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# REACTION OF TYROSYL-MODIFYING REAGENTS WITH THE LIGAND- AND DNA-BINDING DOMAINS OF THE RABBIT LIVER GLUCOCORTICOID RECEPTOR

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**Summary**—We have studied the effects of *p*-nitrobenzenesulfonyl fluoride, 4-fluorosulfonyl-1-hydroxy-2-naphtoic acid, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole and tetranitromethane on the glucocorticoid receptor from rabbit liver. Our results show that all tyrosine modifying reagents inhibit the binding of [$^3$H]dexamethasone to the receptor. Equilibrium binding experiments revealed that only 4-fluorosulfonyl-1-hydroxy-2-naphtoic acid is a competitive inhibitor while the other chemical probes decrease the concentration of binding sites. Transformation of glucocorticoid–receptor complexes was markedly reduced when heat treatment was performed in the presence of tyrosyl-directed reagents. Taken together, these results indicate for the first time that critical tyrosyl moieties may be involved in both hormone binding and transformation of the glucocorticoid receptor.

## INTRODUCTION

A substantial body of information has emerged from studies using glucocorticoid receptor antibodies as probes that have allowed isolation of clones containing glucocorticoid receptor cDNAs [1]. The complete amino acid sequence of human [2, 3], mouse [4] and rat [5] glucocorticoid receptors has revealed three domains. The steroid binding domain contains multiple cysteine residues [6–8], two of which appear essential for steroid binding [9], the DNA binding domain, characterized by a cysteine–lysine–arginine rich region [2–5] and an immunogenic domain [10]. Several recent studies have focused on the characterization of the role of sulhydryl groups in permitting the binding of glucocorticoids and the transformation of the glucocorticoid receptor [11–14]. Furthermore, chemical modification of functional groups in crude systems has demonstrated the involvement of basic amino acids in the binding of the glucocorticoid receptor to DNA [15–17]. But no information is yet available concerning the potential role of the tyrosyl residues that are localized in the steroid- and DNA-binding domains of the receptor protein as deduced from the amino acid sequence [18]. Evidence has accumulated which indicates that tyrosyl residues are involved in the catalytic site of steroid binding proteins like corticosteroid binding globulin [19], progesterone binding globulin [20] or enzymes i.e. estradiol-17$\beta$ dehydrogenase [21]. Furthermore, recent experiments have shown that the estrogen receptor is phosphorylated by a receptor kinase on a tyrosine residue [22] suggesting that tyrosine phosphorylation might be an initial event of steroid hormone action [23]. It was thus pertinent to investigate whether tyrosine residues may play a functional role in the mechanism of action of the glucocorticoid receptor.

Recently, *p*-nitrobenzene sulfonyl fluoride (NBSF), a chemical reagent for modification of the phenol ring of tyrosine, was utilized by Liao *et al.*[24] and Shorr *et al.*[25] demonstrated that 4-fluorosulfonyl-1-hydroxy-2-naphtoic acid (FSNA) modifies selectively tyrosyl residues. In this study, we investigated the effects of NBSF, FSNA and routinely used tyrosyl probes i.e. tetranitromethane $C(NO_2)_4$ and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) to determine a putative role of tyrosine(s) in the steroid- and DNA-binding activities of the glucocorticoid receptor from rabbit liver.

## MATERIALS AND METHODS

### *Chemicals*

[1,2,4(n)$^3$H]Dexamethasone (sp. act. 40–50 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, England). Nonradioactive dexamethasone was purchased from Roussel Uclaf (Romainville, France). NBSF, FSNA, $C(NO_2)_4$ were obtained from Aldrich (Strasbourg, France). NBD and 5,5′-dithiobis(2-nitrobenzoic acid) ($Nbs_2$) were obtained from

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*Abbreviations*: $C(NO_2)_4$: Tetranitromethane; Dexamethasone: 9$\alpha$-fluoro-16$\alpha$-methyl-11$\beta$, 17$\alpha$, 21-trihydroxy-1,4-pregnadiene-3,20-dione; FSNA: 4-fluorosulfonyl-1-hydroxy-2-naphtoic acid; $Nbs_2$: 5,5′-dithio-bis (2-nitrobenzoic acid); NBD: 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NBSF: *p*-nitrobenzene sulfonyl fluoride.

Sigma (St Louis, Mo.). Trisacryl GF 05, DEAE Trisacryl, ssDNA Ultrogel A4R and HA Ultrogel were purchased from Industrie Biologique Française (Villeneuve la Garenne, France). Bio-Gel HTP was obtained from Bio Rad Laboratories (Richmond, Calif.). All other reagents were of analytical grade.

*Preparation and labeling of cytosol*

Cytosol from adrenalectomized rabbits was prepared in 20 mM potassium phosphate, 10 mM sodium molybdate, 20% (by vol) glycerol, pH 8.0 (buffer I) as described previously [26]. Duplicate samples were incubated at 4°C with $2 \times 10^{-8}$ M [$^3$H]dexamethasone for 16 h. Non-specific binding was determined in a parallel incubation with a 1000-fold excess of unlabeled dexamethasone. Binding of [$^3$H]dexamethasone was routinely measured in duplicate using batch assays of hydroxylapatite [27]. Binding parameters were calculated according to Scatchard [28]. Cytosolic preparations contained 6–8 mg/ml protein.

*Reaction of receptor preparations with chemical reagents*

Cytosol was diluted 2-fold with buffer I. Chemical reagents were prepared as stock solutions from 0.050 to 0.1 M in isopropyl alcohol (NBSF and FSNA) or absolute ethanol ($C(NO_2)_4$ and NBD). Controls received equal volumes of isopropyl alcohol or ethanol. After a 15 min reaction at room temperature duplicate samples were assayed for specific binding.

*Separation of the glucocorticoid–receptor complexes*

The extent of receptor transformation was measured by the method of Holbrook *et al.*[29] with minor modifications. This chromatographic procedure employed DNA Ultrogel, DEAE Trisacryl and HA Ultrogel chromatography for binding of transformed, non transformed and meroreceptor complexes respectively. DNA Ultrogel, DEAE Trisacryl and HA Ultrogel columns with packed volumes of 0.2 ml were prepared separately in 1 ml syringes. The three columns were then connected one to the other (DNA Ultrogel on the top, DEAE Trisacryl in the middle and HA Ultrogel on the bottom) and equilibrated with 20 mM potassium phosphate, 10 mM molybdate, 20 mM 2-mercaptoethanol, 5% (by vol) glycerol, pH 7.4 (buffer II). Diluted samples were loaded, gels were washed with 20 ml buffer II and counted. Results are expressed as percentages of total receptor bound radioactivity adsorbed on the three gels.

*Receptors transformation*

Transformation of the [$^3$H]dexamethasone–receptor complex was performed by exposure to high salt concentration (0.3 M KCl) and elevated temperature (20°C) after elimination of sodium molybdate by passage through a column (vol 10 ml; $\phi$ 1.1 cm) filled with Trisacryl GF 05 and eluted at 0.5 ml/min with 20 mM potassium phosphate, 5% (by vol) glycerol, pH 8. The fractions (1 ml) containing the glucocorticoid receptor were pooled and used for binding studies or DNA binding capacity.

*General methods*

Proteins were measured by the Coomassie blue adsorption method [30] using bovine serum albumin as standard.

Radioactivity was determined in a Beckman LS 2800 liquid scintillation spectrometer. Samples (0.8 ml) were counted in 4 ml Beckman Ready-Solv HP/b scintillation fluid with a counting efficiency of ~45%.

## RESULTS

*Effects of various tyrosyl-directed reagents on ligand-free and ligand-bound glucocorticoid receptor*

Different reagents that react with tyrosine were tested for their effects on the binding of [$^3$H]dexamethasone to the glucocorticoid receptor. As shown in Fig. 1, NBSF and FSNA impaired [$^3$H]dexamethasone binding in a dose-dependent manner. This time-course of the inhibition of ligand binding by tyrosyl modifying reagents is shown in Fig. 2. The inhibition of ligand binding induced by all reagents was already obtained after a 15 min reaction time. If one assumes that tyrosine-modifying reagents react according to a bimolecular mechanism, they can interact either with the receptor protein or with the ligand. A putative modification of the ligand was examined. After treatment for 15 min at 20°C in the presence of tyrosyl modifying reagents, no new product was detected by thin-layer chromatography (data not shown). Therefore, the effects observed may be interpreted in terms of reaction of tyrosine modifying reagents with the receptor protein itself or an associated protein. Further experiments were done to determine if NBSF and FSNA might be active on a partially purified glucocorticoid receptor preparation. However, in the absence of ligand, the glucocorticoid receptor protein is unstable and very difficult to purify with good yields. The development of high-performance liquid chromatography has enhanced our ability to obtain a preparation of functionally active steroid free glucocorticoid receptor. As shown previously [31], high-performance liquid chromatography of molybdate stabilized cytosol on a Mono Q column allows a 70-fold purification of the rabbit liver glucocorticoid receptor with a 75–80% recovery. The reaction of NBSF and FSNA (0.1–2 mM) with this partially purified preparation induced a concentration-dependent decrease of the dexamethasone binding activity quite similar to that observed in the cytosolic preparations (data not shown).

However, if cytosol was preincubated with $2.10^{-8}$ M [$^3$H]dexamethasone prior to addition of chemical probes, there was a high protection of

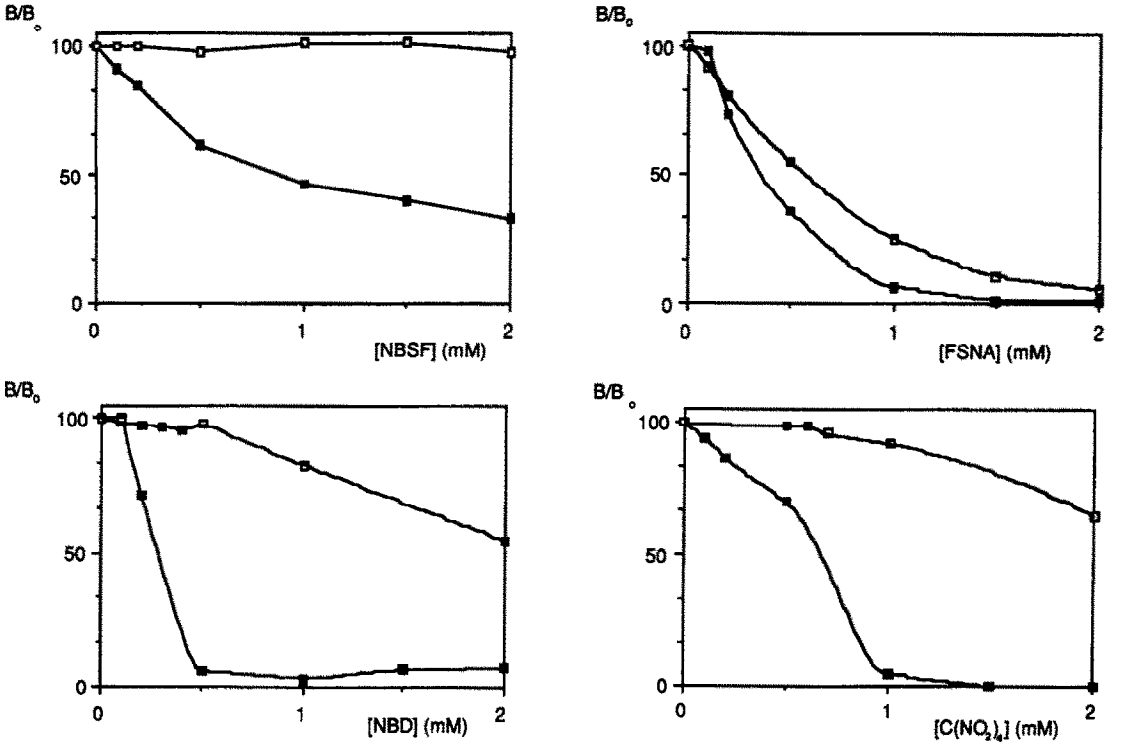


Fig. 1. Relative effects of tyrosyl probes on dexamethasone-free and dexamethasone-bound glucocorticoid receptor. Aliquots of cytosol (1 ml) were incubated for 15 min at 20°C with increasing concentrations of NBSF, FSNA, NBD or $C(NO_2)_4$ prior to labeling for 16 h at 4°C with $2 \times 10^{-8}$ M [$^3$H]dexamethasone (■). Labeled cytosol (□) was treated with varying concentrations of tyrosyl probes for 15 min at 20°C before determination of specific binding. In each case, specifically bound [$^3$H]dexamethasone was estimated by batch assays of hydroxylapatite. 100% control values ($B_0$) were equivalent to 240 fmol/mg of protein and 190 fmol/mg of protein for unbound receptor and bound receptor, respectively.

specific binding in the case of NBSF. On the other hand, dexamethasone did not protect the glucocorticoid receptor against the loss of binding activity caused by FSNA and the degree of loss of bound steroid was proportional to the concentration of FSNA. NBSF and FSNA are believed to be specific reagents of tyrosine residues on proteins [24, 25], but other well known compounds like $C(NO_2)_4$ and NBD are commonly used in the modification of tyrosine. The relative effectiveness of $C(NO_2)_4$ and NBD in inhibiting the binding of [$^3$H]dexamethasone to the receptor is shown in Fig. 1 and the time-course of inhibition is presented in Fig. 2. These two compounds, and more notably NBD, are highly potent in promoting inhibition of steroid binding. Figure 1 also shows that in the same range of concentrations, steroid-bound receptor was less susceptible to the action of NBD and $C(NO_2)_4$ than the steroid free receptor.

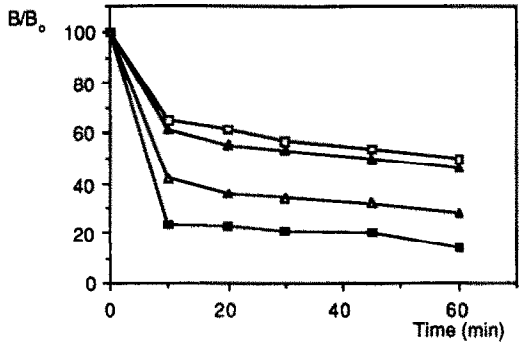


Fig. 2. Time-course of the inhibition of the binding of [$^3$H]dexamethasone by tyrosyl-directed reagents. Samples of cytosol were incubated with 1.25 mM NBSF (▲); 0.5 mM FSNA (■); 0.3 mM NBD (△) or 0.7 mM $C(NO_2)_4$ (□). At each time-point aliquots were removed and labeled for 16 h at 4°C with $10^{-8}$ M [$^3$H]dexamethasone. The amount of specifically bound [$^3$H]dexamethasone was estimated by batch assays with hydroxylapatite. Results are expressed as percentages of a control incubation performed in the presence of vehicle. Control value ($B_0$) was equivalent to 130 fmol/mg of protein.

*Effect of NBSF on the dissociation of ligand-bound receptor*

The time-course of loss of [$^3$H]dexamethasone from the receptor was investigated in the presence and absence of NBSF (Fig. 3). In the absence of NBSF, bound [$^3$H]dexamethasone dissociated from the receptor with a first order kinetics and a dissociation rate constant of $4.8 \times 10^{-3}$ mn$^{-1}$. In the presence of 2 mM NBSF, the dissociation was significantly decreased (dissociation rate constant $1.5 \times 10^{-3}$ min$^{-1}$).

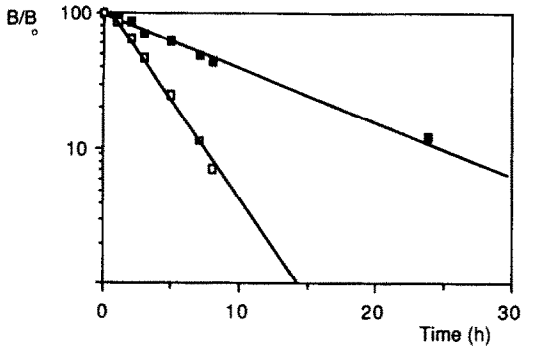


Fig. 3. Influence of NBSF on the dissociation of [$^3$H]dexamethasone from the glucocorticoid–receptor complex. Cytosol (2 ml) was labeled with $2 \times 10^{-8}$ M [$^3$H]dexamethasone at 4°C for 16 h and then aliquots were exposed at 20°C in the presence (■) or absence (□) of 2 mM NBSF. Dissociation was initiated by the addition of a 1000-fold excess of unlabeled dexamethasone. At the indicated times, duplicate samples (0.1 ml) were removed and assayed for residual bound radioactivity (B) using batch assays with hydroxylapatite. The control value ($B_0$) represents the amount of specifically bound [$^3$H]dexamethasone at the beginning of the dissociation and is equivalent to 98 fmol/mg of protein. At each time, the specific binding of the control sample was also determined to take into account the degradation of glucocorticoid–receptor complexes.

*Nature of the inhibition*

The nature of the inhibition induced by tyrosyl modifying reagents was further investigated by Scatchard analysis of the binding data. Figure 4 shows that the inhibition of dexamethasone binding by NBSF was due to a decrease in the concentration of binding sites and not to the production of a protein with altered affinity for the steroid. The inhibition was concentration-dependent. For instance, a 3-fold decrease of the concentration of specific binding sites in the presence of 1.5 mM NBSF was observed as compared to the control (Table 1). On the other hand, FSNA modified the ability of the steroid to bind to the receptor by altering the affinity of the receptor for dexamethasone. Table 1 shows that the ligand's affinity of the receptor was decreased by a factor 20 as compared to the control. NBD and $C(NO_2)_4$ also induced a loss of specific binding sites without influencing the affinity of the receptor for the steroid.

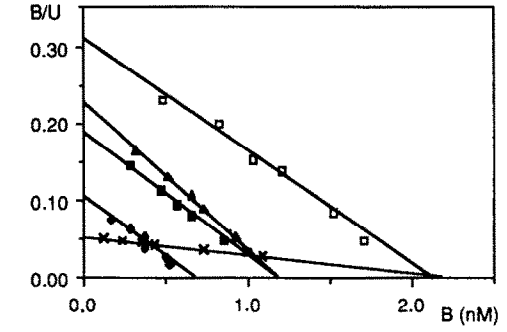


Fig. 4. Inhibition of the binding of [$^3$H]dexamethasone to the receptor by tyrosyl modifying reagents. Aliquots of cytosol (1 ml) were initially treated for 15 min at 20°C in the absence (□) or in the presence of 1.5 mM NBSF (◆), 0.2 mM FSNA (×), 0.25 mM NBD (■) or 0.6 mM $C(NO_2)_4$ (▲). Duplicate samples were further incubated with increasing concentrations ($2.5 \times 10^{-9}$–$3.5 \times 10^{-8}$ M) of [$^3$H]dexamethasone alone or in the presence of a 1000-fold excess of unlabeled steroid. After 16 h at 4°C, duplicate samples were assayed for specific binding. B: bound [$^3$H]dexamethasone; U: unbound [$^3$H]dexamethasone.

Table 1. Influence of tyrosyl probes on the equilibrium parameters of the ligand receptor interaction

| Inhibitor (mM) | $K_a \times 10^9 M^{-1}$ | $R_0$ pmol/mg protein |
|---|---|---|
| None | 0.15 | 0.27 |
| NSBF | | |
| 0.20 | 0.16 | 0.26 |
| 0.75 | 0.17 | 0.15 |
| 1.50 | 0.16 | 0.08 |
| FSNA | | |
| 0.05 | 0.08 | 0.33 |
| 0.20 | 0.02 | 0.31 |
| 0.50 | 0.007 | 0.32 |
| $C(NO_2)_4$ | | |
| 0.60 | 0.19 | 0.22 |
| NBD | | |
| 0.25 | 0.16 | 0.22 |

Samples of cytosol were incubated 15 min at 20°C in the absence or presence of the indicated concentrations of tyrosyl modifying reagents. Afterwards, saturation experiments were performed in duplicate with $2.5 \times 10^{-9}$–$3.5 \times 10^{-8}$ M [$^3$H]dexamethasone for 16 h at 4°C. Equilibrium binding parameters were derived from Scatchard plots of the binding data. $K_a$: equilibrium association constant. $R_0$: concentration of binding sites.

*Reversibility of the inhibition*

Next, experiments were conducted to determine whether the effects of chemical probes were reversible or not. The data presented in Table 2 show that, after removal of excesses of FSNA and NBD by gel filtration, the specific binding activity was recovered to approximately 95 and 100%, respectively. On the

Table 2. Reversibility of the inactivation of rabbit liver glucocorticoid receptor by tyrosyl-modifying reagents

| Inhibitor | Gel filtration Prior Addition of inhibitor | Gel filtration After Addition of inhibitor | Specific binding (%) |
|---|---|---|---|
| NBSF (1 mM) | + | | 41.5 |
| | | + | 42.3 |
| FSNA (0.5 mM) | + | | 18.4 |
| | | + | 95.1 |
| NBD (0.3 mM) | + | | 11.9 |
| | | + | 106 |
| $C(NO_2)_4$ (1 mM) | + | | 5.8 |
| | | + | 11.7 |

Samples of cytosol (3 ml) were subjected to gel filtration and then incubated for 15 min at 20°C in the presence or absence of 1 mM NBSF, 0.5 mM FSNA, 0.3 mM NBD or 1 mM $C(NO_2)_4$ prior to incubation with $2 \times 10^{-8}$ M [$^3$H]dexamethasone for 16 h at 4°C. Aliquots of cytosol (3 ml) were treated for 15 min at 20°C in the presence of the same concentrations of tyrosyl probes and then subjected to gel filtration before incubation with $2 \times 10^{-8}$ M [$^3$H]dexamethasone. Samples undergoing gel filtration without alkylating agent correspond to 100% value equivalent to 212 fmol/mg of protein.

other hand, upon removal of unreacted NBSF and $C(NO_2)_4$, the specific binding activity of the cytosol was lost completely showing the irreversibility of the inactivation induced by NBSF and $C(NO_2)_4$.

*Specificity of the inhibition*

While NBSF, NBD and $C(NO_2)_4$ have been reported to be specific for tyrosine [19, 25, 32, 33], FSNA however might display some reactivity towards lysine and cysteine [25], this latter being known to be implicated in the binding of steroid [6–7]. To envision the eventuality that loss of receptor binding might be due to modification of cysteine and/or tyrosine we performed additional experiments. Cytosol was preincubated with the well-known thiol specific reagent 5,5′-dithio-bis (2-nitro-benzoic acid) ($Nbs_2$). Treatment with 1–2 mM $Nbs_2$, which reacts with thiol groups to produce a disulfide bond [34], induced a complete loss of binding activity (Fig. 5B). Addition of 10 mM dithiothreitol to preparations previously treated with $Nbs_2$ allowed a near complete reversibility of $Nbs_2$ inhibition. This latter result rendered the opportunity to evaluate the specificity of NBSF, NBD, FSNA and $C(NO_2)_4$. Aliquots of cytosolic preparations were preincubated 15 min at 20°C with or without tyrosyl reagents and then 15 min with 10 mM dithiothreitol. Figure 5A shows that dithiothreitol treatment allows a near complete restoration of specific binding for NBSF and NBD. However, no regeneration of specific binding was observed when samples were treated with FSNA or $C(NO_2)_4$.

In a second set of experiments, aliquots of cytosol were first incubated with 2 mM $Nbs_2$ in order to block the −SH groups. The −SH blocked preparations were treated with FSNA, NBSF, NBD or $C(NO_2)_4$ and subsequently with 10 mM dithiothreitol to regenerate free −SH groups. Specific binding was measured after each step of the experiment. As shown in Fig. 5B, the decreased activity (98%) of the SH blocked receptor was partially recovered (60%) when dithiothreitol was introduced after modification by NBSF, NBD and $C(NO_2)_4$. However, only 7% of the original activity was detected when the cytosolic fraction was treated with FSNA.

*Influence of tyrosyl-modifying reagents on receptor binding to DNA*

Examination of the amino acid sequences of the human, rat and mouse glucocorticoid receptors shows the presence of tyrosine residues localized within the DNA-binding domain. It was therefore of interest to check whether tyrosyl probes would influence or not the transformation step and/or the interaction with DNA. Cytosol was incubated up to equilibrium with $2 \times 10^{-8}$ M [$^3$H]dexamethasone. Samples were cleared of molybdate by gel filtration and transformed by warming for 30 min at 20°C with 0.3 M KCl and 2 mM 2-mercaptoethanol in the presence or absence of tyrosyl modifying reagents.

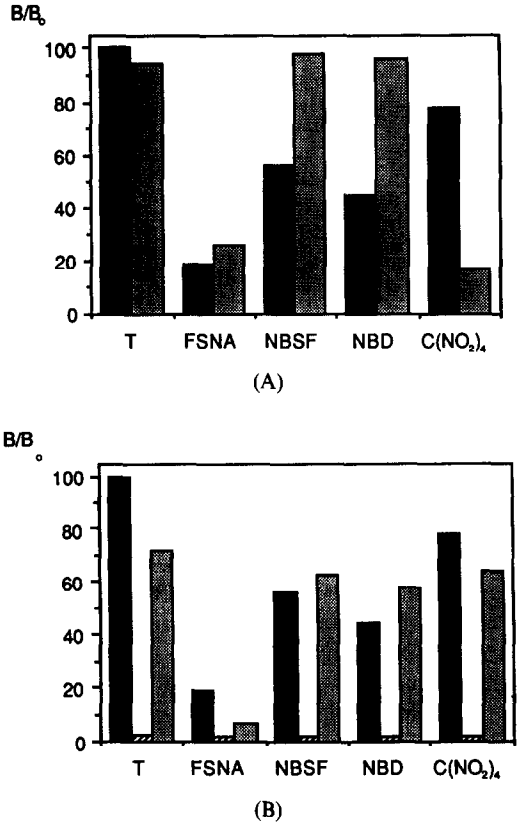


Fig. 5. Modification of native and SH blocked cytosolic preparations with tyrosyl reagents. (A) Combined effects of tyrosine directed reagents and dithiothreitol on ligand binding to the glucocorticoid receptor. Samples of cytosol (1 ml) were incubated for 45 min at 20°C in the absence of tyrosine directed reagents (control) or 30 min at 20°C and then 15 min at 20°C in the presence of 0.5 mM FSNA, 1 mM NBSF 0.25 mM NBD, and 0.6 mM $C(NO_2)_4$ (■). In an other set of experiments, aliquots were incubated for 15 min at 20°C with or without tyrosyl reagents followed by a 15 min exposure to 10 mM dithiothreitol (▨). After each treatment, aliquots were incubated with $2 \times 10^{-8}$ M [$^3$H]dexamethasone for 16 h at 4°C and assayed for specific binding. The control value (T) was equivalent to 195 fmoles/mg of protein. Ordinate: percentage of specifically bound [$^3$H]dexamethasone. (B) Combined effects of tyrosine directed reagents, dithiothreitol and $Nbs_2$ on ligand binding to the glucocorticoid receptor. Aliquots or cytosol (1 ml) were incubated for 45 min at 20°C in the absence of treatment (control) or 30 min at 20°C followed by 15 min at 20°C with tyrosyl reagents (■). In a second set of experiments, samples were incubated for 15 min at 20°C with 2 mM $Nbs_2$ and further modified for 15 min with tyrosyl probes (▨). In the last set of experiments, after a 15 min $Nbs_2$ treatment, samples were further modified by tyrosyl probes for 15 min and free −SH groups were regenerated by a 15 min treatment with 10 mM dithiothreitol (▨). Specifically bound [$^3$H]dexamethasone was estimated as described in the legend of Fig. 5A.

The reducing agent 2-mercaptoethanol is necessary for binding of the transformed glucocorticoid receptor to DNA and it also affects the transformation process itself [12–14]. Under the experimental conditions used there was no substantial reduction of NBSF activity due to 2-mercaptoethanol (not shown). At the concentrations used, most of the

Table 3. Relative effects of various tyrosine modifying agents on [$^3$H]dexamethasone receptor binding to ionic exchangers

| Nature of ionic exchanger | + Molybdate | Binding (%) − Molybdate | | | | |
|---|---|---|---|---|---|---|
| | | Alone | +NBSF | +NBD | +$C(NO_2)_4$ | +FSNA |
| DNA | 10.3 | 61.1 | 20.1 | 52.3 | 26.2 | 60.5 |
| DEAE | 82.0 | 15.2 | 69.5 | 30.5 | 58.0 | 16.7 |
| HA | 7.7 | 23.7 | 10.4 | 17.2 | 15.8 | 22.8 |

Rabbit liver cytosol containing [$^3$H]dexamethasone bound untransformed receptor was chromatographed through Trisacryl GF 05 to remove thiols and molybdate. Fractions containing macromolecular bound radioactivity were pooled and adjusted to $5 \times 10^{-9}$ M [$^3$H]dexamethasone. Samples (1 ml) were incubated for 15 min at 20°C in the presence of 1 mM NBSF, 0.7 mM $C(NO_2)_4$ or 0.2 mM FSNA, followed by incubation 30 min at 20°C with 2 mM 2-mercaptoethanol and 0.3 M KCl. Control preparations were left untreated by tyrosyl reagents but received 10 mM molybdate after gel filtration (untransformed) or the same volume of buffer (transformed). Binding to ionic exchangers was performed as described in Materials and Methods after appropriate dilution of the samples. 100% specific binding in untransformed samples was equivalent to 96 fmol/mg protein.

tyrosyl probes had little effect on dexamethasone binding (Fig. 1). Only FSNA produced a dramatic decrease of receptor bound steroid which led us to choose a concentration of 0.2 mM. Results presented in Table 3 show that, under the *in vitro* conditions used, approximatively 60% of dexamethasone–receptor complexes were transformed as estimated by the minicolumn procedure. Control samples containing 10 mM molybdate exhibited a very low binding to anionic matrixes. Under these conditions, NBSF and $C(NO_2)_4$ produced a marked inhibition on binding of the dexamethasone-receptor complex to DNA Ultrogel (Table 3), whereas NBD produced only a slight inhibition and FSNA was ineffective.

The reduction in DNA binding caused by NBSF and $C(NO_2)_4$ could be due to either blocking the transformation step or inhibition of DNA binding. In order to discriminate these two possibilities, samples of cytosol containing [$^3$H]dexamethasone–receptor complexes were first subjected to transformation by increasing temperature and ionic strength and then treated with NBSF or $(C(NO_2)_4$. In this experiment (Table 4), NBSF did not alter significantly DNA binding of prewarmed complexes but $C(NO_2)_4$ inhibited the binding to DNA. This result supports the conclusion that NBSF inhibits the transformation step itself without influencing the DNA binding capacity.

Table 4. Influence of tyrosyl reagents on binding of transformed glucocorticoid–receptor complex to ionic exchangers

| Nature of ionic exchanger | + Molybdate | Binding (%) − Molybdate | | |
|---|---|---|---|---|
| | | Alone | + NBSF | + $C(NO_2)_4$ |
| DNA | 12 | 67.6 | 59.7 | 34.2 |
| DEAE | 81.5 | 10.4 | 16.7 | 46.5 |
| HA | 6.5 | 22 | 23.6 | 19.3 |

Samples (1 ml) of desalted [$^3$H]dexamethasone bound complexes were first incubated 30 min at 20°C with 0.3 M KCl, 2 mM 2-mercaptoethanol in the presence or absence of 10 mM molybdate. The incubations were then continued for an additional 15 min at 20°C in the presence or absence of 1 mM NBSF or 0.7 mM $C(NO_2)_4$. Specific binding of the control was equivalent to 80 fmol/mg of protein.

## DISCUSSION

The data presented in this study clearly reveal that tyrosyl directed reagents can inhibit the binding of dexamethasone to rabbit liver glucocorticoid receptor. Among the compounds tested NBD was the most potent inhibitor since a 50% loss of binding was observed at 0.3 mM; it was followed by FSNA (0.4 mM), $C(NO_2)_4$ (0.7 mM) and NBSF (1 mM). We have demonstrated that, except for FSNA, inactivation can be prevented by the presence of the ligand which strongly suggests that an essential tyrosine may be implicated in the binding site of the glucocorticoid receptor. Results of Scatchard plots performed in the presence of varying concentrations of NBSF, NBD and $C(NO_2)_4$ revealed that the inhibition of binding was due to a loss of binding sites rather than to an alteration of affinity. In the presence of FSNA inactivation appeared to be due to a decrease of the binding affinity, suggesting a competition near the steroid binding site.

The inhibition of binding due to a loss of binding sites may be explained by the covalent modification of an amino acid, i.e. a tyrosine, implicated in the binding site of the receptor. This would be on account of an irreversible mechanism. However, in the case of NBD, upon removal of unbound reagent the specific binding was recovered (Table 2). To account for this phenomenon, one may envisage the nature of the binding of NBD. At alkaline pH, the nitrobenzofurazan moiety which was attached to a nucleophilic atom may be transferred intramolecularly to a lysine residue [35]. In fact, to our knowledge, this latter residue is not of critical importance for steroid binding. The reduction of the Tyr-*O*-NBD linkage has been described [35] but attempts we made to stabilize the Tyr-*O*-NBD derivative by dithionite treatment were unsuccessful.

Among the alkylating reagents tested only NBSF and $C(NO_2)_4$ were irreversible inhibitors of hormone binding. In these two cases, we can speculate on a stable covalent modification of a residue belonging to the binding site. In view of the irreversible binding of NBSF (Fig. 3, Table 2), it is conceivable that the

synthesis of a steroid analog containing this reagent group would alkylate a tyrosine residue in close proximity to the receptor binding site. Although electrophilic labeling of the glucocorticoid receptor has already been employed with success using dexamethasone mesylate [7, 36, 37], the irreversible interaction of NBSF would permit other instructive findings about the amino acids involved in steroid binding.

The competitive and reversible inhibition observed with FSNA would be consistent with a model where FSNA and the steroid bind to the same site on the receptor. The binding of FSNA might be the consequence of a steric conformation partly similar to that of a steroid hormone. The same phenomenon has been reported for $\beta$-lapachone, a derivative of 1,2-naphtoquinone [38] or antipsychotic phenothiazines like trifluoperazine [39] which influence steroid binding through a direct interaction with the glucocorticoid receptor. A similar mechanism has already been reported by Harrison *et al.*[40] who found that a thiol attacking reagent *p*-chloromercuriphenyl sulfonate promotes a reduction of binding affinity. A possible explanation would be that the binding of FSNA in or near the active site of the receptor induces a conformational change of the receptor leading to a decreased affinity.

A crucial problem concerns the broad specificity of tyrosyl directed reagents which can react with cysteine and lysine [41]. Possible interactions with cysteinyl residues were explored by examining the effects of $Nbs_2$ and dithiothreitol on the ability of tyrosyl probes to inactivate specific binding sites. Modifications induced by $C(NO_2)_4$ and FSNA are not affected by dithiothreitol treatment (Fig. 5A). On the other hand, the inhibition of binding caused by NBSF and NBD can be reversed by dithiothreitol, suggesting that these reagents may in part react with thiol groups. Pretreatment of glucocorticoid receptor with a thiol specific reagent followed by inactivation with tyrosyl specific reagents and then dithiothreitol showed that only FSNA and $C(NO_2)_4$ may be considered as rather specific probes of tyrosyl residues. The results obtained in the presence of FSNA and $C(NO_2)_4$ are unlikely to be due to modification of cysteine residues. This suggests that at least one tyrosine might be localized in the ligand binding site of the rabbit liver glucocorticoid receptor.

From the data presented here, it is clear that the glucocorticoid receptor reacts with tyrosine modifying reagents which differently affect DNA binding of the glucocorticoid receptor. Treatment of the glucocorticoid–receptor complex with 1 mM NBSF or 0.7 mM $C(NO_2)_4$ before transformation impaired subsequent DNA binding. Minicolumn analysis showed that most of specific binding appeared as a non-transformed complex. In contrast, NBD and FSNA did not prevent DNA binding of the glucocorticoid receptor. Because the tyrosyl-modifying reagent was present during all the transformation procedure, it was not possible to determine whether the reagent inhibited transformation to the DNA binding form, or DNA binding of transformed receptor, or whether it prevented both processes. We reexamined this problem and were able to show that when transformation was performed in the absence of tyrosyl modifying reagent and then tested by the minicolumn procedure, NBSF did not inhibit DNA binding. In contrast, $C(NO_2)_4$ impaired DNA binding. One may speculate that $C(NO_2)_4$ binds through a putative tyrosine which participates in the interaction with DNA. Conversely we suggest that NBSF would interact with a tyrosyl residue located outside the DNA binding domain in such a position that dissociation of untransformed complex would be impaired.

The experimental data reported in this study suggest that tyrosine residue(s) may be involved in the binding of glucocorticoids to the glucocorticoid receptor and binding of the glucocorticoid–receptor complex to DNA. Phosphorylation of progesterone, glucocorticoid and estradiol receptors has been observed in intact cells or tissues labelled with [$^{32}$P]orthophosphate under a variety of conditions. Phospho-amino acid analysis showed that estradiol receptor was phosphorylated on tyrosine whereas progesterone and glucocorticoid receptors were mainly phosphorylated on a serine residue [42]. Although it has been proposed that tyrosine(s) would be implicated in the phosphorylation of partially purified glucocorticoid receptor. This hypothesis is supported by the fact that partially purified rat liver glucocorticoid receptor interacts with high affinity with anti-phosphotyrosine antibody 2G8 coupled to Sepharose [43]. Furthermore, Rao and Fox [44] showed that treatment of human breast epithelial cells with [$^{32}$P]orthophosphate followed by phospho-amino acid analysis of glucocorticoid receptor allows the identification of phospho-serine (89%) and phospho-tyrosine (11%). Therefore it will be of considerable interest to check whether tyrosine or serine phosphorylation regulates steroid and DNA binding activities of the rabbit liver glucocorticoid receptor.

Although this interpretation aligns with our current knowledge regarding glucocorticoid receptor structure and function, we are also aware that the present study was carried out on a crude receptor preparation. In these conditions, one cannot exclude the eventuality that modified residues may be localized on an associated non-receptor protein like the well-known heat shock protein hsp 90 [45]. As far as we know glucocorticoid binding occurs in the presence of a complex between a dimer of hsp 90 and the 94 kDa monomer [46, 47]. No proof of the direct involvement of tyrosyl residues in the ligand binding domain can be given except the results of binding experiments (Fig. 4, Table 1). However, it can be argued that when cytosolic preparations are cleared of molybdate and subjected to elevated temperature and ionic strength the associated hsp 90 is released from the glucocorticoid–receptor complex [48]. In

these conditions, modification reagents alter DNA binding suggesting that the receptor monomer is concerned rather than an associated protein. However, the definitive proof of the involvement of tyrosine(s) in glucocorticoid receptor function must await purification of the ligand-free receptor which will permit the identification of the number and the localization of specifically modified tyrosine(s).

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