Bilirubin Binding to Human Liver Ligandin (Glutathione S-Transferase)*

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The number of binding sites and the dissociation constants were determined for the binding of bilirubin to human liver ligandin and to human serum albumin. Albumin has a primary bilirubin binding site ($K_D = 0.03 \mu M$), measured by the peroxidase procedure, and two apparently equivalent secondary binding sites ($K_D = 2 \mu M$), determined by fluorescence quenching experiments. By contrast, ligandin does not have a corresponding high affinity site. The absence of this high affinity site was shown both by the peroxidase procedure and by direct competition between albumin and ligandin for bilirubin. Bilirubin binding to ligandin, measured by fluorescence quenching, is complex. At both pH 6.5 and 7.4, two interacting sites were observed with a Hill coefficient of 1.5, $K^* = 3 \mu M$. Bilirubin binding to ligandin is not independent of glutathione S-transferase activity. Depending upon pH and upon the order in which the reactants are added, bilirubin can markedly alter the transferase activity. The results are interpreted in terms of kinetically stable conformational isomers of ligandin induced by bilirubin or by glutathione.

In addition to their binding properties and transferase properties, these proteins also show $\Delta^1$-2,3-ketosteroid isomerase activity (Benson et al., 1977) and glutathione peroxidase activity (Prohaska and Ganther, 1977).

In view of the multiple properties of glutathione S-transferase (ligandin), several of which are probably physiologically important, structure-function studies of these proteins are of considerable interest. We report here results from a study of human liver ligandin. Specifically, the number of bilirubin binding sites in the dissociation constants were evaluated for ligandin compared to those for albumin. In addition, the question of the relationship between bilirubin binding and glutathione S-transferase activity was evaluated.

**Materials and Methods**

Human serum albumin (Sigma) was treated with charcoal to remove residual lipids according to the procedure of Chen (Chen, 1967). Human liver ligandin was purified by glutathione affinity chromatography as described previously (Simons and Vander Jagt, 1977). The serum albumin and ligandin were used in the purest form possible. Based on their chromatographic behavior on CM-cellulose, these probably correspond to transferases $\alpha$ and $\epsilon$, using the notation of Jakoby (Kamisaka et al., 1975). All of the data in the present study refer to transferase $\epsilon$, the high pl form. However, transferase $\alpha$ was also examined; no essential differences were observed between these two forms of the protein.

In analyzing the fluorescence quenching data, it was assumed that quenching followed a Langmuir adsorption isotherm, namely

$$\Delta F_e = \frac{F_e \left[ br \right]}{K_D + \left[ br \right]}$$

where $\Delta F_e$ and $F_e$ are the corrected quenching and maximum quenching values and $br$ is the free bilirubin. $F_e$ was estimated from visual inspection of plots of $F_e$ versus $[br]$. Dissociation constants, $K_D$, were obtained from plots of $\Delta F_e$ versus $\Delta F_e/[br]$ or from Halfman-Nishida plots of $\Delta F_e/F_0$ versus $[br]/[F_0]$, where $br$ is the total bilirubin concentration and $F_0$ is the total albumin or ligand concentration. (Halfman and Nishida, 1972). For ligandin, which did not show a simple Langmuir isotherm for bilirubin binding, the data also were analyzed by Hill plots in order to obtain the interaction coefficients, $n$, and the composite dissociation constants, $K'$.

The glutathione S-transferase activity of ligandin was measured using 1 mm 1-chloro-2,4-dinitrobenzene as the electrophile and 2.5 mM GSH. The reaction was followed at 340 nm, corrected for the background reaction. Activity is expressed in units of micromoles/min. One unit of activity corresponds to an absorbance change of 3.2 A/min at 340 nm in a 3-ml reaction volume, 1-cm path length, assuming $\Delta A = 9.6 \mu M^{-1}cm^{-1}$ (Habig et al., 1974a). The ligandin sample (transf erase $\epsilon$) used in this study showed a specific activity of approximately 50 units/mg and a single band on polyacrylamide gel electrophoresis at pH 8.9 or 3.6.

The horseradish peroxidase-catalyzed oxidation of bilirubin by $H_2O_2$ (Jacobsen and Wennburg, 1974) but not of bilirubin bound to albumin was used to determine the dissociation constant for bilirubin at the primary binding site of albumin. Initial rates of oxidation of bilirubin were measured at 460 nm in the presence of varying amounts of albumin (or ligandin), using a Cary 219 recording spectrophotometer. 0.02 absorbance units full-scale. Data were analyzed by Scatchard plots. Bilirubin binding to albumin and to ligandin also was measured by fluorescence quenching experiments.

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RESULTS

Bilirubin Binding to the Primary Site of Albumin and to Ligandin—The binding of bilirubin to the primary binding site on albumin was determined by the horseradish peroxidase procedure (Jacobsen and Wennburg, 1974). Scatchard plots generated from the initial rate data showed a single primary binding site for bilirubin, $K_D = 3 \times 10^{-8}$ M. This value is in the middle of the range of values in the literature: $1.6 \times 10^{-7}$ M (Gray and Strouple, 1978), $2.5 \times 10^{-8}$ M (Jacobsen, 1977), and $8 \times 10^{-9}$ M (Berde et al., 1979).

This same peroxidase procedure was applied to a study of the binding of bilirubin to ligandin, pH 7.4. Addition of ligandin, 1.25 to 3 μM, to 1 μM solutions of bilirubin did not alter the initial rate of oxidation of bilirubin by the peroxidase system. This suggests either that bilirubin binding to ligandin is not as tight as binding to albumin or that bilirubin bound to ligandin is not protected from the peroxidase system. To test between these alternatives, direct competition by albumin and ligandin for bilirubin was evaluated. Fig. 1 (left) shows the fluorescence emission spectra of 1 μM albumin compared with 1 μM albumin plus 1 μM bilirubin. Fig. 1 (center) shows the same spectra for ligandin, using the same concentrations and same instrument settings. Fig. 1 (right) shows the emission spectrum for a mixture of 0.5 μM albumin and 0.5 μM ligandin, which is identical to one-half the sum of the spectra for 1 μM albumin and 1 μM ligandin, as expected. When 0.5 μM bilirubin was added to this mixture (Fig. 1, right), the resulting spectrum was identical to that which was calculated by assuming that all of the fluorescence quenching was due to binding of bilirubin to albumin. Thus, in direct competition, it does not appear that ligandin possesses a high affinity site comparable to the primary site on albumin.

A final attempt to measure a high affinity site for the binding of bilirubin to ligandin was made by looking at the effects of ligandin on the oxidation of bilirubin in the presence of albumin. The presence of ligandin in samples of bilirubin plus albumin did not alter the initial rates of oxidation of bilirubin by the peroxidase system, even up to a 3:1 ratio of ligandin to albumin. Thus, no evidence for a high affinity site on ligandin was obtained.

Bilirubin Binding to the Secondary Sites of Albumin—The binding of bilirubin to the secondary binding sites of albumin was determined by measuring the ability of bilirubin to quench the intrinsic protein fluorescence of albumin (Fig. 2). The initial addition of bilirubin results in an essentially stoichiometric quenching of fluorescence until 1 eq of bilirubin has been added. The quenching of the remaining fluorescence by bilirubin could be analyzed by assuming that binding to the secondary site followed a simple Langmuir binding isotherm with $K_D = 45$ (Equation 1). This procedure gave $K_D = 1.6 \pm 0.3$ μM for the average dissociation constant measured for the secondary site (Fig. 2).

In order to estimate the number of secondary binding sites, as well as calculate the dissociation constant by another method, the data in Fig. 2 were analyzed by the method of Halfman and Nishida (Halfman and Nishida, 1972). This procedure is as follows. Some change in a physicochemical

![Figure 1](https://example.com/f1.png)

**Fig. 1.** Fluorescence emission spectra of human serum albumin and human liver ligandin with and without bilirubin. *Left,* fluorescence emission spectrum of 1 μM human serum albumin (HSA) compared with the emission spectrum of 1 μM albumin plus 1 μM bilirubin (br), pH 7.4. *Center,* fluorescence emission spectrum of 1 μM human liver ligandin (HLL) compared with the emission spectrum of 1 μM ligandin plus 1 μM bilirubin, pH 7.4. *Right,* fluorescence emission spectrum of a solution of 0.5 μM albumin and 0.5 μM ligandin compared with the spectrum of this same solution containing 0.5 μM bilirubin, pH 7.4. All of the spectra in the figure were recorded using the same instrument settings (6 nm slits both for excitation and for emission) and are uncorrected spectra; $T = 25°C$.

![Figure 2](https://example.com/f2.png)

**Fig. 2.** Fluorescence quenching curves for bilirubin binding to human serum albumin. *Left,* fluorescence quenching curves, corrected for inner filter effects, for the addition of aliquots of a 0.5 mM stock solution of bilirubin (br) to human serum albumin (HSA), pH 7.4. *Point A,* both for 1 μM albumin and for 4 μM albumin, represents the point where 1 eq of bilirubin has been added. *Right,* plots of the quenching data from the figure (left) using Equation 1 (see "Material and Methods"). *br,* free bilirubin.
Thus, the fluorescence quenching data in Fig. 2 at 1 and 4 μM albumin can be plotted as shown in Fig. 3 to generate a Halfman-Nishida plot. The Scatchard plot of the data (Fig. 3) gives the same dissociation constant as was determined in Fig. 2. However, the Scatchard plot indicates the existence of two essentially equivalent secondary sites for binding bilirubin to albumin.

**Bilirubin Binding to Ligandin**—The binding of bilirubin to ligandin at pH 6.5 and pH 7.4 was followed by measuring the quenching of the intrinsic protein fluorescence. Fig. 4 shows the quenching curves for the binding of bilirubin to 0.5 and 2 μM ligandin, pH 7.4. Analysis of these quenching curves by procedures similar to those used with albumin indicated that the binding of bilirubin to ligandin did not follow the simple binding isotherm of Equation 1 (Fig. 4, right). These plots were derived by assuming a single binding site for bilirubin. If the concentration of free bilirubin was estimated by procedures similar to those used with albumin indicated plots were derived by assuming a two-site model, the plots were not significantly different. In addition, the data at pH 7.4 were similar to those obtained at pH 6.5.

The quenching data, both at pH 6.5 and pH 7.4, were plotted by the method of Halfman and Nishida. The sigmoid characteristics of the data became apparent. This is shown in Fig. 5 for the binding of bilirubin to ligandin at pH 6.5. The data were analyzed using Hill plots which indicated cooperative binding of bilirubin to ligandin, both at pH 6.5 and pH 7.4. Based upon several measurements at two protein concentrations and at two pH values, the Hill coefficient was estimated to be $n = 1.5 \pm 0.3$. The composite dissociation constant, $K'$, is about 5 μM. Fig. 5 shows representative Hill plots of the quenching data. The conclusion is that ligandin binds bilirubin to two interacting sites. Since the cooperativity is modest, the composite dissociation constant gives an approximate indication of the two individual dissociation constants. Those dissociation constants for the binding of bilirubin to ligandin are comparable to the secondary binding sites of albumin.

**Effects of Bilirubin Binding on the Glutathione S-Transferase Activity of Ligandin at pH 6.5**—The major question of interest in this study was the relationship between bilirubin binding and the catalytic activities of ligandin. Recent studies by Arias and co-workers of rat liver ligandin indicated that the enzyme from rat liver is a heterodimer and that the binding of bilirubin is independent of glutathione S-transferase activity (Bhargava et al., 1978a, 1978b).
For human liver ligandin, the effects of bilirubin on the catalytic activity are complex and depend upon the order of addition of the reactants. At pH 6.5, the glutathione S-transferase activity of ligandin was measured using 1 mM 1-chloro-2,4-dinitrobenzene as the electrophile in 0.02 M potassium phosphate buffer with 4% ethanol. GSH, 2.5 mM, was added and the background was recorded for 1 min, after which ligandin (2 µl of a stock solution of 28 units/ml) was added. The enzymatic reaction was followed for several minutes in order to record the curvature associated with substrate depletion and/or product inhibition. The reaction was repeated under the same conditions, except that 16 µM bilirubin was added to the reaction mixture before ligandin was added to initiate the reaction. The initial rate upon addition of ligandin was only slightly less than in the absence of bilirubin. However, during the time that the reaction was monitored, the rate of the reaction in the presence of bilirubin slowed more than the rate of reaction in the absence of bilirubin (Fig. 6). Thus, the catalytic activity of ligandin in the presence of bilirubin is time-dependent, reaching a limiting value after about 5 min. At this point, the catalytic activity of ligandin was about 66% of the initial activity. This time-dependent change in the catalytic activity was not a simple first order process (Fig. 6, inset).

The glutathione S-transferase activity of ligandin at pH 6.5 was then determined after bilirubin, 16 µM, and ligandin were allowed to incubate for several minutes. It was anticipated that allowing bilirubin and ligandin to incubate would produce the enzyme form which showed 66% activity and that this form of ligandin could then be studied kinetically. This was not the case. Incubation of bilirubin and ligandin in the absence of GSH produces a form of the enzyme which shows no catalytic activity if the incubation is allowed to proceed for several minutes as shown in Fig. 7. After 6 min, the catalytic activity does not exceed the background rate of reaction between GSH and 1-chloro-2,4-dinitrobenzene. Up to 6 min, the catalytic activity of ligandin depends only upon the length of time bilirubin and ligandin are together in the absence of GSH. This process also is not first order (Fig. 7).

The simplest explanation for the order of addition dependence which ligandin exhibits toward bilirubin and GSH is that relatively slow conformational events are involved and that the conformational state which exists when bilirubin binds to ligandin alone is devoid of catalytic activity, whereas the conformational state which exists when bilirubin binds to ligandin in the presence of GSH retains two-thirds of the catalytic activity of ligandin by itself. In both cases, the conformation changes (Figs. 6 and 7) require bilirubin. This was demonstrated by examining the effect of GSH on the fluorescence quenching by bilirubin. Regardless of whether GSH was present, when ligandin was treated with 16 µM bilirubin, the quenching was complete as rapidly as the reagents were mixed. There was no time-dependent change in quenching on the time scale of Figs. 6 and 7. Consequently, it appears that bilirubin binds rapidly both in the presence and in the absence of GSH, but the presence or absence of GSH will determine whether the subsequent conformational events yield a high or a low catalytically active form of ligandin.

**Effects of Bilirubin Binding on the Glutathione S-Transferase Activity of Ligandin at pH 7.4**—The dependence of the catalytic activity of ligandin on the order of addition of bilirubin and GSH was examined at pH 7.4. The situation is quite unlike that at pH 6.5. At pH 7.4, incubation of ligandin with 16 µM bilirubin alone or in the presence of 2.5 mM GSH, gives, in both cases, the same catalytic activity as was observed at pH 6.5 when GSH was present before bilirubin was added to ligandin. That is, the activity of ligandin toward 1-chloro-2,4-dinitrobenzene at pH 7.4, in the presence of bilirubin, is 66% of the activity observed in the absence of bilirubin. Unlike the situation at pH 6.5, this conformation is obtained as rapidly as the reagents are mixed. Thus, the time required for adoption of this conformation at pH 7.4 is much shorter than at pH 6.5. When bilirubin and ligandin were mixed together without GSH at pH 7.4, no evidence was obtained to indicate that the time of incubation made any difference in the subsequent activity measured when GSH and 1-chloro-2,4-dinitrobenzene were added.

When ligandin and bilirubin were incubated at pH 6.5 and then the pH was raised to pH 7.4, ligandin had little or no catalytic activity, depending upon the length of the incubation time at pH 6.5. Thus, once the low activity conformation is attained at pH 6.5, it is retained at pH 7.4, if bilirubin is present. If bilirubin is diluted out, the specific activity of ligandin returns to its initial value measured in the absence of bilirubin. Consequently, all of the processes involved in changes of conformation for ligandin appear to be reversible.
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CONFORMATIONAL STATES OF HLL

\[
\begin{align*}
\text{BR} & \quad + \quad \frac{k_1}{k_{-1}} \quad \text{BR} + \text{HLL} \\
\text{HLL}_A & \quad \xrightarrow{k_2} \quad \text{HLL}_B \\
\text{HLL}_A & \quad \text{catalytic activity} \quad \xrightarrow{k_4} \quad \text{HLL}_A
\end{align*}
\]

DISCUSSION

Based upon the studies of the binding of bilirubin to human liver ligandin (HLL) at pH 6.5 and pH 7.4 and based upon the observed effects of bilirubin binding on the glutathione S-transferase activity of ligandin, we propose the existence of kinetically stable conformers. The essential features of this model (Scheme 1) are as follows.

1. In the absence of glutathione, bilirubin (BR) binds to the dimeric ligandin rapidly and reversibly (Steps \( k_1 \) and \( k_{-1} \)) to form a complex with 2 eq of bilirubin apparently being bound. This stoichiometry is estimated from the Hill coefficient \( n \approx 1.5 \) for the cooperative binding of bilirubin. This behavior is observed both at pH 6.5 and pH 7.4.

2. Once bilirubin binds to ligandin, the initial complex is catalytically active. However, this complex undergoes some change (Step \( k_2 \)) to form a conformer, which in the presence of GSH (Step \( k_3 \)) shows no catalytic activity towards 1-chloro-2,4-dinitrobenzene. Thus, conformation HLL is a bilirubin-binding protein but not a glutathione S-transferase. At pH 6.5, Step \( k_2 \) can be followed by measuring the loss of glutathione S-transferase activity as a function of time. Step \( k_2 \) is less complex than a simple two-state interconversion, as shown by the fact that \( k_2 \) does not show first order kinetics (Fig. 7). At pH 7.4, incubation of ligandin with bilirubin does not result in the formation of conformer HLL, suggesting that \( k_2 \) is markedly slowed by the increase in pH.

3. In the presence of 2.5 mM GSH, the addition of bilirubin to ligandin again is rapid and reversible (Steps \( k_2 \) and \( k_{-2} \)). The major effect of the GSH is to lessen the cooperativity of binding and to increase the composite dissociation constant. Similar effects of GSH are observed both at pH 6.5 and pH 7.4.

4. Once bilirubin binds to ligandin in the presence of GSH, a conformational change takes place (Step \( k_3 \)). However, the resulting conformer HLL, not only binds bilirubin but also retains 66% of the glutathione S-transferase activity of intact ligandin. Step \( k_4 \) also does not show simple first order kinetics (Fig. 6). At pH 7.4, conformer HLL is attained much more rapidly than at pH 6.5, suggesting that \( k_4 \) is markedly increased by the increase in pH.

5. If conformer HLL is produced at pH 6.5, it remains intact if the pH is raised to 7.4. The interconversion of conformers HLL and HLL appears to be very slow. Thus, once ligandin adopts either the A or the B conformation, there does not appear to be any pH sensitivity to the interconversion of these conformational states.

The significance of the existence of two conformational states for ligandin, both of which bind bilirubin but only one of which is a glutathione S-transferase, is not clear. Conceivably, any physiological state which results in an increase in the flux of bilirubin passing through the hepatocyte, under conditions where the GSH level is low, may lead to a situation where the predominant conformational state of ligandin is HLL. It would seem reasonable to suggest that conditions may arise wherein one could be especially susceptible to the effects of potential mutagens and carcinogens, owing to the fact that HLL is devoid of glutathione S-transferase activity. This activity is one of the main detoxification activities associated with protection from dangerous electrophilic chemicals. What is not clear is whether or not the pH behavior of ligandin, whereby conformer HLL is not formed directly at pH 7.4 in vitro, is a property of ligandin which affords protection in vivo.

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REFERENCES


