturation by sodium dodecyl sulfate, would bind to a column of immobilized *p*-(chloromercuri)benzoate (a sulfhydryl binding matrix). In addition, tryptic digestion of labeled cytosol resulted in a meroreceptor that, after being denatured by sodium dodecyl sulfate, also bound to the *p*-(chloromercuri)benzoate column. They concluded that there was more than one sulfhydryl group on the receptor and that some of these groups were near the steroid binding domain since the sulfhydryl(s) remained on the meroreceptor. Although our data are consistent with those of Harrison et al., some of our interpretations are different. We agree with them that there is more than one sulfhydryl group on the receptor. However, because we have used nondenaturing conditions and different types of reactive sulfhydryl matrices, we have been able to pinpoint a sulfhydryl group that appears to be located in a region on the receptor associated with a specific biological function—the DNA binding domain. As this sulfhydryl appears to be retained on the meroreceptor, we suggest that it is probably associated with the remnants of the original DNA binding domain. Of course, these sulfhydryl groups (as well as others) may still be in close proximity to each other even though they appear to be associated with different receptor functions.

Acknowledgments

We thank Fiona Fillian for her excellent technical assistance.

References

Banerji, A., & Kalimi, M. (1981) *Steroids 37*, 409.
Bethell, G. S., Ayers, J. S., Hancock, W. S., & Hearn, M. T. (1979) *J. Biol. Chem. 254*, 2572.
Bodwell, J. E., & Meyer, W. L. (1981) *Biochemistry 20*, 2767.
Bodwell, J. E., Holbrook, N. J., & Munck, A. (1984) *Biochemistry 23*, 1392.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Brocklehurst, K. (1979) *Int. J. Biochem. 10*, 259.
Elman, G. L. (1958) *Arch. Biochem. Biophys. 74*, 443.
Goidl, J. A., Cake, N. H., Dolan, K. P., Parchman, L. G., & Litwack, G. (1977) *Biochemistry 16*, 2125.
Harrison, R. W., Woodward, C., & Thompson, E. (1983) *Biochim. Biophys. Acta 759*, 1.
Higgins, S. J., Rousseau, G. C., Baxter, J. D., & Tomkins, G. M. (1973) *J. Biol. Chem. 248*, 5866.
Holbrook, N. J., Bodwell, J. E., Jeffries, M., & Munck, A. (1983) *J. Biol. Chem. 258*, 6477.
Holbrook, N. J., Bodwell, J. E., & Munck, A. (1984) *J. Steroid Biochem. 20*, 245.
Kalimi, M., & Love, K. (1980) *J. Biol. Chem. 255*, 4687.
Kalimi, M., Colman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080.
Kobolinski, M., Beats, M., Kalimi, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7897.
Milgrom, E., Atger, M., & Baulieu, E. E. (1973) *Biochemistry 12*, 5198.
Moudgil, V. K., & John, J. K. (1980) *Biochem. J. 190*, 809.
Munck, A., & Wira, C. H. (1975) *Methods Enzymol. 36*, 255.
Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752.
Parikh, I., March, S., & Cuatrecasas, P. (1974) *Methods Enzymol. 34*, 85.
Rees, A. M., & Bell, P. A. (1975) *Biochim. Biophys. Acta 411*, 121.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533.
Schaumburg, B. P. (1972) *Biochim. Biophys. Acta 261*, 219.
Simons, S. S., Jr., Pons, M., & Johnson, D. F. (1980) *J. Org. Chem. 45*, 3084.
Simons, S. S., Jr., Schleenbaker, R. E., & Eisen, H. J. (1983) *J. Biol. Chem. 258*, 2229.
Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., & Field, D. (1981) *Biochemistry 20*, 6509.
Undenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871.
Young, H. A., Parks, W. P., & Scolnick, E. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3060.