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Organic anion-binding by human hepatic GSH *S*-transferases*

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The glutathione (GSH) *S*-transferases are proposed to have a dual role which includes both enzymatic detoxification and binding of nonsubstrate ligands [1, 2]. Rat hepatic GSH *S*-transferases having the Y_a subunit are known to exhibit high-affinity binding of organic anions, whereas transferases with the Y_b subunit have lower affinities [3]. Therefore, only the former have been designated ligandins. However, only scattered observations about the binding specificities and affinities of various forms of human GSH *S*-transferases have been made [4–7]. We purified to homogeneity two major cationic, an anionic, and a neutral form of human hepatic GSH *S*-transferases and examined the binding affinities of these forms for bilirubin, sulfobromophthalein (BSP), indocyanine green (ICG) and hematin, using two different methods.

Methods

Purification of various GSH S-*transferases.* Purification of human hepatic GSH-transferases was performed according to the approach of Jensson and Mannervik [8] for purification of rat liver enzymes. Two operative liver samples which were apparently normal were used. Briefly, the purification procedure consists of Sephadex G-75 superfine chromatography, affinity chromatography on *S*-octylglutathione bound to epoxy-activated Sepharose 6B, and separate chromatofocusing both in the basic and acidic pH range. Four forms of GSH *S*-transferases were identified in chromatofocusing: two cationic forms were found on both livers (eluting at pH 9.0 and 8.7) whereas one liver contained a neutral (pH 6.8) and the other an anionic form (pH 5.4). The cationic forms are referred to as C1 and C2 in order of elution from chromatofocusing. Homogeneity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Laemmli [9]. With this technique the basic and acidic forms appeared as homodimers (subunit $M_r = 25{,}000$) and the neutral form was a homodimer of a larger subunit ($M_r = 26{,}000$).

1-Anilino-8-naphthalenesulfonate (ANS) fluorescence inhibition technique [10–13]. To cuvettes containing 2 ml of 0.01 M sodium phosphate buffer (pH 7.4) (standard buffer) and purified protein (0.1 to 0.3 μM), various amounts of ANS (up to 30 μl from a 5 mM stock solution) were added, and fluorescence was determined (excitation 400 nm and emission 480 nm) at room temperature (25–27°) with an Hitachi MPF-4 spectrofluorometer. To elucidate the type of inhibition, ANS binding was determined with cationic transferase (C1) in the presence and absence of four organic anions. Results were expressed in a Scatchard plot [14], and the dissociation constants were obtained by a non-linear least squares method using the Michaelis–Menten equation. The inhibition constants (K_i) of the organic anions for ANS binding to transferases were determined by varying organic anion concentrations at a constant ANS concentration (15 μM). K_i values were calculated by the non-linear least squares method as previously described [13].

Intrinsic protein fluorescence quenching method [3, 15]. The decrease in intrinsic protein fluorescence after addition of ligands was determined at 340 nm during excitation at 290 nm with an Aminco J4-8960 spectrofluorometer at room temperature. To cuvettes containing purified protein (0.1 μM) in 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 [15]. The analysis of the quenching data was performed according to the method of Chignell [15]. In this analysis, the transferases are assumed to have a single binding site. The concentrations of free and bound ligand were calculated from the fluorescence of the protein at a give point during the titration and the fluoresence when all sites on the protein are occupied, as previously described [3]. Results were expressed in a Scatchard plot [14], 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, ICG, and hematin by human transferases was determined by the ANS fluorescence inhibition technique and the quenching of the intrinsic fluorescence of each protein. The dissociation constants of ANS for C1, C2, acidic and neutral transferases were 14, 23, 12 and 15 μM respectively. The four organic anions competitively inhibited the binding of ANS to these proteins. Figure 1 shows the results with form C1. The inhibition of fluorescence of ANS bound to C1 by increasing concentrations of four organic anions is shown in Fig. 2.

The results of the determination of the dissociation constants for organic anion binding by the human transferases obtained by the two methods are summarized in Table 1. The dissociation constants for organic anion binding by two rat GSH *S*-transferases are also listed for comparison. K_d values obtained with both techniques were in reasonable agreement (almost within 2-fold difference). Each of the four forms of human transferase bound either bilirubin, BSP or ICG with similar affinity, whereas C_1 exhibited somewhat higher affinity for hematin that the other transferases. It is uncertain if this difference indicates a more specific role for this protein in intracellular heme transfer.

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In comparing human and rat transferases, the human transferases bound bilirubin and BSP with at least an order of magnitude lower affinity than rat GSH *S*-transferase B, whereas human transferases bound ICG and hematin with high affinities comparable to or greater than the rat transferases.

In contrast to the rat, in which two classes of transferases can be identified, one with high and one with low affinity for organic anions, the human transferases do not exhibit heterogeneity with regard to this property. The previous published values of dissociation constants for organic anion binding to human hepatic transferases using the protein fluorescence quenching method are listed in Table 1. Our results for bilirubin and BSP binding to the neutral transferase are in good agreement with those of Warholm *et al.* [7]. Our bilirubin binding results are in reasonable agreement with those of Simons and Vander Jagt [5] and Vander Jagt *et al.* [6] with cationic forms. Our results with hematin, ICG and bilirubin show much higher affinities than reported by Kamisaka *et al.* [4]. The difference in results is not readily explainable. However, it could be due to conditions used. We used a lower protein concentration (0.1 μM) in studying the quenching of the intrinsic protein fluoresence than Kamisaka *et al.* (1.0 to 1.4 μM). In addition, we considered that the difference might be due to our not using GSH in the binding studies. However, we checked the effect of GSH (2 mM) on the inhibition of ANS binding to C1 by bilirubin, but found no difference in the presence or absence of GSH. The lack of influence of GSH is in agreement with Warholm *et al.* [7].

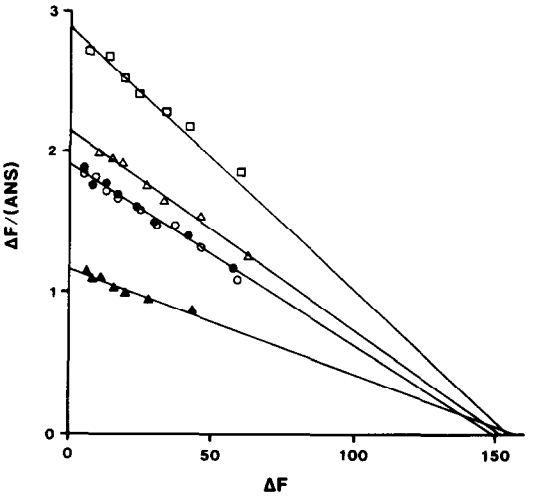

Fig. 1. Scatchard plot of ANS binding to the major cationic human GSH *S*-transferase (C1) in the absence (□) and presence of 2 μM bilirubin (△), 1 μM BSP (●), 0.2 μM ICG (○), and 1.0 μM hematin (▲). The concentration of ANS was varied from 2.5 to 50 μM, while concentrations of inhibitors and the cationic transferase (0.3 μM) were held constant. The binding of ANS was determined by the change of fluorescence (ΔF).

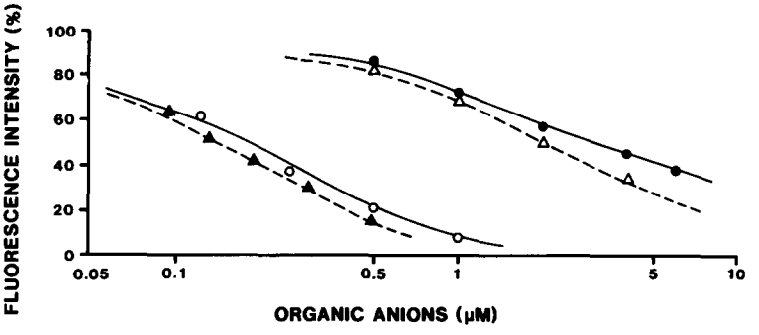

Fig. 2. Inhibition of ANS binding (fluorescence intensity) by varying organic anion concentrations. Cationic GSH-*S* transferase (C1) concentration was 0.15 μM and ANS concentration was fixed at 15 μM. The organic anions include bilirubin (△), BSP (●), ICG (○) and hematin (▲).

Table 1. Dissociation constants (K_d) for organic anion binding to human and rat GSH *S*-transferases

	Human GSH *S*-transferase K_d (μM)				Rat GSH *S*-transferase K_d* (μM)	
Ligand	Cationic 1	Cationic 2	Neutral	Acidic	B (Y_aY_c)	A (Y_bY_b)
Bilirubin	1.4 (1.6) [18–110†, 5‡, 3.3§]	1.7 (2.9)	2.8 (1.1) [10‖]	2.1 (6.8)	(0.13)	(0.69)
BSP	2.0 (3.7)	3.0 (3.9)	1.6 (2.2) [1‖]	3.2 (4.2)	(0.37)	(0.94)
ICG	0.12 (0.14) [20†]	0.20 (0.16)	0.16 (0.09)	0.24 (0.61)	(0.76)	
Hematin	0.05 (0.13) [10†]	0.08 (0.35)	0.12 (0.26)	0.25 (0.41)	(0.07)	(0.20)

Dissociation constants were obtained by the ANS fluorescence inhibition technique and by the quenching of the intrinsic protein fluorescence (in parentheses). The values by the ANS technique are means of two experiments. Some experiments by the fluorescence quenching method were repeated twice, and the values are in good agreement.

* Our previous report [3]. † Kamisaka *et al.* [4]. ‡ Simons and Vander Jagt [5]. § Vander Jagt *et al.* [6]. ‖ Warholm *et al.* [7].

In summary, using protein fluoresence quenching and competitive displacement of ANS, we have assessed organic anion binding to human hepatic GSH *S*-transferases. The cationic, neutral and acidic forms showed comparable binding properties. Bilirubin and BSP were bound with much lower affinity than by rat ligandin, whereas ICG and hematin were bound with comparable high affinity as by rat ligandin.

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Transport of a fluorescent antifolate by methotrexate-sensitive and methotrexate-resistant human leukemic lymphoblasts

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Interest in fluorescent inhibitors of dihydrofolate reductase (DHFR*) has grown over the decade since the original report [1] of the synthesis of MTX-F from methotrexate (MTX), fluorescein isothiocyanate (FITC), and 1,5-pentanediamine. It was shown [2, 3] that MTX-F could serve as an intracellular marker for DHFR, and this led to impressive uses of the compound in studies of DHFR overproduction in MTX-resistant cells by flow cytometry [4–8]. A compound of related but simpler structure, PT430 (Fig. 1), was synthesized in our laboratory from FITC and the lysine analogue of MTX [9, 10]. other fluorescent DHFR ligands were synthesized independently by reaction of the lysine and ornithine analogues of MTX with dansyl chloride [11–14]. Flow cytometric studies have been performed with PT430 as the fluorescent marker [9, 10, 15, 16], but have not been reported for the dansyl derivatives.

In this paper, we present an improved synthesis of PT430 and describe flow cytometric measurements of its uptake by MTX-sensitive human leukemic lymphoblasts (CEM cells) [17] and a resistant subline (CEM/MTX) with normal DHFR levels but a marked defect in MTX transport [18]. The influence of MTX on PT430 uptake by these cells is also compared. Our results demonstrate that cells with a defect in their transport mechanism for MTX can be distinguished from otherwise normal cells by flow cytometric analysis following incubation with PT430.

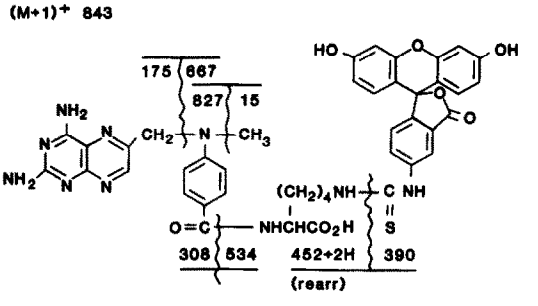

Fig. 1. Structure and fast atom bombardment mass spectral fragmentation pattern of PT430. Sites of fragmentation are shown by wavy lines.

Materials and methods

Spectra. Infrared spectra were obtained on a Perkin–Elmer model 781 double-beam spectrophotometer, and ultraviolet absorbance spectra were recorded on a Cary model 210 UV/visible instrument. Proton NMR spectra were obtained on a Varian T60A spectrometer. Fast atom bombardment mass spectra (FABMS) were determined on a Varian MAT 311A instrument through the courtesy of Dr. James Piper, Southern Research Institute, Birmingham, AL. Samples were dissolved in a mixture of *N,N*-dimethylformamide (DMF) and glycerol with 10% *p*-toluenesulfonic acid added to a final concentration of 10%. The probe tip was bombarded with Xe atoms of 8 keV energy, at a beam current setting of 1.2 mA [19].

N$^{\alpha}$-(4-*Amino*-4-*deoxy*-N^{10}-*methylpteroyl*)-N$^{\epsilon}$-(tert-*butyloxycarbonyl*)-L-*lysine* tert-*butyl ester.* To a suspension of 4-amino-4-deoxy-*N*^{10}-methylpteroic acid monohydrate

* Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); MTX, methotrexate, 4-amino-4-deoxy-*N*^{10}-methylpteroyl-L-glutamic acid; MTX-F, fluorescein-diaminopentane-methotrexate; PT430, *N*$^{\alpha}$-(4-amino-4-deoxy-*N*^{10}-methylpteroyl)-*N*$^{\epsilon}$-(4′-fluoresceinthiocarbamyl)-L-lysine; FITC, fluorescein isothiocyanate; DEAE-cellulose, *N,N*-diethylaminoethylcellulose; FBS, fetal bovine serum; DME, Dulbecco's modified Eagle's medium; and PBS, phosphate-buffered saline.