Liver Cytosol Corticosteroid Binder IB, a New Binding Protein*

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A new binding protein named corticosteroid Binder IB elutes just after ligandin in DEAE-Sephadex chromatograms. It has been partially purified to about 2500-fold over cytosol proteins. Calculation of the number of steroid binding sites, assuming one site per molecule of Binder IB fraction after DEAE-Sephadex chromatography, suggests a concentration of the binding protein of about 0.0004% of cytosol proteins. Its pl value is judged to be 7.5 to 8 from its elution position on DEAE-Sephadex chromatograms. IB has an apparent molecular weight of 30,500 ± 10% by gel filtration and a Stokes radius of 20 Å. Binder IB binds radioactive dexamethasone, cortisol, and corticosterone in vitro with estimated $K_d$ values of 1, 13, and 25 nM, respectively. Saturation curves are abnormal, showing two phases. The saturation curves within the physiological range of concentrations of steroid are abnormal and suggestive of cooperativity. The second phase, at concentrations of glucocorticoids above saturation and physiological levels, shows extensive binding. After fractionation from other steroid binding proteins, the specificity of binding from competition studies in vitro is dexamethasone > cortisol = corticosterone = estradiol-17β > deoxycorticosterone = dihydrotestosterone > aldosterone = cortolone > testosterone. Other steroids tested are less efficient ligands. The binding is probably noncovalent, but strong; and the complex becomes more dissociable as purification proceeds, suggesting a conformational change in the protein. Storage and rebinding with steroid are possible throughout the purification process, although extensive ligand dissociation and desaturation of the protein occur after the final purification step. Binding in vitro is temperature-sensitive and binding is sharply pH dependent with an optimum at 7.5. The ligand is the unmethylated steroid as judged by extraction of steroid-IB complex with methylene chloride and subsequent thin layer chromatography. The physiological function of this protein is unknown at present and purification of the major corticosteroid hormone receptor to homogeneity may be required before the function of Binder IB is fully understood.

Previously, we have reported the presence of four corticosteroid binding proteins in adrenalectomized rat liver cytosol separable after intraperitoneal administration of radioactive corticosteroid (1, 2). Recently, we also noted the occurrence of another binding protein, designated corticosteroid binder IB, which elutes just after ligandin (Binder I) in DEAE-Sephadex A-50 column chromatograms (3, 4). Significantly, this protein binds dexamethasone in addition to other glucocorticoids and there are unusual phenomena in its binding saturation curves in vitro. In this communication we describe the partial purification and properties of Binder IB. A preliminary report of this work has been made (5).

MATERIALS AND METHODS

Animals—Adrenalectomized male Sprague-Dawley rats (Charles-River Breeding Labs) were used 7 to 12 days after surgery. The 130- to 140-g rats were fed a normal chow diet and maintained on 1% saline. The animals were killed by decapitation and the livers were perfused in situ with 0.9% saline. The livers were then removed and homogenized in an equal volume of 0.25 M sucrose in 50 mM Tris-HCl, pH 7.5 (6). For in vitro preparations [1,2-3H]corticosterone was dried in a stream of $N_2$, suspended in 0.9% sterile saline, and stirred for 30 min at 4°C before intraperitoneal injection. The mixture (250 μCi per rat) was injected and the animals were killed 10 min later.

Isotopes and Chemicals—[1,2,4-3H]corticosterone (specific activity, 40 Ci/mmol) and [1,2-3H]cortisol (specific activity, 46.3 Ci/mmol) were obtained from New England Nuclear Corp. [1,2,4-3H]Dexamethasone (specific activity, 10 Ci/mmol) was obtained from Amer- sham/Searle. The isotopes were checked for purity on precoated silica gel plates purchased from Brinkmann. The solvent systems are described below under "Steroid Extraction." Dexamethasone, cortisol, deoxycorticosterone, 17β-estradiol, corticosterone, δ-aldosterone, 5α-dihydrotestosterone, and androstanolone were obtained from Sigma; progesterone, cortisone acetate, and testosterone were from Nutritional Biochemicals, and cortolone was from Pfaltz and Bauer. Unlabeled steroids were subjected to thin layer chromatography similarly to radioactive steroids. The molecular weight marker proteins; cytochrome c (type VI horse heart) and ovalbumin were purchased from Sigma, bovine serum albumin Fraction V from Armour Laboratories, and α-chymotrypsinogen from Boehringer/Mannheim. All the reagents for disc gel electrophoresis were obtained from Eastman Kodak. Column chromatography resins: Sephadex G-25, G-100, DEAE-Sephadex A-50, and CM-Sephadex C-50 were obtained from Pharmacia. Radioactivity was measured using Cal-U-O-Sil-sph-
thallium-dioxane scintillation mixture described previously (7) in an Intertechnique scintillation spectrometer at 12°C.

Column Chromatography—Livers from 4 rats injected intraperitoneally with [1,2-3H]corticosterone (290 μCi per rat) were perfused, removed, and homogenized in 1 volume of 0.25 M sucrose in 50 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 105,000 x g for 1 hour in a Beckman model L-2 ultracentrifuge. All operations were conducted at 4°C (8). The cytosol thus obtained was immediately applied to a Sephadex G-25 column (3 x 55 cm) equilibrated with 1 mM Tris-HCl, pH 7.5. The column was eluted with the same buffer into protein-bound radioactivity and unbound pools (9), and the bound radioactive pool was lyophilized. The concentrated, but not dry, bound pool was diluted with 50 mM Tris-HCl, pH 7.5, to a protein concentration of 100 mg/ml. This solution was applied to a DEAE-Sephadex A-50 column (3 x 76 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. The column was eluted with a continuous three-chamber (500 ml each) gradient of KCl (0 to 1 M) in 50 mM Tris-HCl, pH 7.5, and 4-ml fractions were collected at a flow rate of 60 ml/hour. Fractions of bound radioactivity, eluting just after ligandin in buffer at 0 mM KCl, were collected and concentrated by lyophilization. The concentrated Binder IB was resuspended in 1 mM Tris-HCl, pH 7.5, to a protein concentration of 4 to 5 mg/ml. The sample was applied to a Sephadex G-100 column (1.8 x 46 cm), previously calibrated with marker proteins and charged with 1 mM Tris-HCl, pH 7.5. The sample was eluted with the same buffer into protein-bound radioactivity and unbound pools (9), and the bound radioactive pool was lyophilized. The concentrated Binder IB was resuspended in 1 mM Tris-HCl, pH 7.5, to a protein concentration of 4 to 5 mg/ml. The results of specificity of binding are indicated in Fig. 2.

Molecular Weight—The apparent molecular weight of the binder was estimated by gel filtration on a Sephadex G-100 column (1.8 x 47 cm) following the method of Andrews (12), using cytochrome c (12,400), ovalbumin (44,000), α-chymotrypsinogen (25,000), and bovine serum albumin (67,000) as molecular weight markers. The column was eluted with 1 mM Tris-HCl, pH 7.5, and 2-ml fractions were collected. The void volume was determined by blue dextran, Vₐ/ₐ, molecular weight of protein was plotted (Vₑ, elution volume; Vᵥ, void volume).

Steroid Extraction—To extract the steroid from the in vivo-labeled IB from DEAE-Sephadex A-50, equal volumes of cold methylene chloride and sample were added to a conical, glass-stoppered test tube. This mixture was mixed in a vortex for 5 min, centrifuged for 5 min at 700 x g, and the organic layer was removed. The sample was extracted twice more and the pooled extracts were concentrated in a stream of N₂ to about 0.5 ml. An aliquot was applied to a precoated silica gel plate and developed in benzene/acetone (1/1), and in a second determination, methylene chloride/acetone/methanol (7/2/1). The plates were air-dried, scraped, and counted in Cal-O-Sil scintillation fluid. Unlabeled corticosterone was used as a standard and its zone was determined under ultraviolet light. All procedures up to the application to the plate were carried out at 4°C.

Temperature Studies—The temperature-dependent binding curve of IB was determined in Tris-HCl, pH 7.5, buffer Aliquots (5 ml) of the pooled IB from DEAE-Sephadex were incubated at temperatures ranging from 0 to 45°C for 90 min before being applied to a Sephadex G-25 minicolumn to separate bound from unbound fractions. The minicolumns were run at 4°C. The per cent binding was determined and plotted versus temperature.

pH Studies—The pH activity curve of Binder IB from DEAE-Sephadex A-50 was obtained using potassium phosphate and Tris-HCl buffers at concentrations of 50 mM. The routine binding assay was followed as described above except that 5-ml aliquots of Binder IB pool were incubated with 1 ml of the appropriate buffer at the prescribed pH. All incubations were carried out at 4°C. Buffers of the same pH were used to elute the column.

RESULTS

Chromatographic Behavior of IB after Labeling with Steroid in Vivo—Ten min after injection of [3H]corticosterone, animals are killed using a guillotine, bled from the neck, and the livers perfused in situ with cold isotonic KCl. Cytosols are prepared (8) and the steroid-macromolecule complexes are separated by gel filtration on columns of Sephadex G-25 (8, 13). The macromolecules are concentrated by lyophilization about 4 to 6-fold, applied to a column of DEAE-Sephadex A-50, and eluted with a KCl gradient (3). A typical pattern is shown in Fig. 1. Binder IB is resolved from ligandin (Binder I) and is eluted after it. This procedure was used for the purification of the steroid-protein complex and for preparation of uncomplexed binder for in vitro experiments. By virtue of its elution position, we conclude that the kᵢ value of IB is between pH 7 to 8, since ligandin has a kᵢ value of 8.9 determined by electrophoresis (14, 15). The fractionation procedure described above is sufficient to prepare IB for in vitro studies, since further purification, described later, enriches only one steroid-binding protein fraction and at the stage of ion exchange chromatography, Binder IB is resolved from the other known corticosteroid binding proteins.

Specificity of Binding of IB in Vitro by Competition with [3H]Corticosterone—For these experiments the Binder IB is used directly from the DEAE-Sephadex A-50 column as indicated in Fig. 2. The protein concentration in this pool is usually 1 to 1.6 mg/ml. The results of specificity of binding are summarized in Table I. Dexamethasone appears to be the favored steroid of those tested, followed closely by the natural glucocorticoids. Curiously, 17β-estradiol competes with radio-
TABLE I
Specificity of steroid binding to binder IB

| Steroid (unlabeled) | Average % inhibition (7 to 8 experiments/value) | Range of inhibition |
|---------------------|-----------------------------------------------|-------------------|
| Control*            | 0                                             |                   |
| Dexamethasone       | 83                                            | 80-86             |
| Cortisol            | 80                                            | 78-84             |
| Corticosterone      | 80                                            | 78-84             |
| 17β-estradiol       | 80                                            | 78-84             |
| Deoxycorticosterone | 78                                            | 76-82             |
| Dihydrotestosterone | 77                                            | 77-78             |
| n-Aldosterone       | 67                                            | 61-70             |
| Cortexolone         | 67                                            | 61-70             |
| Testosterone        | 63                                            | 58-65             |
| Cortisone acetate   | 60                                            | 60                |
| Progesterone        | 49                                            | 45-56             |
| Androstanolone      | 30                                            | 28-32             |

Active corticosterone binding as well as the glucocorticoids, and dihydrotestosterone is a more efficient ligand than testosterone. This specificity of binding is somewhat similar to that of the hormone receptor (Binder II) (3). It also appears to be different from transcorin (10). Although the specificity of IB towards the functional glucocorticoids might appear to be lower than expected is when the ability of anti-glucocorticoids, such as estradiol, testosterone, and cortisone is examined, the secondary increase in the saturation of binding curves (Fig. 3) needs to be borne in mind. Thus at 500-fold the concentration of radioactive dexamethasone, dexamethasone, for example, would be expected not only to compete with the higher-specificity, low-capacity sites, but would also occupy some of the sites of the second phase of the saturation curve, making it appear that the potent glucocorticoids are only slightly more active than steroids which are anti-inducers.

Saturation Curves with Glucocorticoids in Vitro—To obtain a dissociation constant, Kd, for each glucocorticoid, the unlabeled IB fraction was prepared as described above. Saturation curves with [3H]dexamethasone, [3H]corticosterone, and [3H]cortisol are shown in Fig. 3. The initial portions of the saturation curves appear to be sigmoidal for the three steroids. As the levels of the glucocorticoids are increased, the curves level off and at still higher levels the binding is greatly increased. The large increases in binding at high levels of steroids may probably indicate a second set of low affinity,
poorly saturable sites (17). The dissociation constants can be estimated roughly from the saturation phase of each curve to be: dexamethasone, about 1 nM; corticosterone, 25 nM, and cortisol about 13 nM. The complexity of these curves represents a significant difference from those observed with the hormone receptor (Binder II) (3), although it is possible that sigmoidicity might be a function of protein concentration and that supplementation with a nonsteroid binding protein might alter apparent sigmoidal behavior, as in the case with the chick oviduct progesterone receptor (18). Protein concentrations used here were between 1 to 4 mg/ml and were in the same range as similar studies with the hormone receptor which did not give sigmoidal curves (3). However, when ovalbumin, a protein which does not bind steroid (18), was added to aliquots of binder IB so that the total protein concentration was raised from 4 to 10 mg, the sigmoidal nature of the saturation curve with radioactive corticosterone was unchanged so that sigmoidicity is not a function of protein concentration within this range of protein concentration. The data of Fig. 3 suggest, in addition to the data in Table I, that the same binding site is involved in the binding of the three steroids since, at saturation, 3 to 4 pmol of steroid are bound per mg of protein in each case.

Effects of Temperature and pH on Binding—Unlabeled Binder IB pool was used directly for temperature and pH studies as described above. To determine the effects of temperature, 5-ml portions of the IB pool were incubated with 25 nM [3H]corticosterone at the temperatures indicated in Fig. 4 for 90 min. Thereafter the incubation mixtures were rapidly cooled in ice and binding was measured. The resulting binding is obviously temperature-dependent as the inverse linear relationship between binding and increase of temperature of the association of steroid and protein is virtually nil.

The results of incubating Binder IB and radioactive corticosterone at different pH values are shown in Fig. 5. Five milliliters of Binder IB pool from DEAE-Sephadex chromatography were incubated with 25 nM [3H]corticosterone together with phosphate or Tris buffer so that the final buffer concentrations were 50 mM at the pH specified in Fig. 5. The means of three experiments with the standard error of the mean in Fig. 5 show sharp pH dependence of binding with an optimum at pH 7.5.

Estimation of Molecular Weight by Gel Filtration—After labeling in vivo by administration of [3H]corticosterone, IB can be taken from the DEAE-Sephadex fraction and chromato-
G-25 column ("Materials and Methods"). The bound pool is chromatographed on a column of DEAE-Sephadex A-50 and the labeled pool eluting just after ligandin is collected (Fig. 1). This pool is lyophilized to achieve a 4- to 6-fold concentration of protein. During thawing a precipitate of nonspecific protein forms and is centrifuged off. The supernatant is chromatographed on a column of Sephadex G-100 (Fig. 8). The pool from Sephadex G-100 is chromatographed on a column of CM-Sephadex C-50 (Fig. 9) as a final step. The purification sequence is summarized in Table II.

Since there are four other binding proteins in cytosol, specific radioactivity cannot be used as an index to purification of IB until it has been resolved from the other binding proteins. This is achieved at the DEAESephadex chromatographic step and we assume quantitative recovery of bound steroid up to that point. In terms of protein fractionated away there is a 22-fold purification after the DEAE-Sephadex step. Precipitation of nonspecific protein after lyophilization and thawing, and chromatography on a column of Sephadex G-100 gives additional 17-fold purification. Subsequent chromatography on CM-Sephadex gives a further purification of 6- to 7-fold to give an over-all purification of about 2500-fold. If IB were homogeneous at this point, its concentration would be about 0.04% of cytosol proteins. Examination of purity by disc gel electrophoresis at each major fractionation step is shown in Fig. 10. The disc gel pattern of the 2500-fold purified material shows one major band and a faint minor band suggesting that IB is about 0.0004% of the cytosol proteins under these conditions. One binding site per molecule (30,000 daltons) has been assumed, although the suggestion of cooperativity in the saturation curves may subject this assumption to later revision. This would represent about 1/30 the concentration of the hormone receptor (Binder II). Unfortunately, we have been unable to apply disc gel electrophoresis or isoelectrofocusing in sucrose gradients to this protein and maintain the steroid-protein complex up to the present time. The binder may be frozen after any of the chromatographic steps and still retain its binding capacity for steroid. The purified binder (CM-Sephadex fraction) is stable and may be stored frozen for several weeks without loss of radioactivity or binding capacity. Fractionation steps subsequent to CM-Sephadex reveal extensive dissociation of the steroid-protein complex and increased lability of the protein. The behavior of IB described above is very different from our experiences with the hormone receptor (3).

**DISCUSSION**

The physiological role of Binder IB is unknown and we may not have insight into this problem until the hormone receptor,
protein and accounts for the large purification factor together with the Sephadex G-100 chromatographic step.

Because ligandin (Binder I in Fig. 1) has recently been proposed to be identical with GSH S-alkyl transferase B, one of the glutathione transferases which catalyzes the transfer of GS- to the methyl group of methyl iodide or to other cosubstrates (20), IB could be an enzyme. Should it have such an activity, the effects of nonsubstrate ligands upon enzymatic reaction would be of utmost interest. Militating against this possibility, however, is the fact that these transferases uniformly appear to have molecular weights of 45,000 with two equal size subunits, whereas IB is about 30,000. It would be difficult to reconcile this property with ligandin or the GSH transferase enzymes. There is the possibility that IB may be one of two equally-sized subunits of the hormone receptor (67,000 daltons) because of its apparent molecular weight, binding specificity, and apparent Kd values for binding of dexamethasone, corticosterone, and cortisol. We have observed a second corticosteroid-bound macromolecule, in addition to receptor, in the nucleus in vivo experiments with a PI value by isoelectrofocusing of 7.5 (3). This opens the possibility that this second macromolecule may be IB and that it occurs in the nucleus because it may have some steroid receptor activity in its own right or it may derive from the receptor. Up to now, the progesterone receptor from chick oviduct has been shown to contain a steroid binding subunit which can be dissociated from the receptor by calcium ions (18). We have repeated similar experiments with Binder II which did not dissociate a steroid binding subunit either as measured by ion exchange chromatography or by gel filtration (Sephadex G-100) chromatography. This leads to the conclusion that if IB is the steroid binding subunit of the receptor, it is much more tightly bound than is the case with the chick oviduct progesterone receptor.

The association between steroid and IB is our observation that radioactive steroid becomes increasingly extractable into cold methylene chloride with increasing purity of IB. This can be interpreted as either progressive denaturation or a conformational change in the protein. The latter possibility seems most likely since reassociation with radioactive steroid can be accomplished at all stages of purification. This explanation is supported by the complex saturation curves for steroid binding (Fig. 3). At the final stage of purification here, there is a great increase in dissociation of steroid upon application of further purification procedures.

Table II shows that as much as 25% of bound corticosterone is associated with this protein fraction in vivo. With such a low level of protein in the cell, extremely large scale experiments will be required to prepare a homogeneous protein. However, even at low protein concentrations in the cell, IB might store small amounts of hormone, releasing steroid slowly as the cellular concentration continued to fall, if the cooperative phenomena observed in vitro are operative in the physiological...
context. This mechanism could be especially important in view of the growth-promoting effects of adrenal corticosteroids (21), but it remains for future investigation to delineate the physiological role of the binding protein.

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