Bilirubin Binding to Rat Liver Ligandins (Glutathione S-Transferases A and B)

RELATIONSHIP BETWEEN BILIRUBIN BINDING AND TRANSFERASE ACTIVITY*

David L. Vander Jagt, Selma P. Wilson, Valerie L. Dean, and Peter C. Simons
From the Department of Biochemistry, The University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

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Glutathione S-transferases A and B (ligandins) from rat liver were purified by GSH-affinity chromatography. The peroxidase procedure was used to determine the dissociation constants for the binding of bilirubin at pH 6.5 to the primary binding sites; \( K_D = 0.11 \) and 0.13 \( \mu M \) for transfersases A and B, respectively. The binding of bilirubin to the secondary sites, determined by fluorescence-quenching experiments, indicated dissociation constants of 5 and 8 \( \mu M \) for transfersases A and B. The two binding sites on either transferase A or transferase B show no cooperative interactions, unlike the situation with human liver ligandin (Simons, P. C., and Vander Jagt, D. L. (1980) J. Biol. Chem. 255, 4740-4744). The transferase activity of these proteins is sensitive to the presence of bilirubin and GSH. At pH 6.5, the addition of bilirubin to transferase A or B results in the formation of apparent conformations which exhibit very little catalytic activity. The presence of GSH forces the transferases to adopt different conformations upon addition of bilirubin. These conformations retain their transferase activities. Circular dichroic spectra support the conclusion that different conformational isoforms are obtained, depending upon the order of addition of the reactants.

The binding of a series of hydrophobic ligands to transferase A or transferase B was analyzed by Dixon plots of the initial rate data. In general, these ligands are strict competitive inhibitors of the electrophile. However, for electrophiles that resemble bilirubin, binding to a second site is apparent. Hematin, for example, exhibits high affinity, competitive inhibition of the transferase activity, but also exhibits secondary, noncompetitive inhibition which shows the same order of addition behavior that is observed with bilirubin. The results suggest that liver ligandins have a unique bilirubin-binding site which is separate from the transferase-catalytic site.

The existence of enzymes capable of conjugating GSH with a variety of electrophiles has been known for many years (Combes and Stakelum, 1961; Booth et al., 1961). These proteins also are effective in complexing bilirubin and other low solubility compounds (Litwack et al., 1971; Habig et al., 1974a; Kamisaka et al., 1975a). In addition, they show \( \Delta^{15,3} \)-ketosteroid isomerase activity (Benson et al., 1977) and glutathione peroxidase activity (Prohaska and Ganther, 1977).

The names used in the literature for these proteins vary according to the function under study, but generally they are called glutathione S-transferases or ligandins.

Earlier, we described some studies of bilirubin binding to human-liver ligandin which showed that this ligandin can exist in either of two kinetically stable conformational states (Simons and Vander Jagt, 1980). These different conformers are obtained depending upon the order with which bilirubin and glutathione are added to solutions of ligandin. Both conformers are bilirubin-binding proteins, but only one of the conformers retains significant transferase activity. Thus, it is apparent that bilirubin binding is not independent of transferase activity for human-liver ligandin. In the present paper we expand these studies to rat liver ligandin, specifically glutathione S-transferases A and B, using the notation of Jakoby and co-workers (Habig et al., 1974b). Bilirubin binding and transferase activity have been reported to be independent processes for glutathione S-transferase B (Bhargava et al., 1978).

MATERIALS AND METHODS

Rat liver ligandin (glutathione S-transferases A and B) and human liver ligandin were purified by glutathione-affinity chromatography as described previously (Simons and Vander Jagt, 1977). Transferrases A and B were separated from each other and from the other liver transferases by chromatography on CM-cellulose, as described by Jacoby (Habig et al., 1974b). Transferrases A and B used in this study showed specific activities of 55 and 15 pmol/min/mg, respectively. The glutathione S-transferase activity was measured by following the reaction between 2.6 \( \mu M \) GSH, and 1 \( \mu M \) 1-chloro-2,4-dinitrobenzene at 340 nm. Generally, the assay buffer was prepared by dissolving the electrophile in 0.02 \( M \) potassium phosphate containing 0.1 \( M \) NaCl and 4% ethanol, pH 6.5. Protein concentrations were measured by a dye-binding procedure (Bradford, 1976).

The binding of bilirubin to the primary binding sites of transferrases A and B was measured using the peroxidase procedure of Jacobson. This procedure, developed for measuring the binding of bilirubin to albumin, utilizes the fact that free bilirubin is rapidly oxidized by \( H_2O_2 \) in the presence of horseradish peroxidase while bilirubin bound to albumin is protected from oxidation (Jacobson and Wennberg, 1974). Bilirubin bound to ligandin is also protected from oxidation. Initial rates of oxidation of bilirubin were measured at 460 nm in the presence of varying amounts of transferrase A or B, using a Cary 219 recording spectrophotometer. Data were analyzed by Scatchard plots. Fluorescence-quenching data for the binding of bilirubin to the secondary site of transferrase A or B were analyzed using Equation 1.

\[
\Delta F = \frac{F_0[br]}{K_D + [br]}
\]

\( \Delta F \) and \( F_0 \) are the corrected quenching and maximum quenching values and \( br \) is the free bilirubin. Dissociation constants were obtained from plots of \( \Delta F \), versus \( [br]. \) Fluorescence measurements were made using a Perkin-Elmer MFP 44a spectrofluorometer. All measurements of bilirubin binding were made at 25°C in 0.02 \( M \) potassium-phosphate buffer, 0.1 \( M \) NaCl, at pH 6.5 or 7.4. For both transferrases A and B, \( \lambda_{em} = 280 \) nm; \( \lambda_{m} = 325 \) and 320 nm for A and
B, respectively. Sites were 6 nm both for excitation and for emission. Correction was made for inner filter effects of bilirubin (McClure and Edelman, 1966). Bilirubin (Sigma) stock solutions were prepared fresh daily in 0.01 M NaOH.

The binding of bilirubin to transferases A and B also was examined by circular dichroism using a Jasco J-40C spectrometer. These measurements used the same buffer systems as were used for the fluorescence measurements.

RESULTS

Bilirubin Binding to Glutathione S-Transferases A and B from Rat Liver—Fig. 1 shows the quenching of the intrinsic protein fluorescence of transferase A (top left) and transferase B (bottom left) upon addition of bilirubin to 2 μM solutions of transferase, pH 7.4. Addition of the first equivalent of bilirubin results in a nearly stoichiometric quenching of fluorescence suggesting that both transferase A and transferase B have a high affinity binding site for bilirubin. The quenching of the intrinsic fluorescence that remains after bilirubin has been bound to the primary site can be analyzed as shown in Fig. 1 (right) for transferase A (top right) and transferase B (bottom right). Both transferases show similar dissociation constants for the secondary sites of 6 and 4 μM for transferase A and transferase B, respectively. These values agree quite well with the value of 3 μM reported by Arias and co-workers for transferase B (Bhargava et al., 1978). The plots of ΔF versus ΔF/br [br] in Fig. 1 were made by assuming that there are single primary binding sites.

The dissociation constants for the binding of bilirubin to transferases A and B were also measured at pH 6.5. In addition, the constants were determined at 6.5 and 7.4 in the presence of 2.5 mM GSH. As shown in Table I, the dissociation constants for both transferases are not very sensitive to pH or to GSH in this pH range. All of the dissociation constants for the secondary sites are in the range 4 to 13 μM.

Bilirubin Binding to the Primary Sites of Glutathione S-Transferases A and B—Transferase A or B, 0.4 to 2.3 μM, was added to 1-MM solutions of bilirubin, pH 6.5, containing fixed amounts of horseradish peroxidase. Initial rates of oxidation of free bilirubin upon addition of H₂O₂ were analyzed by Scatchard plots (Fig. 2). Single high affinity sites were observed for transferases A and B with dissociation constants 0.11 and 0.13 μM, respectively.

Relationship between Bilirubin Binding and Catalytic Activity of Glutathione S-Transferases A and B from Rat Liver—Fig. 3 shows the effects of bilirubin on the glutathione S-transferase activity of rat-liver transferases A and B at pH 6.5. Depending upon the length of time that bilirubin and transferase A or transferase B are incubated in the absence of GSH, the resulting activity measured by initiating the reaction with GSH decreases and approaches the nonenzymatic background rate if the incubation period exceeds several minutes. Close examination of the plots in Fig. 3 indicates that the conversion of transferase A or transferase B into an apparent conformation with low catalytic activity is not a first order process. The activity drops rapidly initially but then more slowly. If a lower concentration of bilirubin was used in the incubation with transferase A or B, then the rate of the conformational change is slower than shown in Fig. 3. It is important to note that the time dependence shown in Fig. 3 was measured at pH 6.5.

Fig. 4 shows the results of changing the order of addition of the reactants. If GSH and bilirubin are added to the assay buffer at pH 6.5 and then transferase A or transferase B is added to initiate the catalytic reaction, the resulting initial rate curve slows continuously until a limiting value is reached. Fig. 4 is a plot of the tangents to the initial rate curves at various time points, corrected for any slowdown in rate that can be attributed to substrate depletion or product inhibition. Both transferase A and transferase B adopt stable states after several minutes; these apparent conformations retain significant transferase activities of 80% and 70% for transferase A and B, respectively.

The order of addition experiments shown in Figs. 3 and 4 were repeated at pH 7.4. At this pH, the activities of transferases A and B toward 1-chloro-2,4-dinitrobenzene in the presence of GSH and bilirubin are independent of the order of addition of GSH, bilirubin, and transferase. Regardless of the order of addition of the reactants, a curve similar to Fig. 4 is obtained. Thus, at pH 7.4 it appears that transferases A and B adopt the same high activity states that were observed at pH 6.5 only when GSH was present with bilirubin (Fig. 4).

If transferase A or transferase B is incubated with bilirubin
were analyzed by Scatchard plots. Transferase oxidation of bilirubin by \( \text{H}_2\text{O}_2 \), catalyzed by horseradish peroxidase, thiol S-transferases in the presence of variable amounts of transferase reaction. \[ \text{bilirubin} \]

one primary site, added last to initiate the reaction. Left, transferase and bilirubin. Each experimental point is the initial rate measured after the indicated time of incubation. GSH was and transferase B, pH same as in

there are differences in the ellipticities stable conformers of transferases A and B can be produced. Therefore, we examined the order of addition dependence on the circular dichroic spectrum of bound bilirubin with a well defined circular dichroic spectrum of bound bilirubin with a strong ellipticity band near 460 nm (Kamisaka et al., 1975b). Therefore, we examined the order of addition dependence on the circular dichroic spectra in order to obtain spectral evidence to support the conclusion that different kinetically stable conformers of transferases A and B can be produced. Fig. 5 shows the circular dichroic spectrum at pH 6.5 of bilirubin bound to transferase A; tracing A shows the spectrum of bilirubin plus protein; tracing B shows the effect of adding 2.5 mM GSH to the solution used to obtain spectrum A; tracing C shows the spectrum where all reactants are the same as in tracing B except that GSH was added to transferase A first and bilirubin was added last. It is apparent that there are differences in the ellipticities (tracing B versus tracing C) depending upon the order of addition of the reactants. Transferase B shows a similar order of addition dependence except that the spectral differences are not quite as large as those observed for transferase A. At pH 7.4, however, no spectral differences were observed with the different orders of addition of the reactants. Thus, the circular dichroic spectra are entirely consistent with the kinetic results and provide spectral evidence that the two states, which can be obtained by varying the order of addition of GSH and bilirubin to transferase A or to transferase B, are two different conformations.

Inhibition of Glutathione S-Transferase A and B by Hydrophobic Ligands—The effects of ligands other than bilirubin on the transferase activity of transferases A and B were examined using a series of hydrophobic compounds. Bromcresol green and sulfobromophthalein both exhibited strict com-

FIG. 2. Bilirubin binding to the primary binding site of glutathione S-transferases A and B. The initial rate data for the oxidation of bilirubin by \( \text{H}_2\text{O}_2 \), catalyzed by horseradish peroxidase, in the presence of variable amounts of transferase A or transferase B were analyzed by Scatchard plots. Transferase A (△) shows one primary bilirubin binding site, \( K_D = 0.11 \mu M \), transferase B (○) shows one primary site, \( K_D = 0.13 \mu M \), pH 6.5, 25 °C.

FIG. 3. Glutathione S-transferase activities of transferase A and transferase B, pH 6.5, as a function of the time of incubation of transferase and bilirubin. Each experimental point is the initial rate measured after the indicated time of incubation. GSH was added last to initiate the reaction. Left, transferase A; right, transferase B. The dashed line shows the rate of the nonenzymic background reaction. [bilirubin] = 34 µM, 25 °C.

at pH 6.5 as in Fig. 3, and then if the pH is raised to 7.4 by addition of NaOH, the resulting solution still shows the same low activity as was measured at pH 6.5. Thus, although the low activity states are not obtained directly at pH 7.4, they are kinetically stable if they are first produced at pH 6.5 and then are subjected to a change in pH. In addition, if transferases A and B are incubated with bilirubin to produce the low activity states and then the solutions are diluted with buffer in order to lower the bilirubin concentration, transferase activity returns, indicating that these are reversible processes.

Circular Dichroism Studies of the Conformational States of Rat Liver Glutathione S-transferases A and B—The binding of bilirubin to transferase B is known to produce a well defined circular dichroic spectrum of bound bilirubin with a strong ellipticity band near 460 nm (Kamisaka et al., 1975b). Therefore, we examined the order of addition dependence on the circular dichroic spectra in order to obtain spectral evidence to support the conclusion that different kinetically stable conformers of transferases A and B can be produced. Fig. 5 shows the circular dichroic spectrum at pH 6.5 of bilirubin bound to transferase A; tracing A shows the spectrum of bilirubin plus protein; tracing B shows the effect of adding 2.5 mM GSH to the solution used to obtain spectrum A; tracing C shows the spectrum where all reactants are the same as in tracing B except that GSH was added to transferase A first and bilirubin was added last. It is apparent that there are differences in the ellipticities (tracing B versus tracing C) depending upon the order of addition of the reactants. Transferase B shows a similar order of addition dependence except that the spectral differences are not quite as large as those observed for transferase A. At pH 7.4, however, no spectral differences were observed with the different orders of addition of the reactants. Thus, the circular dichroic spectra are entirely consistent with the kinetic results and provide spectral evidence that the two states, which can be obtained by varying the order of addition of GSH and bilirubin to transferase A or to transferase B, are two different conformations.

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FIG. 4. Time course of the glutathione S-transferase activity of transferase A and transferase B, pH 6.5. Each point represents the tangent on a continuous tracing of transferase activity in the presence of bilirubin. The reaction was initiated by adding the enzyme last, unlike in Fig. 3 where enzyme and bilirubin were incubated before the reaction was initiated with GSH. Left, transferase A; right, transferase B. The limiting activities for transferases A and B are 80 and 70%, respectively, relative to the activities measured in the absence of bilirubin. [bilirubin] = 16 µM, 25 °C.

FIG. 5. Circular dichroic spectra of bilirubin complexes with rat liver glutathione S-transferase A, pH 6.5. The order of addition of bilirubin and glutathione to the transferases, which leads to different conformations showing low activity (Fig. 3) or high activity (Fig. 4), also results in different ellipticities for bilirubin bound to protein. Tracing A is the dichroic spectrum measured after addition of 32 µM bilirubin to 20 µM transferase A. Tracing B is the spectrum measured after 2.5 mM GSH was added to the solution used in tracing A; tracing C is the spectrum measured by using the same concentrations of transferase A, bilirubin, and GSH as were used for tracing B except that GSH was added to transferase A before bilirubin was added. Similar order of addition spectral differences were observed with transferase B. At pH 7.4, neither transferase A nor transferase B showed any order of addition dependence in the circular dichroic spectra. Ellipticities are in millidegrees.
Multiple Functions of Glutathione S-Transferase

Fig. 6. Dixon plots of the inhibition of glutathione S-transferase B by bromcresol green, biliverdin, and hematin. Top, inhibition of transferase B by bromcresol green at 1 (○) and 0.5 mM (■) 1-chloro-2,4-dinitrobenzene, pH 6.5, GSH = 2.5 mM. The inhibition is competitive, $K_i = 6 \mu M$. Transferase A gave a similar pattern, $K_i = 9 \mu M$. Inhibition of transferase B and A by sulfobromophthalein is also competitive, $K_i = 18$ and 28 $\mu M$, respectively. The inhibition of transferases A and B by these two inhibitors is not dependent upon the order of addition of the reactants. Center, inhibition of transferase B by biliverdin at 1 (○) and 0.5 mM (■) 1-chloro-2,4-dinitrobenzene, pH 6.5, GSH = 2.5 mM. The binding of biliverdin appears to be competitive inhibition of transferases A and B with respect to the binding of the electrophilic substrate 1-chloro-2,4-dinitrobenzene. A representative plot of the data is shown in Fig. 6 for the inhibition of transferase B by bromcresol green. The inhibition constants are listed in Table I. These two ligands were chosen as examples of hydrophobic compounds with potentially different properties in that sulfobromophthalein is also a substrate for the transferases. However, its substrate properties are poor compared with 1-chloro-2,4-dinitrobenzene, thereby allowing sulfobromophthalein to be treated as an inhibitor. There was no order of addition effect with either of these ligands. That is, the initiation of the reaction by adding enzyme last or GSH last produced the same results. There is a slight deviation from linearity in the Dixon plots at low inhibitor concentration (Fig. 6). This was more noticeable for inhibition of transferase B than transferase A.

The inhibition of transferases A and B by biliverdin is more complicated than the inhibition by bromcresol green or sulfobromophthalein. Fig. 6 shows a Dixon plot of the inhibition of transferase B. There appear to be two classes of binding sites for biliverdin. The first is a high affinity site where the binding is competitive, $K_i = 1 \mu M$. The second is a site where the binding is noncompetitive, $K_i = 25 \mu M$. There was no order of addition dependence for any of these data. Transferase A also exhibited two sites for biliverdin (Table I).

The inhibition of transferase A and B by hematin is even more complicated than the inhibition by biliverdin, as shown in Fig. 6, for the binding of hematin to transferase B. There is a slight deviation from linearity in the Dixon plots at low inhibitor concentration (Fig. 6). This was more noticeable for inhibition of transferase B than transferase A.

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The inhibition of transferase A and B by hematin is even more complicated than the inhibition by biliverdin, as shown in Fig. 6, for the binding of hematin to transferase B. There

Fig. 7. Dixon plots of the inhibition of glutathione S-transferase from human liver. For all plots, pH = 6.5, [GSH] = 2.5 mM, and [1-chloro-2,4-dinitrobenzene] = 1 (○) or 0.5 mM (■).
are two sites available, one competitive with electrophile and one noncompetitive with electrophile, similar to the situation with biliverdin. However, the binding of hematin to the second site showed the order of addition dependence exhibited by bilirubin. This is shown in Fig. 6 by the open triangles, where GSH was added last, compared with the closed circles, where enzyme was added last. This order of addition effect was not observed for the binding of hematin to the first site. Transferase A showed similar behavior. Thus, it appears that as the structure of the ligand begins to resemble bilirubin, the inhibition of transferases A and B begins to show some of the inhibition properties produced by bilirubin.

**Inhibition of Glutathione S-Transferase from Human Liver by Hydrophobic Ligands.**—The inhibition of glutathione S-transferase from human liver by the same compounds used with transferases A and B was determined. Human liver transferase was shown previously to exhibit the same time-dependent, order of addition dependence of activity upon binding bilirubin as shown in Figs. 3 and 4 for transferases A and B (Simons and Vander Jagt, 1980). Fig. 7 shows Dixon plots of the data for inhibition of human liver transferase by the four hydrophobic ligands. Three of these show strict competitive inhibition with no deviation from linearity and no order of addition behavior. Inhibition by hematin appears to be noncompetitive, although this is difficult to determine by this analysis in view of the very tight binding of this ligand (Table I). Again, inhibition plots are linear and show no order of addition behavior. Thus, human liver transferase reflects even more clearly than rat liver transferases that the binding of bilirubin is fundamentally different than the binding of other ligands. This supports the conclusion that the binding of bilirubin to liver transferases occurs at a unique site which is highly specific for bilirubin and is distinct from the transferase-catalytic site. Nevertheless, depending upon the experimental conditions, the binding of bilirubin to its unique site can influence the transferase properties of liver ligands.

**DISCUSSION**

The inhibition of glutathione S-transferase A and B from rat liver, as well as the inhibition of human liver transferase by a variety of ligands (Table I), is a straightforward type of inhibition which generally is competitive with binding of the electrophile. The results in Table I are in general agreement with the extensive data of Jakoby and co-workers (Ketley et al., 1975). For example, our inhibition studies of transferase A and B by sulfobromophthalein gave k, values 28 and 18 µm for A and B, respectively, similar to their values of 40 and 12 µm obtained using the similar compound 3,6-dibromosalicyl-fopthalein. Our dissociation constants for the binding of bilirubin to the secondary sites of transferases A and B are in the range 4 to 13 µm, depending upon pH and upon the presence or absence of GSH. Their values, based upon fluorescence and kinetic studies, are 2 to 15 µm. It is only in the more detailed analysis of the binding of bilirubin to liver transferases that it becomes apparent that the binding of bilirubin is fundamentally different than the binding of other ligands.

We suggest, based upon this study of rat liver transferases A and B and upon earlier studies of human liver transferase, that the binding of bilirubin to its primary binding site involves binding to a specific site. Three of these show strict dependence of the binding of bilirubin or other ligands to a secondary, nonspecific site which is part of the transferase-catalytic site. The conclusion is based upon the following: 1) bilirubin has the unique ability to induce all of the liver transferases examined thus far to adopt different conformations depending upon the order of addition of GSH and bilirubin (Figs. 3 and 4; also Simons and Vander Jagt (1980)); 2) the conformational changes that take place can be followed using conventional spectrophotometry owing to the time scale for these changes; 3) these conformational changes that follow the binding of bilirubin to liver transferases are pH dependent, and 4) the ellipticity of bilirubin bound to transferase A or B differs depending upon the order of addition of bilirubin and GSH, supporting the idea that the observed changes reflect the formation of distinct, kinetically stable conformations.

Our observation of the existence of a single high affinity site for the binding of bilirubin to transferases A and B, measured by the peroxidase procedure (Fig. 2), is in agreement with recent studies of rat liver transferase B which indicate that this transferase is a heterodimer and that only one of the two subunits has a high affinity site for bilirubin (Bhargava et al., 1978, 1980a). Interestingly, the transferase from rat testis appears to be a homodimer which lacks the high affinity binding site for bilirubin (Bhargava et al., 1980b). It will be of interest, therefore, to examine whether the transferase from rat testis lacks the time-dependent, order of addition properties exhibited by the liver transferases upon binding bilirubin. Studies are underway to evaluate the binding properties of human and rat transferases from a variety of tissues.

In our earlier studies of human liver transferase, we suggested that the ability of bilirubin to bind to transferase and to induce the formation of a conformational state with very little transferase activity may have serious consequences. This appears to be a general property of liver transferases. Conceivably, situations may arise whereby the flux of bilirubin that must be transported through the hepatocyte is large under conditions where GSH may be depressed. This may result in the conversion of much of the liver transferases into conformations which have little ability to protect against those carcinogenic and mutagenic electrophiles that normally are detoxified by the glutathione S-transferases. Admittedly, these changes under in vitro conditions are observed at pH 6.5 but not at pH 7.4. The consequences in vivo remain unknown.

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