A subclass of glutathione *S*-transferases as intracellular high-capacity and high-affinity steroid-binding proteins

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The distribution of glucocorticoids incubated with rat liver cytosol preparations or administered *in vivo* to adrenalectomized rats was analysed by chromatographic procedures. Corticosterone or dexamethasone was co-eluted with Yb-type GSH *S*-transferases in anion-exchange and gel-permeation chromatography systems, and these glucocorticoids also were bound to Yb forms in analyses by immunoadsorbent and lysyl-GSH affinity matrices. Pretreatment of cytosol with lysyl-GSH to extract GSH *S*-transferases or incubation with excess bilirubin, which is expected to compete with steroids for binding to the protein, yielded preparations that were devoid of this major steroid-binding component. In mixtures of the multiple rat GSH *S*-transferases, corticosterone preferentially interacted with Yb forms rather than Ya and Yc subgroups. All of the multiple Yb forms resolved by chromatofocusing procedures retained the steroid-binding capacity. It is suggested that these abundant proteins can account for a considerable share of intracellular glucocorticoid binding and represent a high-affinity non-saturable binding component with potential to function in steroid-hormone metabolism and action.

INTRODUCTION

GSH *S*-transferases catalyse conjugation reactions between the thiol moiety of GSH and compounds with electrophilic centres (Boyland & Chasseaud, 1969; Jakoby, 1978; Chasseaud, 1979; Mannervik, 1985) and have the capacity to bind lipophilic substances (Kamisaka *et al.*, 1975; Ketterer *et al.*, 1978; Smith & Litwack, 1980; Bhargava & Arias, 1981; Maruyama *et al.*, 1983). The proteins are thus considered to function in detoxification and intracellular transport processes. Mammalian GSH *S*-transferases are dimeric, with M_r values of about 50000, and combinations of three classes of subunits, Ya, Yb and Yc, account for microheterogeneity of rat GSH *S*-transferases (Ishitani *et al.*, 1976; Bass *et al.*, 1977; Bhargava *et al.*, 1978*a,b*, 1980*a,b*; Hayes *et al.*, 1981; Scully & Mantle, 1981; Mannervik & Jensson, 1982; Frey *et al.*, 1983; Jakoby *et al.*, 1984; Maruyama *et al.*, 1984). The Ya and Yc subunits are structurally related, and combine to form homo- and hetero-dimers, but originate from different gene transcripts, and they may be regulated independently (Beale *et al.*, 1982; Kalinyak & Taylor, 1982; Daniel *et al.*, 1983; Tu *et al.*, 1984*a,b*; Lai *et al.*, 1984; Pickett *et al.*, 1984; Sheehan *et al.*, 1984). The Yb forms represent a distinct subclass of these proteins and do not assemble as heterodimers with Ya or Yc (Kitahara & Sato, 1981; Mannervik & Jensson, 1982; Maruyama *et al.*, 1983).

In contrast with Ya and Yc forms, several classes of steroids bind to the Yb GSH *S*-transferases in the presence or in the absence of GSH (Maruyama & Listowsky, 1984). In view of the selective high-affinity binding of steroids to pure Yb forms, these proteins have the potential to function in formation, transport, metabolism and perhaps even in the action of steroid hormones. These considerations would be more meaningful if this protein could be shown to be involved in steroid

binding in cell extracts or with steroids administered *in vivo*. In the present paper Yb GSH *S*-transferase components of rat liver are implicated in binding of glucocorticoids administered to rats or added to cytosol preparations.

EXPERIMENTAL

Materials

GSH was obtained from Sigma Chemical Co., 1-chloro-2,4-dinitrobenzene was from Aldrich Chemical Co. and SDS, acrylamide and bisacrylamide were from Bio-Rad Laboratories. [$1\alpha,2\alpha(n)^3$ H]Corticosterone (42 Ci/mmol) and [1,2,4- 3 H]dexamethasone (40 Ci/mmol), obtained from Amersham in toluene/ethanol (9:1, v/v) solution, were dried and redissolved in ethanol. Chromatofocusing gel PBE 94, Polybuffer PB 96, DEAE-Sephacel, Sephadex G-100, CNBr-activated Sepharose 4B and Protein A-Sepharose CL-6B were from Pharmacia, and Bio-Gel P-2 was from Bio-Rad Laboratories. Buffers and other chemicals were reagent grade, obtained from Sigma Chemical Co. and Fisher Scientific Co. DNA-cellulose, prepared by the procedure of Alberts & Herrick (1971), contained 5.7 mg of double-stranded calf thymus DNA/g of matrix.

Protein preparations

The GSH *S*-transferases were prepared by methods described previously (Maruyama *et al.*, 1984; Habig & Jakoby, 1981). Gel-permeation- and anion-exchange-chromatographic steps combined with lysyl-GSH affinity chromatography (Maruyama *et al.*, 1984) yielded pure preparations of Yb forms of the protein. Enzymic activities were measured with 1-chloro-2,4-dinitrobenzene (1.0 mM) and GSH (5.0 mM) as substrates (Habig & Jakoby, 1981).

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Glucocorticoid binding

Liver cytosol preparations (105000 g supernatants) were fractionated by methods described previously (Maruyama *et al.*, 1984). Protein samples were incubated with small volumes (usually 1–5 μ l) of [3 H]corticosterone or [3 H]dexamethasone for 30 min at 4 °C before initiation of chromatographic analyses. Because of the sufficiently fast dissociation rates of protein-bound steroids, and adsorption of the lipophilic steroids by chromatographic matrices used in this study, most of the column-chromatographic analyses reflected re-equilibration of protein-bound steroid with steroid adsorbed on these

resins during elution. Columns packed with adsorbent plugs such as dextran-charcoal eliminated most of the free steroids that were eluted, but in some cases also decreased the amounts of protein-bound components. The procedures were therefore used only to identify binding components, since non-specific adsorption precluded estimations of stoichiometric ratios on the basis of these binding studies. Equilibrium chromatography was performed by the method of Hummel & Dreyer (1962). Details of individual experimental conditions are cited in the text and appropriate Figure legends.

Fig. 1. Glucocorticoid binding in rat liver cytosol: analysis by DEAE-Sephacel chromatography

Rat liver cytosol (105000 g supernatant) preparations (80–90 mg of protein in 2 ml) in 10 mM-Tris/HCl buffer containing 1.0 mM-EDTA, 1.0 mM-thioglycerol and 0.1 mM-phenylmethanesulphonyl fluoride, pH 8.0, were incubated with 5.0 mM-[3 H]glucocorticoids and applied to 1.4 cm $\times$ 30 cm columns of DEAE-Sephacel. Each 3 ml fraction collected was analysed for A_{280} (protein) ($\bullet$), GSH S-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate ($\circ$) and radioactivity ([3 H]corticosterone) (Δ). (a) Cytosol incubated with [3 H]corticosterone at 4 °C for 30 min. (b) [3 H]Corticosterone was administered to adrenalectomized rats in accordance with the protocol cited in the Experimental section. Liver cytosolic components were obtained and applied directly to the DEAE-Sephacel columns. The insets indicate NaCl gradients used to elute the columns.

Corticosterone binding *in vivo*

Male Sprague-Dawley rats (200–250 g) were used for all studies. Bilateral adrenalectomies were performed by posterior incisions while the animals were anaesthetized with diethyl ether. The rats were maintained on liquid diets containing 0.9% NaCl and 1.0% glucose for 5 days, at which time 100 μ Ci of [3 H]corticosterone, in sterile 0.154 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4, was administered intraperitoneally 1 h before the rats were killed. A second dose of [3 H]corticosterone (100 μ Ci) was administered, and after 10 min livers were excised, perfused with 10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-phenylmethanesulphonyl fluoride, and homogenized in the same buffer. The homogenate was centrifuged at 20000 *g* for 30 min and then at 105000 *g* for 2 h to prepare the supernatant fraction.

Immunoabsorbent

Rabbit anti-(rat Yb GSH S-transferase) sera were obtained as described previously (Maruyama &

Listowsky, 1984). IgG fractions were prepared by passing the antisera through a Protein A-Sepharose CL-6B column, and the IgG fraction was coupled to CNBr-activated Sepharose 4B by procedures of Marcus & Zinberg (1974). Monoclonal antibody to the rat glucocorticoid receptor was given by Dr. G. Litwack (Temple University, Philadelphia, PA, U.S.A.).

Analytical methods

SDS/polyacrylamide gels were prepared by procedures described previously (Bhargava *et al.*, 1978*a,b*). Radioactivity was measured with a Beckman LS-7500 scintillation counter, and absorbances with a Cary 15 spectrophotometer.

RESULTS

The partition of [3 H]corticosterone added to a rat liver 105000 *g* supernatant fraction is represented by the chromatographic pattern on DEAE-Sepharose shown in

Fig. 2. Corticosterone binding in rat liver cytosol: analysis by gel-permeation and lysyl-GSH affinity chromatography

Rat liver cytosol preparations (42 mg of protein/ml) in 10 mM-Tris/HCl buffer containing 1.0 mM-EDTA and 1.0 mM-thioglycerol, pH 8.0, were incubated with 4.0 mM-[3 H]corticosterone, and 5 ml portions of the mixture were applied to a Sephadex G-100 column (1.6 cm $\times$ 90 cm; V_t = 180 ml) (a) and an analytical lysyl-GSH affinity matrix (1 cm $\times$ 10 cm) (b). Symbols are the same as those in Fig. 1.

Fig. 1(a). The elution positions of corticosterone and 'anionic' or Yb-type GSH *S*-transferase activities with 0.08–0.09 M-NaCl coincided in the retained components on the anion-exchange resin. The glucocorticoid-binding component that was eluted with higher salt concentrations (0.15 M) has not been identified, but amounts were usually small and varied in different preparations. [³H]Dexamethasone distributed in a similar manner. Quantities of labelled steroids that were eluted together with basic proteins in the flow-through fractions could be decreased substantially if dextran-charcoal plugs were used in the column. [³H]Corticosterone was also administered to adrenalectomized rats and the label was traced. Here, too, a significant portion of the radioactivity was co-eluted with the anionic GSH *S*-transferase component (Fig. 1b). The overall distribution of steroid found by anion-exchange methods is similar to that observed by Litwack and co-workers by DEAE-Sephadex chromatography (Litwack *et al.*, 1973; Parchman & Litwack, 1977; Litwack *et al.*, 1978; Schmidt *et al.*, 1982), and the anionic component eluted with 0.08 M-NaCl may correspond to the elution position of 'binder II' identified by those workers (Litwack *et al.*, 1973), but the anionic GSH *S*-transferase did not cross-react with monoclonal antibodies to rat glucocorticoid receptors or binder II.

Cytosol preparations were subjected to lysyl-GSH affinity chromatography (Maruyama *et al.*, 1984) to extract all of the multiple forms of rat GSH *S*-transferase, and the remaining components were devoid of the anionic (0.08–0.09 M-NaCl fraction) steroid-binding component. The steroid binding in this fraction was also displaced if cytosol was preincubated with 10 μ M-bilirubin; the GSH *S*-transferases exhibit high-affinity binding capacity for bilirubin. Steroid binding also accompanied GSH *S*-transferase activities in rat liver cytosol fractionated by gel-permeation chromatography (Fig. 2a) and lysyl-GSH affinity chromatography (Fig. 2b). In addition, [³H]corticosterone incubated with liver cytosol adhered to an immunoadsorbent column prepared with IgG fractions from rabbit anti-(rat Yb GSH *S*-transferase) sera (Maruyama & Listowsky, 1984), which had been shown to adsorb specifically transferases with the Yb-type subunits. All of the multiple Yb forms resolved by chromatofocusing procedures exhibited steroid-binding capacity.

The multiple forms of rat liver GSH *S*-transferase were isolated as a group by gel-permeation chromatography (Sephadex G-100), followed by lysyl-GSH affinity chromatography. This family of proteins was incubated with [³H]corticosterone and fractionated by high-resolution preparative anion-exchange chromatography. In this system the first flow-through fractions contained the basic GSH *S*-transferases, composed of Ya- and Yc-type subunits, which were partially resolved from the basic Yb forms (Fig. 3). Steroid that was not bound to protein came through the column shortly after the Ya and Yc fractions in the 'breakthrough' volume. The anionic GSH *S*-transferase and bound [³H]corticosterone were co-eluted with 0.08 M-NaCl.

DISCUSSION

Glucocorticoid-binding capacity of rat liver cytosolic proteins paralleled Yb or anionic-type GSH *S*-transferase activities in gel-permeation- (Fig. 2a) and anion-exchange-chromatographic systems (Fig. 1), as well as in lysyl-GSH

affinity chromatography (Fig. 2b). Pretreatment of cytosol with lysyl-GSH to extract GSH *S*-transferases, or addition of bilirubin, which is expected to compete with steroids for binding to the protein (Kamisaka *et al.*, 1975), eliminated this discrete glucocorticoid-binding component. These data appear to implicate the Yb GSH *S*-transferase as a prominent steroid-binding protein in liver cytosol.

The data in Fig. 3 show that cationic Ya- or Yc-type GSH *S*-transferases or ligandins, which were previously considered to be steroid binders (Litwack *et al.*, 1971; Smith & Litwack, 1980), are probably not effective under those conditions. Earlier studies also showed that steroid binding to these forms of the transferases is of lesser affinity than that of Yb forms, and their binding capacity for steroids is abolished in the presence of GSH (Maruyama & Listowsky, 1984). The GSH *S*-transferases have the capacity to bind a broad range of lipophilic compounds, so that bile acids, bilirubin, haem, fatty acids and related endogenous metabolites (Mannervik, 1985) may be expected to compete with steroids for binding to these proteins.

It may be premature to ascribe definitive functions for the anionic Yb GSH *S*-transferase in steroid-hormone metabolism and action. Earlier evidence for binding of steroids to the purified Yb protein (Maruyama & Listowsky, 1984) has now been extended in scope by demonstrating that the protein retains its steroid-binding capacity even in the presence of other cytosolic components that probably include glucocorticoid receptors. Extrapolation of these results to cellular or physiological levels requires further experimentation. The potential effects of this protein, however, should not be disregarded. This protein is abundant in many cell types, and estimates based on immunological or enzymic data (Maruyama & Listowsky, 1984) suggest that concentrations of the protein in liver cytosol preparations are usually greater than 1 μ M. That greatly exceeds physiological concentrations of steroid hormones. Technical limitations preclude direct measurements of dissociation constants for the glucocorticoids because the protein is easily denatured at very low (nanomolar) concentrations, but the data from spectroscopic studies indicate high-affinity steroid binding with dissociation constants less than 10^{-7} M (Maruyama & Listowsky, 1984). Therefore studies that define binding or receptor proteins as 'specific' on the basis of saturability, or experiments contingent on displacement of bound radiolabelled ligands, may exclude the Yb GSH *S*-transferase because large excesses of total ligand would be required to exceed the binding capacity of such an abundant protein. In sufficiently diluted cytosol preparations, radiolabelled steroids are displaced from the protein by excess of unlabelled forms.

In many tissues this protein would be categorized as 'non-saturable' or 'non-specific' in such binding studies, yet the Yb GSH *S*-transferase has the potential to account for a considerable share of intracellular binding of steroids because of its affinity and high concentration. The protein could possibly be involved in transport and/or metabolic conversions of the steroids because, in addition to its presence in cytosol, it also occurs in subcellular organelles such as microsomal fractions (Morgenstern *et al.*, 1980, 1982) and the nucleus (J. Baron, H. Homma & I. Listowsky, unpublished work). Alternatively, because of its abundance, the

Fig. 3. Analysis of corticosterone binding to the multiple forms of rat GSH *S*-transferase

The family of GSH *S*-transferases was purified from rat liver cytosol by gel-permeation chromatography on Sephadex G-100, followed by lysyl-GSH affinity chromatography. These preparations were shown to consist of three subunit types, Ya, Yb and Yc, of M_r 25000, 23500 and 22000 respectively, by SDS/polyacrylamide-gel electrophoresis, and there were no other protein bands visible on the gels. The mixture of GSH *S*-transferases (1.6 mg in 2 ml) in 10 mM-Tris/HCl buffer containing 1.0 mM-EDTA, pH 8.0, was applied to a 1.4 cm × 50 cm column of DEAE-Sephacel and eluted with the same buffer. ○, GSH *S*-transferase activity; △, radioactivity (³H]corticosterone). The SDS/polyacrylamide-gel (11.5% acrylamide) electrophoretogram in the top inset shows some representative peak fractions (numbered according to elution volumes in ml).

protein may function as a safeguard to prevent undesired effects of steroid hormones. The interplay between this protein and the glucocorticoid receptor of rat liver is at present unclear, but kinetic considerations may be important. Dissociation rates from the Yb forms appear to be sufficiently fast to permit exchange of bound steroids between this protein and other components involved in their metabolism and action.

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