Ligandin

BILIRUBIN BINDING AND GLUTATHIONE-S-TRANSFERASE ACTIVITY ARE INDEPENDENT PROCESSES*

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Physical methods and chemical modifications were used to discriminate between the bilirubin-binding capacity and glutathione-S-transferase activity of ligandin which was purified from rat liver. Binding of bilirubin occurs at a primary high affinity site \( (K_a = 5 \times 10^4 \text{ M}^{-1}) \) and at a secondary, lesser affinity site \( (K_a = 3 \times 10^6 \text{ M}^{-1}) \). Circular dichroism and fluorescence quenching methods were used to distinguish between these sites. Cross-linked as well as reduced and alkylated ligandin lost high affinity bilirubin-binding capacity, but retained glutathione-S-transferase activity, bilirubin binding at a secondary site, and immunological reactivity. Succinylation of ligandin abolished catalytic activity and bilirubin binding at high and low affinity sites, but not immunological reactivity. Catalytic activity was unaffected by concentrations of bilirubin which saturated the primary binding site. These results suggest that the high affinity site at which bilirubin is bound to ligandin is independent from the site at which catalytically reactive substrates bind. The latter substrates probably interact at the secondary bilirubin binding site where bilirubin competitively inhibits glutathione-S-transferase activity.

Rat liver ligandin binds various organic anions and also exhibits glutathione-S-transferase activity (1, 2). Nonsubstrate ligands, such as bilirubin, competitively inhibit glutathione-S-transferase activity of ligandin which suggests an interrelationship between binding and catalytic functions (3, 4). In this report, several lines of evidence indicate that primary high affinity binding of bilirubin to ligandin and its glutathione-S-transferase activity are independent and occur at different domains in the protein. A secondary low affinity site for bilirubin binding is either identical or overlaps with substrate binding at the catalytic site.

MATERIALS AND METHODS

Preparation of Ligandin—Ligandin was isolated from adult male rat liver (5) and its purity was verified by sodium dodecyl sulfate gel electrophoresis in Tris/acetate, phosphate, and glycine gel systems (6), specific catalytic activity (7 to 8 umol of 1-chloro-2,4-dinitrobenzene GSH complex formed/min/mg) (4), quantitative bilirubin binding \( (K_a = 5 \times 10^4 \text{ M}^{-1}) \) (7), electrofocusing (6), immunodiffusion and immunoelectrophoresis (5).

Assay of Glutathione-S-Transferase Activity—Ligandin was incubated with reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene in 0.01 M phosphate buffer, pH 6.6, and increases in absorption at 343 nm were measured (8). In inhibition studies, ligandin was incubated at room temperature for 5 min with bilirubin in the phosphate buffer containing GSH, and the reaction was started by addition of 1-chloro-2,4-dinitrobenzene.

Alkylation of Ligandin—Ligandin was dissolved in 0.01 M Tris buffer, pH 8.2, containing 6 M urea. Nitrogen was bubbled through and maintained as a slow stream above the surface of the solution. β-Mercaptoethanol was added in a 50- to 100-fold molar excess relative to the protein (9). pH was maintained at 8.2 by addition of small amounts of 2 N NaOH, and the reaction proceeded for 30 min to 1 h at room temperature. Recrystallized iodoacetamide was dissolved in 0.01 M Tris buffer at pH 9.2 and added to give a final molar ratio of 1:100 of iodoacetamide to ligandin. The reaction was performed in the dark to prevent liberation of free iodine. The pH was maintained at 8.2 and the reaction proceeded for another 30 to 90 min. The reaction mixture was dialyzed against 0.01 M Tris, pH 8.2, for 1 h at room temperature and for 12 h at 2°C to remove excess iodoacetamide and urea. Alkylated ligandin had the same isoelectric point and molecular weight as did modified ligandin. Titration of modified ligandin with 5,5′-dithiobis(2-nitrobenzoic acid) showed that alkylation was essentially complete; less than 5% reaction occurred when compared to that of the native protein.

Succinylation of Ligandin—Four to five milligrams of lyophilized ligandin was dissolved in 1 ml of 6 M urea 0.2 M borate solution, and 6 N HCl was added to adjust pH to 9.0. A slow stream of nitrogen was bubbled at the top of the solution throughout the treatment. A 50 molar excess of succinic anhydride (based on the number of free amino groups available for modification) was added in five or six equal portions over 1 h (10) and pH was maintained at 8.0 by addition of 2 N NaOH as needed. Fifteen minutes after the last addition of succinic anhydride, the mixture was dialyzed against 0.05 M NH₄HCO₃ and lyophilized.

Cyanogen Bromide Cleavage—Five milligrams of lyophilized, reduced, and alkylated ligandin was dissolved in 70% formic acid. CNBr was added in a 50- to 100-fold molar excess of methionine residues and the reaction continued for 30 h at room temperature in a stoppered flask that was flushed with N₂ and continuously stirred. After the reaction, the mixture was diluted 10- to 20-fold with distilled water and lyophilized.

CovaLently Cross-linked Ligandin—Cross-linking was performed according to methods described by Davise and Stark (11). Dimethylsulfoximide and ligandin solutions in 2 M triethanolamine hydrochloride buffer, pH 8.5, were mixed to give final concentrations of approximately 0.2 mg/ml of protein and 5 mg/ml of dimethylsulfoximide in volumes of 200 ml. The reaction mixture was left at room temperature for at least 3 h.

Binding of Bilirubin to Ligandin by Circular Dichroism—A Cary model 60 spectropolarimeter with 6001 circular dichroism attachment was used to detect bilirubin binding by characteristic circular dichroism spectra between 520 and 400 nm (12); the extent of binding was quantitated on the basis of molar ellipticity values at these wavelengths (7). Measurements were made in a 1-cm pathlength cell at 27°C. Absence of extrinsic bands in the region of bilirubin absorption were interpreted to reflect drastic alterations or lack of binding capacity. To study binding, ligandin was present at 1 to 2 mg/ml and small increments of 0.02 M bilirubin solution in 20 mM NaOH were added (7).

Secondary Structure of Ligandins—Conformational features of modified ligandins were estimated from ellipticity values in the pep-
tide absorption region of 240 to 190 nm. The data were analyzed according to methods described earlier (12).

**Immunoelectrophoresis of Ligandin—**Immunoelectrophoresis was performed for 2 h in barbital buffer, pH 8.6 (α = 0.01) at 80 V per plate. Immunoecplates obtained from Boehringer were used. Incubation with anti-rat liver ligandin IgG, washing, staining, and destaining were performed as described previously (5).

**Determination of Binding by Fluorescence Changes—**A Perkin-Elmer Spectrophotofluorimeter model MPF-3 was used. Cuvettes contained 100 to 150 µg of ligandin in 0.01 M phosphate buffer, pH 7.4, and 20-µl increments of 10⁻⁴ M bilirubin in 20 mM NaOH were added. Corrections for dilution were made. Decrease in intrinsic fluorescence after addition of bilirubin was measured at 336 nm on excitation at 295 nm. Bilirubin absorption at 295 nm did not give rise to inner filter effects, and therefore, no corrections were made for this factor. The stoichiometry binding of bilirubin was determined from the fluorescence data on the basis of Scatchard plots.

**RESULTS**

Ligandin preparations were reduced and alkylated with iodoacetamide, or cross-linked using dimethylsuberimidate. The cross-linked protein consisted primarily of a 46,000 molecular weight dimer that could not be dissociated further by denaturants. Additional data to characterize the cross-linked protein are described in the accompanying paper. The modified proteins were examined by circular dichroism spectroscopy. At saturating concentrations of bilirubin, reduced, alkylated, or cross-linked ligandin exhibited ellipticity bands centered at 515 nm and at 465 nm (Fig. 1). The ellipticity magnitudes near 515 nm were comparable to those of unmodified protein, but ellipticity values at 465 nm were much less than those of native ligandin. The band at 405 nm is not detectable for either cross-linked or alkylated ligandin. In spite of the loss in bilirubin-binding capacity, reduced and alkylated ligandin retained substantial glutathione-S-transferase activity and the cross-linked protein retained full activity (Table I). The overall secondary structure of the modified proteins was similar to that of the native protein since no appreciable circular dichroic changes occurred in the region of peptide absorption after these modifications (Table I).

Immunological reactivity was retained by the alkylated and cross-linked proteins as well as by succinylation. After succinylation, bilirubin binding at both the high and low affinity sites was abolished since no extrinsic ellipticity bands were generated (Fig. 1). Succinylated ligandin was also inactive catalytically and exhibited less ordered secondary structure than did native ligandin (Table I).

The quenching of intrinsic tryptophan fluorescence of ligandin by bilirubin was used as an index of binding. Scatchard plots of the data are shown in Fig. 3. The effect of bilirubin binding at the high affinity site on quenching of tryptophan fluorescence appears to be complex. The apparent intercept on the abscissa which reflects about 0.3 to 0.4 high affinity binding sites and the fluorescent changes attendant to binding at the high affinity site were much less than the major changes that occur after binding to the secondary site. The data are thus more difficult to interpret and determination of absolute number of high affinity binding sites are subject to large experimental errors and cannot be derived from the present studies. The primary objective of this experiment was to study the overall effect of reduction and alkylation on the two

![Fig. 1. Circular dichroism spectra of bilirubin ligandin complexes.](http://www.jbc.org/)

![Fig. 2. Immunoelectrophoretic pattern of native and chemically modified ligandins.](http://www.jbc.org/)
The results show that the binding of bilirubin to its primary high affinity site on ligandin and the GSH-S-transferase activity of the protein are mutually independent processes. Binding at a secondary site of lesser affinity influences the catalytic action. Both reduced and alkylated, and cross-linked ligandins, are catalytically functional but devoid of the high affinity binding capacity for bilirubin.

Ligandin-bilirubin complexes exhibit a characteristic circular dichroic spectrum with positive ellipticity extrema centered at 515 nm and 405 nm and a negative band at 465 nm. The 405 nm band attains maximal values and the 465 nm band reaches saturating values after addition of 1 mol of bilirubin/mol of ligandin (7). The 515 nm band requires addition of higher concentrations of bilirubin to reach saturating levels. Titration experiments employing ellipticities at these wavelengths were performed to calculate binding constants for bilirubin (values of $5 \times 10^7$ M$^{-1}$ were previously obtained using the data at 405 or 465 nm) (7) and $3 \times 10^5$ M$^{-1}$ obtained using data at 515 nm (7). The band at 515 nm probably reflects binding at a site other than the primary high affinity site (7, 12). At the latter site, a bilirubin binding constant of $5 \times 10^7$ M$^{-1}$ was estimated on the basis of competitive binding studies (7). Studies of chemically cross-linked and reduced and alkylated ligandins permit clear distinction between the binding sites. Binding of bilirubin to the modified proteins generated circular dichroic bands only at 515 and 465 nm, with no detectable ellipticity at 405 nm. This suggests that the band at 405 nm is exclusively associated with bilirubin bound at the primary high affinity site as is the major part of ellipticities near 465 nm.

Cross-linked and reduced and alkylated ligandins retained enzymatic activity but not binding capacity, and differentiated these two functions of ligandin. The data also suggest that the integrity of cysteine residues as sulfhydryl groups (or disulfide bridges) of ligandin must be maintained for bilirubin binding to occur at the primary site. These sulphydryl groups are not essential for catalytic function or for binding of bilirubin at the secondary lower affinity site.

Other evidence suggesting that binding at the primary site does not influence catalytic function is derived from bilirubin inhibition studies (Table II). Incubation with saturating amounts and up to 100-fold excess of bilirubin did not affect enzymatic activity. 1-Chloro 2,4-dinitrobenzene (CDNB), which has an affinity constant for ligandin of about $10^7$ M$^{-1}$ (6) as compared to $5 \times 10^7$ M$^{-1}$ for bilirubin (7), would not be expected to displace bilirubin at equimolar or concentrations only 10-fold greater than bilirubin ($10^{-5}$ and $10^{-6}$ M, respectively). In earlier studies, this was demonstrated experimentally by competitive binding studies using circular dichroism analyses (6). In the presence of a greater excess of bilirubin, glutathione-S-transferase activity is competitively inhibited with respect to 1-chloro-2,4-dinitrobenzene. These results are in agreement with the data of Jakoby and co-workers (4). The current study shows that competitive inhibition is attributed to bilirubin bound to the lower affinity site on ligandin. The inhibition constants obtained by Jakoby et al. (4) approximate the binding constant of bilirubin at the secondary site as determined by fluorescence and circular dichroism (7, 12).

REFERENCES

![Graph](image-url)
Separation of Ligandin's Binding and Catalytic Activities

Ligandin. Bilirubin binding and glutathione-S-transferase activity are independent processes.

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