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The Identity of Glutathione S-Transferase B with Ligandin, a Major Binding Protein of Liver

(organic anion binding protein/Y protein)

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ABSTRACT **Evidence is presented that ligandin, an intracellular protein involved in the binding of such anions as bilirubin, indocyanine green, and penicillin, is identical to glutathione S-transferase B (EC 2.5.1.18), an enzyme catalyzing the conjugation of glutathione with such electrophiles as 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, iodomethane, ethacrynic acid, and bromosulfophthalein. The proteins, isolated by distinct methods, have the same specificity for substrates and for ligands, react in identical fashion to antibody produced against ligandin, bear entirely similar physical characteristics and amino acid composition, and are both induced in response to phenobarbital. Indocyanine green, one of the ligands that is not effective as a substrate, was shown to competitively inhibit the conjugation reaction. It is suggested that specificity is directed toward compounds with electrophilic sites.**

Ligandin is a cytoplasmic protein found in abundance in the liver of rat, man, and other species. This protein is capable of binding noncovalently a large number of compounds, which includes bilirubin, heme, benzyl penicillin, certain steroids, and such dyes as bromosulfophthalein and indocyanine green (1, 2). Phylogenetic, ontogenetic, induction, and competition studies support the hypothesis that ligandin is a major determinant of the net flux of organic anions from plasma into the liver (3–7). Fractionation of the protein on the basis of any one of its binding activities has resulted in apparently identical, highly purified preparations (7–9). Thus, the term, ligandin (2), is synonymous with that of azo-dye carcinogen-binding protein (8), corticosteroid binding I protein (9), and Y protein (7)‡, §.

The glutathione S-transferases (EC 2.5.1.18) from rat liver (10–13) have several physical properties in common with rat liver ligandin (2). When crude liver extracts were subjected to filtration on Sephadex G-75, the fraction containing ligandin also served as a source of enzymatic activity for the conjugation of glutathione (GSH) with 1,2-dichloro-4-nitrobenzene (14). However, subsequent fractionation resulted in removal of almost all GSH transferase activity with this substrate despite virtually complete recovery of ligandin (‡, 15).

Since four of the GSH transferases of rat liver (transferases A, B, C, and E) have been purified to homogeneity (11–13), specific tests of the relationship between these enzymes and ligandin are now possible. The present study indicates that ligandin serves as a catalyst in the conjugation of a variety of electrophiles with GSH in a pattern of specificity that implies identity between this protein and GSH transferase B.

Abbreviation: GSH, glutathione.

‡ G. Fleischner, A. J. Levi, Z. Gatmaitan, and I. M. Arias, in preparation.

§ Kirsch, R., Fleischner, G., Kamisaka, K., Gatmaitan, Z. & Arias, I. M. (1974) *J. Clin. Invest.*, submitted.

MATERIALS AND METHODS

The preparations referred to as GSH transferase B were isolated as described by Habig *et al.* (12) and the enzyme was crystallized with ammonium sulfate at 70% of saturation by the general method previously described (16). Preparations referred to as ligandin were isolated as described by Fleischner *et al.* ‡. Each protein was homogeneous, as demonstrated by acrylamide gel electrophoresis with and without sodium dodecyl sulfate, sedimentation equilibrium centrifugation, and the ability to produce monospecific precipitating antibody.

Conditions for enzymatic analysis of each of the substrates have been presented (12). The concentration of ligandin was determined by radial immunodiffusion (17). Protein was determined chemically (18).

The capacity of GSH transferase B and of ligandin to bind [^{3}H]bilirubin [prepared from [^{3}H]3,5-γ-aminolevulinate (19)], potassium 6-phenyl-1-[^{14}C]acetamido-penicillanate (Amersham/Searle), and indocyanine green was studied by gel filtration. After incubation of 100 μg of protein in 0.5 ml of 0.01 mM potassium phosphate at pH 7.4 for 5 min with 0.5 μmoles of bilirubin (1 μCi), 1 μmole of penicillin (1 μCi), or 3 μmoles of indocyanine green, each mixture was subjected to gel filtration on a 0.5 × 50-cm column of Sephadex G-75 with the same phosphate buffer. Radioactivity was measured in a liquid scintillation system; indocyanine green was determined spectrophotometrically (7).

The preparation and properties of goat antibody to ligandin have been described (20). The precipitin curves with this antibody were obtained by incubating protein and antibody at pH 7.4 in phosphate-buffered saline overnight at 4°; thereafter, polyethylene glycol 6000 (Matheson, Coleman and Bell) was added to a final concentration of 2% (w/v) and the precipitate was removed by centrifugation after an additional 4 hr at 4°. The supernatant fluid was used directly for enzyme assays with 1,2-chloro-4-nitrobenzene. The precipitate was washed twice with phosphate-buffered saline at pH 7.4, containing 2% polyethylene glycol. The residue was dissolved in 0.5 M NaOH, and absorbance at 280 nm was determined.

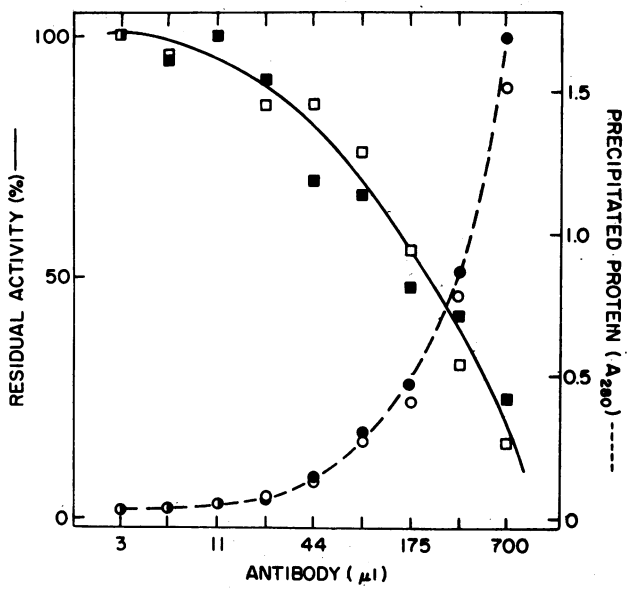

FIG. 1. Quantitative precipitin curve for glutathione transferase B and ligandin. The antigens, 40 μg, were incubated for 16 hr with the immunoglobulin G fraction of goat antiserum to ligandin in a total volume of 0.8 ml. After addition of 22% polyethylene glycol in 80 μl of phosphate-buffered saline (w/v) and centrifugation, the supernatant fluid was assayed for enzyme activity while the pellet was washed twice with phosphate-buffered saline containing 2% of the glycol and dissolved in 0.5 M sodium hydroxide. Solid and open symbols, ligandin and transferase B, respectively. Circles, precipitated protein; squares, enzyme activity.

Litter-mate Sprague–Dawley rats (150–175 g) were tested for the effect of phenobarbital after subcutaneous injection of 8 mg of the drug per 100 g of body weight daily for 10 days. Liver homogenates were subjected to centrifugation at 100,000 × *g*, and the supernatant fluid was fractionated on a column (3.2 × 55 cm) of Sephadex G-75 (7). The fraction bearing the highest concentration of ligandin from each liver extract was used for subsequent assay of enzyme activity.

RESULTS

Catalytic Activity. Ligandin was found to possess GSH transferase activity with the identical spectrum of substrates that characterize transferase B. This parallelism in catalytic activity is demonstrated in Table 1. Both proteins are active in conjugating 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride, iodomethane, and ethacrynic acid with GSH. The absence of activity in conjugating GSH with either 4-nitropyridine *N*-oxide or 1,2-epoxy-3-(*p*-nitrophenoxy) propane differentiates this enzyme species from transferases A, C, and E (10, 12). Bromosulfophthalein is a substrate for both transferase B and ligandin, but substrate inhibition and low intrinsic catalytic rate preclude reporting of accurate kinetic data.

Although the K_m for 1-chloro-2,4-dinitrobenzene is essentially the same for ligandin and transferase B, i.e., 0.6 mM and 0.8 mM, respectively, there is a significant difference in the two proteins: transferase B has a specific activity for all of the substrates that is 2- to 3-fold greater than the species isolated as ligandin. We interpret the observation of higher catalytic activity of transferase B to be the result of refrigeration and enzyme-stabilizing agents, i.e., 30% glycerol, 1 mM EDTA, and 2 mM GSH, used in its preparation. These precautions did not seem to be as important for ligand binding and were not used in the preparation of ligandin.

Antigenicity. When tested by the Ouchterlony immunodiffusion techniques or by immunoelectrophoresis, monospecific goat immunoglobulin G obtained by immunization with ligandin (20) yielded a single line of identity with transferase B and ligandin; GSH transferases A, C, and E did not crossreact with this antibody. Antibody prepared against transferase A (12) did not react with either ligandin or transferase B.

In the precipitin reaction, ligandin and transferase B reacted identically with antibody prepared against ligandin despite differences in their catalytic activity. Within experimental error, the single precipitin curve shown in Fig. 1 fits the experimental data for both protein preparations. The residual supernatant enzyme activity also follows a single curve for both preparations. The removal of enzyme activity from solution correlates well with the precipitation of protein throughout the range of antibody concentration that was used.

Binding Activity. Ligandin that is exposed to bilirubin, indocyanine green, or benzyl penicillin elutes from columns of Sephadex G-75 associated with the ligand; dissociation constants of the order of $< 10^{-6}$, and 10^{-3} M, respectively, have been recorded for these compounds (20, 21). Parallel experiments performed on a qualitative basis with GSH transferase B yielded entirely similar results. Furthermore, quantitative studies on the effect of indocyanine green on the conjugation of 1-chloro-2,4-dinitrobenzene disclosed that the dye is a competitive inhibitor, with a dissociation constant of 5×10^{-6} M.

Physical Properties. GSH transferase B (12) and ligandin (2, ‡) are both proteins of about 45,000 molecular weight, composed of two subunits of equal molecular weight, and have isoionic points above pH 9.0. Amino-acid analyses of the two preparations (performed by George Poy of the National Institutes of Health) were identical and the same as previously reported for transferase B (12).

Effect of Phenobarbital. Treatment of rats with phenobarbital induces ligandin in liver (6, 7). An attempt was made to correlate the induction of ligandin with GSH transferase activity after phenobarbital administration to rats. Reference to Table 2 shows that treatment with phenobarbital increased the activity of liver extracts with 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, and iodomethane. There was no significant increase in activity with the three other compounds that are not substrates of transferase B. Increased enzyme activity resulting from phenobarbital treatment was not due to changes in the concentration of transferases A and C. Antibody to transferase A, which also reacts with transferase C (12), precipitated about the same amount of enzyme activity from the four samples listed in Table 2: 0.23, 0.32, 0.26, and 0.33 μmoles of conjugate formed from 1-chloro-2,4-dinitrobenzene per ml/min.

The amount of activity due to transferase B was determined by treating each of the liver extracts with antibody to ligandin and, after removal of precipitated protein, comparing the remaining enzyme activity with that of the same extract after treatment with normal immunoglobulin G (Table 3).

TABLE 1. *Substrate specificity for glutathione transferase B and ligandin*

Substrate	GSH transferase activity (μmoles min^{-1} mg^{-1})		Transferase/ ligandin
	Transferase B	Ligandin	
1-Chloro-2,4-dinitrobenzene	16.0	7.2	2.2
1,2-Dichloro-4-nitrobenzene	0.014	0.005	2.9
p-Nitrobenzyl chloride	0.25	0.13	1.9
Iodomethane	0.77	0.34	2.3
Ethacrynic acid	0.26	0.12	2.2
4-Nitropyridine *N*-oxide	0	0	—
1,2-Epoxy-3-(*p*-nitrophenoxy) propane	0	0	—

Homogeneous preparations of GSH transferase B and ligandin were tested for GSH transferase activity with the substrates listed (12).

TABLE 2. *Effect of phenobarbital treatment on glutathione transferases of rat liver*

Substrate	GSH transferase activity (μmoles min^{-1} mg^{-1})			
	Control rats		Phenobarbital-treated rats	
	1	2	1	2
1-Chloro-2,4-dinitrobenzene	4.8	3.4	7.6	6.7
1,2-Dichloro-4-nitrobenzene	0.25	0.20	0.31	0.30
1,2-Epoxy 3-(*p*-nitrophenoxy) propane	0.05	0.03	0.04	0.06
Menaphthyl sulfate	0.01	0.01	0.01	0.01
Nitropyridine *N*-oxide	0.07	0.05	0.08	0.08
trans-4-Phenyl-3-buten-2-one	0.09	0.06	0.08	0.06
Iodomethane	0.07	0.07	0.12	0.32

Data were obtained from measurements of enzyme activity of the peak fraction after homogenization and passage through Sephadex G-75. Details are presented in *Materials and Methods*.

Both transferase B activity and ligandin were increased by treatment of the animal with phenobarbital. Significantly, the ratio of transferase B activity to ligandin, i.e., the specific activity, remained essentially constant.

DISCUSSION

The present examination of ligandin and GSH transferase B with regard to catalytic activity, antigenicity, and binding behavior provides compelling evidence for the identity of the two proteins. Conflicting results have been presented previously regarding the possibility that ligandin possessed GSH transferase activity with 1,2-dichloro-4-nitrobenzene as substrate (14, 15); however, this reaction is catalyzed by at least three enzymes in rat liver and ligandin is only one of them. Because the specific activity of ligandin for dichloronitrobenzene (0.003 μmole/min per mg) is three orders of magnitude lower than the specific activity of 4.3 for GSH transferase A (13) and 2.0 for GSH transferase C (12), testing ligandin for enzyme activity results in a value sufficiently low as to question its presence. Contamination of ligandin preparations with as little as 0.1% GSH transferase A or C leads to a doubling in measurable enzyme. Resolution of the problem came from studies utilizing ligandin and GSH transferases A, B, and C that were purified to homogeneity. The precipitin study performed with antibody to ligandin (Fig. 1) confirms the conclusion that preparations of ligandin and GSH transferase B are of the same high degree of purity.

In view of the inherent catalytic activity of ligandin, the specificity for binding to ligandin may be reevaluated. Although ligandin has been described as binding organic anions (2), a neutral compound such as iodomethane serves as a ligand as well as a substrate (10). In evaluating the spectrum of active compounds, we note that the enzyme reaction is one in which a nucleophile, glutathione, attacks a compound with an electrophilic site. Since neutral compounds can be bound, we suggest that the protein's affinity is directed toward electrophilic sites rather than limited to anions. Not all ligands are effective substrates; bilirubin, 3,6-dibromophthalein disulfonate (22), penicillin, and indocyanine green are examples. However, each inhibits catalytic activity and, in the one case examined quantitatively, indocyanine green was a competitive inhibitor with a dissociation constant of the order of 10^{-6} M whether determined by binding or by inhibition of the enzymatic reaction.

The low specific activity of ligandin with dichloronitrobenzene does not suggest that ligandin is an ineffective catalyst. Comparison with other GSH transferases (12, 13) reveals that ligandin is highly active with 1-chloro-2,4-dinitrobenzene and has the highest specific activity of any of the five GSH transferases for the diuretic, ethacrynic acid (12).

TABLE 3. *Correlation of ligandin with glutathione transferase after treatment with phenobarbital*

Treatment	GSH transferase activity (μmoles of product min^{-1} ml^{-1})			Ligandin concentration (mg ml^{-1})	Precipitated GSH transferase activity/ ligandin (μmoles min^{-1} mg^{-1})
	Normal IgG	Anti-ligandin IgG	Activity precipitated		
Control 1	1.84	1.24	0.60	0.086	7.0
Control 2	3.06	1.66	1.40	0.203	6.9
Phenobarbital 1	4.83	1.97	2.86	0.354	8.1
Phenobarbital 2	6.64	2.49	4.15	0.485	8.6

Liver extracts from normal and phenobarbital-treated rats were mixed with normal rabbit immunoglobulin G (IgG) or with excess antibody to ligandin. Antigen–antibody complexes formed were precipitated with 35% ammonium sulfate. Enzyme activity in the supernatant fluid was assayed with 1-chloro-2,4-dinitrobenzene. The concentrations of ligandin were determined by radial immunodiffusion (17).

By micropuncture techniques, ethacrynic acid has been shown to be secreted by the proximal convoluted tubule of the kidney as a GSH conjugate (23); immunofluorescent studies localize ligandin in the kidney to this part of the nephron (24), where it constitutes about 5% of the soluble protein (19). Ligandin is also abundant in parenchymal liver cells and nongoblet mucosal cells of the small intestine, where it constitutes 5% and 2%, respectively, of the soluble protein (20, 24). Since liver extracts contain at least three times as much ligandin as all of the other GSH transferases combined, we predict that the protein performs a physiological role as a catalyst as well as in the binding function of cellular transport.

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