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RAPID COMMUNICATION

Dissociation of a new glutathione S-transferase activity in human erythrocytes

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Human glutathione S-transferases (GST) can be distiguished according to their isoelectric point (pI); a distinction being made between "basic", "neutral", and "acidic" enzymes. These correspond to the three gene families α, μ and π. Isolation of these enzymes is generally performed by affinity chromatography on different sepharose matrices, using 1-chloro-2,4-dinitrobenzene (CDNB) as test substrate [1].

By use of this method, various GST have been identified in human tissues: the cationic enzymes α, β, γ, δ and ε (liver), neutral μ (liver) and φ (lymphocytes), and anionic λ (lung) and π (placenta). Recently, a near neutral class of enzymes named θ has been identified, which could not be distinguished by affinity chromatography and showed no affinity for CDNB [2]. Experiments on cosubstrate specifity showed that glutathione may be substituted by structural analogues, but not by cysteine or thiol compounds [3,14,19].

In human erythrocytes, an anionic GST ρ with an acidic pI was isolated using CDNB as a substrate [3]. Later, other authors characterized another "minor" GST σ in human erythrocytes with a highly basic pI [4].

GSTs are able to conjugate a wide variety of compounds with glutathione, including small molecules such as monohalogenated methanes. Methyl iodide was formerly used as a test substance for the characterization of isoenzymes of GST [5]. A conjugation of methyl chloride to glutathione was observed in human erythrocytes [6].

Previous investigations in our laboratory not only confirmed enzymatic conjugation of methyl chloride to glutathione in human erythrocytes, but also disclosed that among the 45 persons investigated, 27 possessed this enzyme activity whereas 18 did not. The former were called "conjugators", the latter "non-conjugators" [7]. Using erythrocytes from the same persons, an enzymatic conjugation to glutathione by the "conjugators" was likewise observed for methyl bromide and methyl iodide [8], while the "non-conjugators" again lacked enzyme activity for the substrates in erythrocytes. Among these three substrates, the highest enzyme activity was found towards methyl bromide.

In other experiments, human blood samples were incubated with ^{14}C-ethylene oxide. "Conjugators" and "non-conjugators" for methyl bromide showed a distinctly different distribution of radioactivity in these experiments [9]. "Conjugators" and "non-conjugators" also showed striking differences in the distribution of radioactivity in analogous experiments with methyl bromide [10] and methylene chloride [11].

These results involving a number of heterogenous C_1- and C_2- substrates suggest a common enzymatic factor, most likely a GST, in erythrocytes, which is expressed by only a part of the human population. Thus, the aim of the investigations presented here was a description and characterization of this factor and a comparison of the results with data from the hitherto identified enzymes.

MATERIAL AND METHODS

Preparation of blood samples. Individual whole blood samples from human volunteers ("conjugators", v.s.) were drawn into 10 ml heparinized vials. After centrifugation at 400 g for 10 min the plasma was discarded and the sedimented cells were washed three times into saline. Erythrocytes were separated from white blood cells and platelets using the cellulose column as described by Boyum [12]. The erythrocytes were lysed with an equal amount of distilled water (1 h at 4°C). The hemolysate was then dialysed overnight against 20 mM phosphate buffer, pH 7 with 0.15 M KCl and 2mM EDTA (buffer A). To remove the membranes, the hemolysate was centrifuged for 30 min at 5000g. The pellet was discarded. The erythrocyte cytoplasm was then subjected to either of two procedures, A or B.

Procedure A. Glutathione-S-transferases were separated from the supernatant by affinity chromatography using the glutathione (GSH) coupled epoxyactivated sepharose 6B according to Simons and van der Jagt [13]. This affinity column was washed with buffer A until no protein was detectable at 280 nm; the effluent was collected. The column was then eluted with 50 mM Tris-buffer, pH 9.1, containing 5 mM GSH. Furthermore, the effluent of the affinity column was applied to a cation exchange resin (CM-sepharose C-50, Pharmacia) equilibrated with 20 mM phosphate buffer, pH 6.4 (buffer B). The effluent of this column was pooled and tested for GSH transferase activity and methyl bromide conjugating activity as described below.

Procedure B. The supernatant was dialysed against buffer B overnight. Hemoglobin was removed from the erythrocyte cytoplasm by using a batch technique with a CM-sepharose 50 (Pharmacia) equilibrated with buffer B. The hemolysate-free fraction was obtained by centrifugation at 400 g for 5 min.

This fraction was directly applied to a hydroxyapatite column (Calbiochem) equilibrated with 20 mM phosphate buffer, pH 6.6 (buffer C). The column was washed with buffer C until no absorbance was observed at 280 nm. Five ml fractions were collected. The protein bound to the column was eluted with 100 ml of a linear gradient ranging from buffer C to a 200 mM phosphate buffer, pH 6.6; 2.5 ml frations were collected.

Determination of glutathione-S-transferase activity. Transferase activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Hundred µl of each fraction obtained by procedure A or B were added to 1 ml of a solution containing 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CDNB. The formation of the CDNB-conjugate was followed at 340 nm and 25°C. Enzyme units were calculated using $\Delta\varepsilon = 9.6\ mM^{-1}cm^{-1}$ [5].

Determination of activity against methyl bromide. Activity against methyl bromide was measured using the head space technique described by Peter et al. [7] and Hallier et al. [8]. Aliquots of the fractions were added to head space vials (22ml) containing a buffer system (2.5 ml) of 0.125 M phosphate buffer (pH 7.4) with 20 mM GSH. After incubation at 37°C for 10 min under constant rolling, 4 µmol methyl bromide were injected into each head space vial resulting in an initial concentration in the gas phase of the vial of 5000 ppm. Every 15 min, the disappearance of methyl bromide from the gas phase of the vials was monitored by gas chromatography. Control incubations contained heat denaturated samples. In some experiments, GSH was substituted by L-cysteine, β-mercaptoethanol or dithiothreitol.

Inhibition experiments. Experiments on inhibition of the disappearance of methyl bromide from the gas phase were carried out with sulfobromophthaleine (BSP) (0-0.1 mM), deoxycholate (DOC) (0-1mM), S-methylglutathione (SMG) (0-1 mM), oxidized glutathione (GSSG) or N-ethylmaleimide (NEM) (0-1 mM), added to the head space vials prior to incubation. The vials contained 5 ml of buffer (v.s.) with 1 ml of hemolysate (Hb 7.1) each. Eight µmol of methyl bromide were added to each vial to give a concentration of about 10.000 ppm.

RESULTS

Erythrocyte cytoplasm of "conjugators" was subjected to the affinity chromatography, as described above. The results of "procedure A" for the hemolysate from two individual persons are summarized in table 1.

The pooled eluted fractions containing the GSH-transferase activity towards CDNB showed no activity towards methyl bromide. On the other hand, the activity towards methyl bromide was found in the void volume of the affinity column. In order to characterize whether the GST activity towards methyl bromide was an acidic or basic protein, the void volume from the affinity column was applied to a cation exchange resin (Pharmacia CM C-50). This resin, which also retains hemoglobin, did not retain the methyl bromide directed activity (data not shown).

When the erythrocyte cytoplasm was subjected to "procedure B", the fraction obtained from the cation exchange resin was separated on a hydroxyapatite column. The resulting chromatogram is presented in figure 1. Activities towards CDNB pass the column mainly unretained (section A). Only limited activity towards methyl bromide appears in the void volume fractions. The major portion of activity towards methyl bromide appears as a distinct peak in the eluate (section B).

For further evaluation, inhibition experiments were performed with erythrocyte hemolysate, as shown in figure 2. Deoxycholate, up to concentrations of 0.5 mM, did not inhibit the enzymatic conjugation of methyl bromide, but inhibited at higher concentrations. Sulfobromophthaleine inhibited even at low concentrations. Oxidized glutathione was a poor inhibitor. S-Methylglutathione revealed no inhibitory effects, up to concentrations of 1mM. N-Ethylmaleimide inhibited at 0.5mM the activity to 20%.

Experiments on cosubstrate requirement using L-cysteine, β-mercaptoethanol or dithiothreitol instead of glutathione showed that glutathione may be substituted by L-cysteine, in contrast to β-mercaptoethanol and dithiothreitol (data not shown).

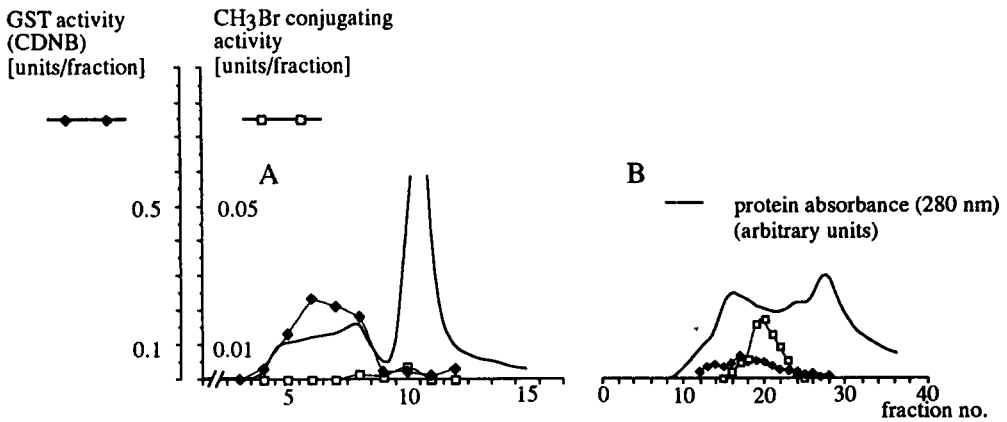

figure 1: separation on a hydroxyapatite column of GSH-transferase activities from activity towards methyl bromide in human hemolysate ("conjugator") (procedure B):
A) isocratic elution with buffer B ("void volume")
B) linear gradient, phosphate buffer, pH 6.6, 20mM - 150mM

DISCUSSION

Previous studies on the conversion of monohalogenated methanes have shown that these are metabolized to S-methylglutathione in human erythrocytes [6,7,8]. The existence of two human subpopulations was demonstrated: "conjugators" metabolize methyl halides to S-methylglutathione, whereas non-conjugators do not. These interindividual differences could be shown not only for monohalogenated methanes, but also for ethylene oxide (EO) [9] and methylene chloride (DCM) [11]. Due to these interindividual differences in the metabolism of methyl halides, an enzyme polymorphism on the basis of GST was postulated.The metabolism of the above named substrates in human erythrocytes is inhibited by CDNB and BSP, which led to the proposal that the enzymatic factor responsible is GST σ [7,8]. In addition, experiments with human GST π from human placenta excluded that this isoenzyme was identical with the isoenzyme in question.

The results presented here demonstrate that GST σ and GST ρ, recently isolated from human erythrocytes, can also be excluded: Both erythrocyte GST are separated from hemolysate by affinity chromatography on GSH sepharose [4]. The eluate containing both GST σ and ρ does not show activity towards methyl bromide. Furthermore, NEM could not activate the turnover of methyl bromide, as described by Singh and Awasthi for GST σ [15]. GST ρ, which was first separated using isocratic HAP-chromatography [3], could thus be separated from the activity towards methyl bromide. A strong inhibition by NEM, as described for the closely related GST π, could not be observed.

Inhibition studies with different inhibitors of the GSTs were carried out to identify the class of the new activity. DOC, NEM and BSP are effective inhibitors of the class μ [16]. An inhibition could only be observed with BSP, not with the other two inhibitors. SMG did not inhibit the turnover from methyl bromide. A subtype of the class μ is therefore improbable.

Habig et al. [5] isolated two GST, B and E, from rat liver, both active towards methyl iodide. A relationship to GST B may be excluded since GST B binds to CM-cellulose, whereas the GST active towards methyl bromide does not. In contrast to GST B, GST E also passes the CM cellulose cation exchanger, as the GST active towards methyl bromide does. Interestingly, GST E is not identified by the activity towards methyl iodide, but by its activity towards epoxides. A relation between GST E and the GST with methyl bromide activity seems possible on the basis of experiments using the substrate ethylene oxide [9,17].

The enzyme activity towards methyl bromide shows some resemblance to GST θ [2]. This isoenzyme does not bind to any GSH affinity resin and shows activity towards an epoxide, 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), but not towards CDNB. This also holds true for the closely related GST 5-5 and 12-12 from rat liver, which also show enzyme activity for methylene chloride. This substance has been shown to be suited for differentiation between our "conjugators" and "non-conjugators" [11]. Immunochemical analyses have shown that antisera of the GST E were positive against GST 5-5, 12-12 from rat liver and θ from human liver [2].

From these comparisons it can be concluded that the enzyme activity in erythrocytes of human "conjugators" directed towards methyl bromide and some other C_1- and C_2-substrates is a hitherto unknown isoenzyme in erythrocytes, possibly related to GST θ.

table 1: dissociation of GST activities towards CDNB and towards methyl bromide in human hemolysate ("procedure A")

	sample volume (ml)	protein conc. (mg/ml)	total protein (mg)	GST activity (units) CDNB	GST activity (units) CH_3Br
person A (conjugator)					
hemolysate	10	1030	10300	*	0.46
affinity column					
(i) void volume	20	370	740	*	0.11
(ii) transferase fractions eluted	7.5	0.06	0.43	0.65	0.0
person B (conjugator)					
hemolysate	10	1230	12300	*	0.15
affinity column					
(i) void volume	16.5	740	12210	*	0.14
(ii) transferase fractions eluted	7.2	0.03	0.19	0.66	0.0

* photometrical determination of CDNB turnover not possible due to hemoglobin interference

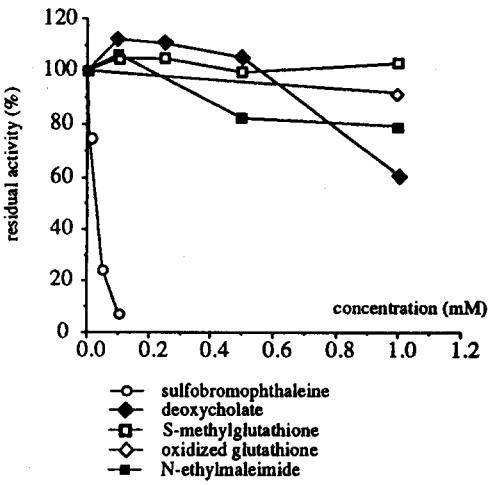

figure 2: inhibition experiments with human hemolysate

This new enzyme activity has not been identified in the past, probably because it differs from classic GST isoenzymes in terms of its substrate specifity. Furthermore, this isoenzyme activity is found so far only in human erythrocytes, not in various animal species [18].

REFERENCES

1. Hayes JD, McLellan LI, Stockman PK, Howie AF, Hussey AJ, Beckett GJ, Human glutathione S-transferases; a polymorphic group of detoxification enzymes, in: Mantle TJ, Pickett CB, Hayes JD (eds) Glutathione S-transferases and carcinogenesis, pp. 3-19, Taylor and Francis,1987
2. Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B, Theta, a new class of glutathione transferases purified from rat and man, *Biochem J* **274**: 409-414, 1991
3. Marcus CJ, Habig WH, Jakoby WB, Glutathione transferase from human erythrocytes, *Arch Biochem Biophys* **188**: 287-293
4. Awasthi YC,and Singh SV, Purification of a new fonn of glutathione S-transferase from human erythrocytes, *Biochem Biophys Res Comm* **125** (3):1058-1060, 1984
5. Habig WH, Pabst MJ, Jakoby WB, Glutathione S-transferases, The first enzymatic step in mercapturic acid formation, *J Biol Chem* **249**: 7130-7139, 1974
6. Redford-Ellis M, Gowenlock AH, Studies on the reaction of chloromethane with human blood, *Acta Pharmacol Toxicol* **30**: 36-48, 1971
7. Peter H, Deutschmann S, Reichel C, Hallier E, Metabolism of methyl chloride in human erythrocytes, *Arch Toxicol* **63**: 351-355, 1989
8. Hallier E, Deutschmann S, Reichel C, Bolt HM, Peter H, A comparative investigation of the metabolism of methyl bromide and methyl iodide in human erythrocytes, *Int Arch Occup Environ Health* **62**: 221-225, 1990
9. Föst U, Hallier E, Ottenwälder H, Bolt HM, and Peter H, Distribution of ethylene oxide in human blood and its implications for biomonitoring, *Hum Exp Toxicol* **10**: 25-31, 1991
10. Gansewendt B, Thier R, Schroeder KR, Hallier E, Peter H, Enzyme polymorphism in the metabolism of methylene chloride, ethylene oxide and methyl bromide and its implications for epidemiology, *J Cancer Res Clin Oncol* **117** (Suppl): S 16, 1991
11. Thier R, Föst U, Deutschmann S, Schröder KR, Westphal G, Hallier E, and Peter H, Distribution of methylene chloride in human blood, *Arch Toxicol*, Suppl. **14**: 254-258, 1991
12. Boyum A, Separation of white blood cells, *Nature* **204**: 793-794, 1964
13. Simons PC, van der Jagt, DL, Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography, *Anal Biochem* **82**: 334-341, 1977
14. Principato GB, Danielson UH, and Mannervik B, Relaxed thiol substrate specifity of glutathione transferase effected by a non-substrate glutathione derivative, *FEBS Lett* **231**: 155-158, 1988
15. Singh SV, and Awasthi YC, Cationic glutathione S-transferase of human erythrocytes has unique kinetic characteristics among human glutathione S-transferases, *Biochem Biophys Res Comm* **137**: 1174-1180, 1986
16. Warholm M, Guthenberg C, Mannervik B, Molecular and catalytic properties of glutathione transferase μ from human liver: an enzyme efficiently conjugating epoxides, *Biochem* **22**: 3610-3617, 1983
17. Leutbecher M, Langhof Th, Peter H, Föst U, Ethylene oxide: metabolism in human blood and its implication to biological monitoring, *Arch Toxicol*, Suppl. **15**: 293, 1991
18. Deutschmann S, Peter H, Reichel C, Bolt M, Hallier E, Kinetik der Metabolisierung von Methylbromid und Methyliodid in Erythrocyten des Menschen und verschiedener Tierspezies, *Verh Dtsch Ges Arbeitsmed (Gentner, Stuttgart)* **29**: 517-519, 1989
19. Adang AEP, Brussee J, van der Gen A, Mulder GJ, The glutathione-binding site in glutathione S-transferases. Investigation of the cysteinyl, glycyl and γ-glutamyl domains, *Biochem J* **269**: 47-54, 1990