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SHORT COMMUNICATIONS

Comparison of the binding affinities of five forms of rat glutathione *S*-transferases for bilirubin, sulfobromophthalein and hematin*

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The glutathione (GSH) *S*-transferases are a family of dimeric cytosol proteins that have been divided into two groups on the basis of subunit composition: Y_a and/or Y_c versus Y and/or $Y_{b'}$ combinations [1, 2]. A dual role has been proposed for these proteins which includes both enzymatic detoxification and binding of nonsubstrate ligands. Most work on the latter function has focused on one particular form, GSH *S*-transferase B, which has been referred to as ligandin. This protein is a heterodimer with a Y_aY_c subunit composition [1]. Recently the relationship between transferase B and ligandin has been challenged, the latter having been separated as a homodimer with a Y_aY_a subunit composition [3]. In addition, recent work has suggested that the Y_aY_a protein binds ligands with greater affinity than Y_aY_c and that the Y_a subunit has a higher affinity site [2]. Ketley *et al.* [4] in earlier work found comparable binding affinities for various ligands by transferases A, B, and C. Thus, in contrast to the claims by several groups that the Y_a subunit determines high-affinity binding, their work suggests that the Y_b subunit is also capable of high-affinity binding. However, they did not study Y_aY_a protein or the recently purified transferases C_2 or D ($Y_{b'}Y_{b'}$) [1]. Therefore, since controversy exists as to the relative binding affinities of the various forms of GSH *S*-transferases for nonsubstrate ligands, we investigated the binding of three organic anions, bilirubin, hematin and sulfobromophthalein (BSP), to five purified basic transferases from rat liver.

Methods

Purification of various GSH S-transferases. Purification of GSH *S*-transferases was performed according to the method of Mannervik and Jensson [1]. Briefly, the purification procedure consists of affinity chromatography on *S*-hexylglutathione bound to epoxy-activated Sepharose 6B followed by chromatofocusing. Five homogeneous forms of GST were isolated. Subunits were identified by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to Laemmli [5]. Nomenclature for each of the subunits (Y_a, Y_b, $Y_{b'}$, Y_c) is also according to Mannervik and Jensson [1]: GSH *S*-transferases, Y_aY_a (ligandin or L_2), Y_aY_c (ligandin, or B or BL), Y_bY_b (A or A_2), $Y_bY_{b'}$ (C or AC), $Y_{b'}Y_{b'}$ (D or C_2). Y_cY_c (AA or B_2) was not homogeneous and was not evaluated further.

Binding studies. Binding studies were performed with a Perkin-Elmer MPF-44 spectrophotofluorometer at room temperature (25–27°). The decrease in intrinsic protein tryptophan fluorescence after addition of ligands was determined at 330 nm during excitation at 290 nm. To cuvettes containing 2 ml of purified protein (0.08 μM) in 0.01 M sodium phosphate buffer, pH 7.4 (standard buffer), was added a maximum of 30 μl of stock solution of ligands in 1- to 5-μl aliquots. When necessary, the fluorescence intensities were corrected for inner filter effect by the method of Chignell [6]. The analysis of the quenching data was performed according to the method of Chignell [6]. In this analysis, the transferases are assumed to have a single binding site. This assumption is supported by previous studies [2, 4, 7] which have shown that GSH *S*-transferases have a single high-affinity binding site for various ligands. From titration studies, the concentration of free and bound ligand can be calculated from equations 1, 2 and 3:

$$Q = (100 - F)(100 - F_D) \quad (1)$$

$$[LP] = QP_t \quad (2)$$

$$[L_f] = [L_t] - QP_t \quad (3)$$

where F = fluorescence of the protein at a given point during the titration, and F_D = fluorescence of the protein when all sites on the protein are occupied, P_t is the total concentration of the protein $[LP]$ is the concentration of the complex, namely the concentration of bound ligand, $[L_f]$ is the concentration of free ligand, and $[L_t]$ is the concentration of total ligand. Results were expressed in a Scatchard plot [8], and the dissociation constant was calculated by a non-linear least squares method using the Michaelis–Menten equation.

Results and discussion

The binding of bilirubin, BSP and hematin to Y_aY_a, Y_aY_c, $Y_bY_{b'}$, $Y_bY_{b'}$, and $Y_{b'}Y_{b'}$ was determined by the quenching of the intrinsic tryptophan fluorescence of each protein. Examples of two results for bilirubin and hematin are expressed in a Scatchard plot (Fig. 1). The binding stoichiometries with hematin were close to unity and somewhat lower with bilirubin. The explanation for the latter is uncertain but may reflect overestimation of the monomeric bilirubin concentration due to self-aggregation. For either compound, Y_aY_a had the highest affinity, followed by Y_aY_c. $Y_bY_{b'}$, Y_bY_b and $Y_{b'}Y_{b'}$ had similar affinities for either compound (hematin, bilirubin), but the affinities were more than an order of magnitude lower than that of Y_aY_a. The dissociation constants thus obtained are summarized in Table 1 together with the results for BSP binding. Where known, the published values from other laboratories and the techniques employed are indicated in the table.

The Y_aY_c protein has been reported to have a single high-affinity binding site for bilirubin [2, 4, 7, 15] and BSP [11]. While there are a few studies suggesting that each Y_a subunit has a single high-affinity binding site and therefore that the Y_aY_a protein has two binding sites [16, 17] other studies have identified only a single binding site for bilirubin on the Y_aY_a protein [2, 4, 7, 15]. Our results (Fig. 1) support the view that there is a single high-affinity binding site on the Y_aY_a protein as well as on the other four transferases studied with each ligand (bilirubin, hematin, and BSP). Boyer *et al.* [2] reported that Y_aY_a protein had approximately two times higher affinity for both bilirubin and indocyanine green than the Y_aY_c protein. Our present studies support their findings and, in addition, we observed that Y_aY_a and Y_aY_c proteins had much higher affinity for organic anions than the other proteins (Y_bY_b, $Y_bY_{b'}$, $Y_{b'}Y_{b'}$).

Using the fluorescence quenching technique, we have found higher affinity binding than reported by Ketley *et al.*

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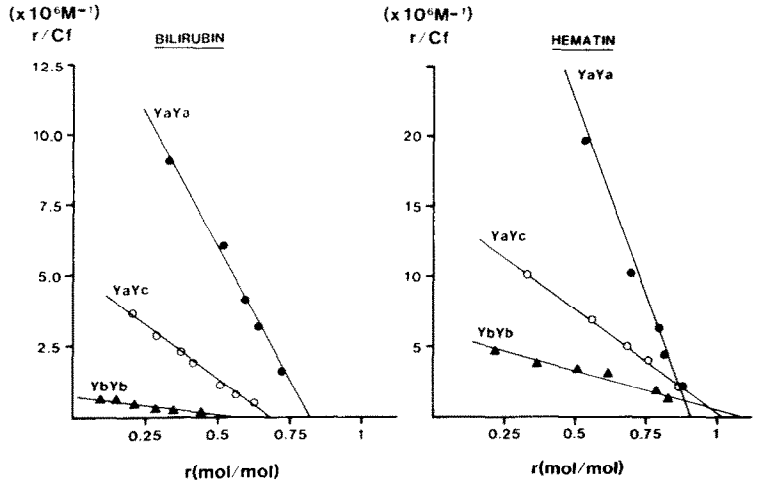

Fig. 1. Scatchard plot of the binding of bilirubin and hematin by GSH *S*-transferases Y_aY_a, Y_aY_c and Y_bY_b. Binding was determined by the quenching of intrinsic protein tryptophan fluorescence as described in Methods. Binding stoichiometry was estimated for the protein dimers.

Table 1. Summary of dissociation constants (K_d) obtained by the fluorescence quenching method

	GSH *S*-transferases K_d (μM)				
Ligand	Y_aY_a	Y_aY_c	Y_bY_b	$Y_bY_{b'}$	$Y_{b'}Y_{b'}$
Hematin	0.020	0.068 (0.03,* 0.05,† 0.1,‡ 1§)	0.20 (0.03,* 2‡)	0.41 (7‡)	0.25
Bilirubin	0.053 (0.34‖)	0.13 (0.02,§ 0.13,¶ 0.14,** 0.68,‖ 2‡)	0.69 (0.11,¶ 15‡)	0.78 (2‡)	1.10
BSP	0.23	0.37 (0.10,†† 1,§ 18*)	0.94 (0.44,‡‡ 28*)	0.83	1.07

* Vander Jagt *et al.* [9], enzyme inhibition.
† Tipping *et al.* [10], fluorescence quenching.
‡ Ketley *et al.* [4], fluorescence quenching.
§ Kamisaka *et al.* [11], circular dichroism.
‖ Boyer *et al.* [2], fluorescence quenching.
¶ Vander Jagt *et al.* [9], peroxidase method.
** Tipping *et al.* [12], difference spectra.
†† Tipping *et al.* [13], equilibrium dialysis.
‡‡ Jakobson *et al.* [14], equilibrium dialysis.

for transferases Y_aY_a, Y_bY_b and $Y_bY_{b'}$ [4] and somewhat higher affinity binding of bilirubin to transferases Y_aY_a and Y_aY_c than Boyer *et al.* [2] using this technique. The explanation for these differences is uncertain but could be due to different conditions; we used a lower protein concentration (0.08 μM) than the other groups (0.5 to 10 μM) and, in contrast to the others, we did not do our studies in the presence of GSH. However, our binding constants for bilirubin and BSP binding to transferases Y_bY_b and Y_aY_c agree with the findings of others (see Table 1). In general, our results indicate that the Y_aY_a transferase has somewhat higher affinity than the Y_aY_c transferase and that both of these Y_a subunit-containing proteins bind nonsubstrate ligands at a single site with much higher affinity than the Y_b or $Y_{b'}$ subunit-containing enzymes. The newly described $Y_{b'}Y_{b'}$ transferase has similar binding affinities for organic anions as Y_bY_b and $Y_bY_{b'}$ enzymes.

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Adriamycin—a potent inhibitor of Ca^{2+}–cardiolipin interaction

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Adriamycin (doxorubicin) is a potent antineoplastic agent, effective against a broad spectrum of leukemias and solid tumors [1, 2]. The therapeutic utility of the drug is limited, however, by its cumulative, and apparently irreversible, cardiotoxicity [3, 4].

The oncolytic activity of adriamycin is believed to result from intercalation of the anthracycline ring into the DNA double helix and subsequent interference with DNA replication and/or transcription [1, 5], which may be enhanced by localized generation of activated oxygen species [6]. Cardiotoxicity has been correlated with disruption of mitochondrial function [7]. Ultrastructurally, myelin-like figures indicative of drug–lipid interaction are seen [8, 9], and it has been shown that adriamycin interacts stoichiometrically with acidic phospholipids [10]. Complexation with cardiolipin (diphosphatidylglycerol), a lipid almost totally restricted in eukaryotic cells to the inner mitochondrial membrane, is particularly strong: $K_a = 1.6 \times 10^6\ M^{-1}$ [11].

Involvement of cardiolipin in Ca^{2+} transport across the mitochondrial inner membrane has been proposed based on the finding that Ca^{2+} induces cardiolipin to adopt inverted, non-bilayer, configurations [12, 13] and that cardiolipin can mediate the extraction of Ca^{2+} from an aqueous to an organic phase [14, 15]. Goormaghtigh *et al.* [16] have demonstrated, using ^{31}P-NMR, that adriamycin prevents both phenomena and have, therefore, suggested that the cardiotoxic side effects of adriamycin may result from altered mitochondrial Ca^{2+} fluxes.

Investigations of lipid organization using ^{31}P-NMR are limited to the use of high (millimolar) lipid concentrations and similarly high concentrations of ligand. The physiological relevance of the observations can therefore be questioned. We have described recently a two-phase organic extraction system which utilizes micromolar concentrations of both Ca^{2+} and lipid [17], and have shown that cardiolipin interacts with Ca^{2+} selectively and with apparent high affinity (K_d = 1–4 μM). I report here that adriamycin concentrations of 10 μM or less eliminate Ca^{2+}–cardiolipin interaction in this system at cytosolic Ca^{2+} concentrations (0.1 to 10 μM; [18]), and that drug effectiveness appears to be enhanced by positive cooperativity.

Materials and methods

The two-phase organic extraction system was a modification of that previously described [17]. Cardiolipin (4.9 to 9.5 nmoles) was dissolved in 1.0 ml toluene. The 2.0 ml aqueous phase contained 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-KOH, pH 8.0; $^{45}CaCl_2$ (1–300 μM); and adriamycin–HCl (0–50 μM). The two phases were vortexed together for 1 min and then separated by low-speed centrifugation. Samples of the organic and aqueous phases, the latter collected by puncturing the polypropylene tubes, were analyzed by standard liquid scintillation counting techniques. The concentration of Ca^{2+} bound to cardiolipin ($[Ca^{2+}]_b$) was calculated from the amount of $^{45}Ca^{2+}$ in the organic phase, and the free Ca^{2+} concentration ($[Ca^{2+}]_f$) from $^{45}Ca^{2+}$ remaining in the aqueous phase. The proportion of the total Ca^{2+} recovered, calculated from the sum of $[Ca^{2+}]_b$ + $[Ca^{2+}]_f$, increased with increasing total Ca^{2+} concentration and accounted for more than 80% of the label added to the tubes for Ca^{2+} concentrations >2 μM. Interfacial accumulation of a lipid–Ca^{2+} complex was, therefore, not considered in analyzing the data. No Ca^{2+} could be detected in the organic phase in the absence of cardiolipin, nor did adriamycin mediate the extraction of Ca^{2+}. The 1 min vortexing time was sufficient to maximize Ca^{2+} extraction into the organic phase. The data shown are representative of at least three experiments and are corrected for the Ca^{2+} content of the Hepes buffer (0.58 μM) determined by atomic absorption spectrophotometry.

Bovine heart cardiolipin (Na^+-salt) was purchased from Sigma and $^{45}CaCl_2$ from New England Nuclear. Toluene was Photrex grade from J. T. Baker. Adriamycin–HCl was provided by Dr. N. Bachur, University of Maryland Cancer Research Center.

Results

The effects of adriamycin on cardiolipin-mediated extraction of Ca^{2+} from an aqueous phase into an organic (toluene) phase are summarized in Fig. 1 and Table 1. Toluene was selected as an organic phase analog to the interior of