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Enzyme kinetics and substrate selectivities of rat glutathione *S*-transferase isoenzymes towards a series of new 2-substituted 1-chloro-4-nitrobenzenes

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1. Four different rat glutathione *S*-transferase (GST) isoenzymes, belonging to three different classes, were examined for their GSH conjugating capacity towards 11 2-substituted 1-chloro-4-nitrobenzene derivatives. Significant differences were found in their enzyme kinetic parameters K_m, k_{cat} and k_{cat}/K_m.

2. Substrates with bulky substituents on the *ortho*-position appeared to have high affinities (low K_m's) for the active site of the GST-isoenzymes, suggesting that there is sufficient space in this area of the active site. A remarkably high K_m (low affinity) was found for 2-chloro-5-nitropyridine towards all GST-isoenzymes examined.

3. GST 3-3 catalysed the reaction between GSH and the substrates most efficiently (high k_{cat}) compared with the other GST-isoenzymes. Moreover, GST 3-3 showed clear substrate selectivities towards the substrates with a trifluoromethyl-, chlorine- and bromine-substituent. 1-Chloro-2,4-dinitrobenzene and 2-chloro-5-nitrobenzonitrile were most efficiently conjugated by all four GST-isoenzymes examined.

4. When the rate of the conjugation reactions was followed, a linear increase of formation of GS-conjugate could be seen for 2-chloro-5-nitrobenzonitrile during a much longer period of time than for 1-chloro-2,4-dinitrobenzene with all GST-isoenzymes examined. Therefore, it is suggested that 2-chloro-5-nitrobenzonitrile might be recommended as an alternative model substrate in GST-research.

Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) constitute a group of multifunctional cytosolic isoenzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of electrophilic alkylating compounds, thereby protecting cells against their potential toxicity (for reviews, see Mannervik and Danielson 1988, Armstrong 1991, Commandeur *et al.* 1995). They are also involved in the reduction of organic hydroperoxides (Tan *et al.* 1986), the isomerization of 3-ketosteroids (Benson *et al.* 1977) and the binding of non-substrate hydrophobic ligands such as bile acids, bilirubin, and a number of drugs and thyroid hormones (Ishigaki *et al.* 1989). Considerable evidence indicates that GSTs are, in addition to other factors, involved in cellular drug resistance (Hayes and Wolf 1990, Waxman 1990). In particular, GSTs are implicated in the resistance to alkylating agents such as chlorambucil, melphalan and nitrosoureas (Brakenhoff *et al.* 1994).

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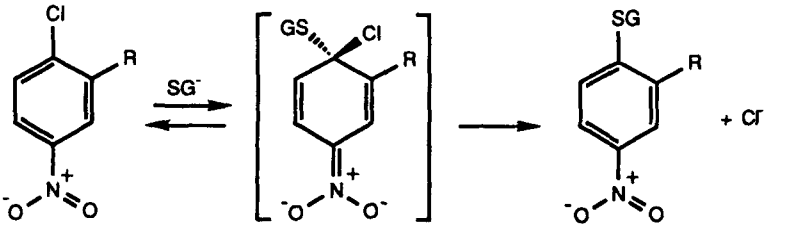
GST-isoenzymes consist of two subunits that can be identical (homodimers) or different (heterodimers). GST-subunits possess a binding site for GSH (G-site) and a separate binding site for hydrophobic electrophilic substrates (H-site) (Mannervik and Danielson 1988). The selectivity of GSTs for GSH is relatively high (Adang *et al.* 1991), whereas the selectivity for electrophilic substrates is rather broad. Based on gene structure, primary amino acid sequence similarities, immunological reactivities, isoelectric points, inhibition properties and substrate selectivities, the cytosolic GSTs are generally grouped into at least four distinct gene classes, named alpha, mu, pi (Mannervik *et al.* 1985) and theta (Meyer *et al.* 1991). Twelve distinct subunits have been identified in rat tissues and combinations of these subunits result in at least 15 homo- and heterodimeric GSTs. In the past several years various GSTs have been investigated by chemical modifications (Asaoka and Takahashi 1989, Xia *et al.* 1993), site-specific mutagenesis (Stenberg *et al.* 1991, Johnson *et al.* 1993, Wang *et al.* 1993, Ploemen *et al.* 1994) and more recently by crystal structures of GSTs (Reinemer *et al.* 1991, 1992, Ji *et al.* 1992, Sinning *et al.* 1993). These studies have led to considerable insight into the structure–function relationships of these enzymes.

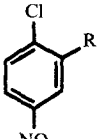
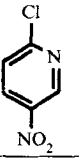
1-Chloro-2,4-dinitrobenzene (CDNB) is the most widely used model substrate for determination of GST-activities and GST-inhibition patterns (Habig *et al.* 1974). The addition of GSH to CDNB proceeds via a nucleophilic aromatic substitution (S_nAr) reaction and is generally considered to occur via a Meisenheimer or σ-complex intermediate (Miller 1968), as illustrated in scheme 1. Chen *et al.* (1988) used several 4-substituted 1-halo-2-nitrobenzenes to investigate the catalytic mechanism of rat GST 4-4 in nucleophilic aromatic substitution reactions. Substituent and leaving group effects on k_{cat} and k_{cat}/K_m for the 4-substituted 1-halo-2-nitrobenzenes were consistent with a mechanism involving rate-limiting or partially rate-limiting formation of a Meisenheimer intermediate between GSH and the haloarene. Furthermore, a linear correlation was found between $\log k_{cat}$ and the Hammett substituent constant (σ^-), suggesting that the electron-withdrawing capacity of the 4-substituent determines the stability of the Meisenheimer intermediate and thus the catalytic efficiency. More recent investigations (Liu *et al.* 1992, Zhang *et al.* 1992) provided indirect evidence that the turnover of rat GST 3-3 in nucleophilic aromatic substitution reactions (i.e. in CDNB) may be limited by product release. Moreover, Johnson *et al.* (1993) showed that the dissociation of the GS–DNB conjugate from GST 3-3 (k_{off}) is equivalent to k_{cat}, which strongly indicates that the physical step of product dissociation apparently limits turnover of CDNB by GST 3-3. Despite this drawback, CDNB is the most frequently used model substrate for studying activities, inhibition patterns and mechanisms of action of GST-isoenzymes.

In the present study, four rat GST-isoenzymes from three different classes (alpha, mu and pi) were examined for their ability to conjugate GSH to a series of new 2-substituted 1-chloro-4-nitrobenzene derivatives (table 1). Some of the substrates were specifically designed for this study to investigate if it was possible to introduce GST-isoenzyme selectivity. Substrate selectivities and enzyme kinetics led to a proposal to use an alternative substrate as model substrate in GST-research.



Scheme 1. Reaction scheme for the nucleophilic addition of GS^- to 2-substituted 1-chloro-4-nitrobenzenes. The extra negative charge in the Meisenheimer intermediate is stabilized by electron-withdrawing substituents.

Table 1. Chemical structure and reference number of substrates used. The maximal absorbance wavelengths (λ_{max}) of the substrates and the corresponding GS-conjugates and the extinction coefficients (ε) of the GS-conjugates are shown. ε's are the mean ± SD of three independent

Substrate		λ_{max} substrate (nm)	λ_{max} GS-conjugate (nm)	ε ($mM^{-1}.cm^{-1}$)
1	NO_2	250	340[a]	9.6[a]
2	CHO	271	350	20.1 ± 0.3
3	COC_6H_5	274	342	8.9 ± 1.0
4	CO_2CH_3	274	342	14.1 ± 1.2
5	$CO_2(CH_2)_3CH_3$	274	341	13.7 ± 0.1
6	$CO_2C(CH_3)_3$	274	344	10.0 ± 0.7
7	CN	271	338	9.6 ± 0.1
8	CF_3	300	336	10.1 ± 2.7
9	Cl	277	345[a]	8.5[a]
10	Br	278	348	9.5 ± 0.1
11		281	333	15.3 ± 1.8

[a] Taken from Habig and Jakoby (1981).

Materials and methods

Chemicals

GSH was obtained from Boehringer (Mannheim, Germany) and 1-chloro-2,4-dinitrobenzene (**1**) (all substrates used are depicted in table 1) was obtained from Sigma Chemical Co. (St Louis, MO, USA) and recrystallized from ethanol before use. 1-Chloro-2-trifluoromethyl-4-nitrobenzene (**8**; 98% pure), 1,2-dichloro-4-nitrobenzene (**9**; 99% pure), 2-chloro-5-nitrobenzonitrile (**7**; 99% pure), 2-chloro-5-nitrobenzophenone (**3**; 98% pure), and 2-chloro-5-nitrobenzoic acid from Aldrich-Chemie (Steinheim, Germany), and 2-chloro-5-nitropyridine (**11**; > 97% pure), and 2-chloro-5-nitrobenzaldehyde (**2**; > 97% pure) from Fluka Chemie AG (Buchs, Switzerland). Buffers and chemical reagents were of the highest quality commercially available.

Syntheses

Methyl 2-chloro-5-nitrobenzoate (**4**) and *n*-butyl 2-chloro-5-nitrobenzoate (**5**) were synthesized by esterification of 2-chloro-5-nitrobenzoic acid with the corresponding alcohol. In short, a mixture of 4 g

(20 mmol) 2-chloro-5-nitrobenzoic acid, 1·6 ml sulphuric acid (95–98 %) and 120 ml methanol or *n*-butanol respectively for **4** or **5**, was refluxed while stirring for 18 h. After cooling, 75 ml water was added. For purification of compound **4**, the methanol was evaporated. The residual water phase was neutralized with sodium bicarbonate in which the product is insoluble. The crystals were filtered, washed with cold water and dried. Recrystallization from methanol gave white crystals of **4** in a yield of 94 %, m.p. 69 °C. ^{1}H-nmr (^{2}H-dimethylsulphoxide): δ 3·93 (s, CH_3, 3H), 7·91 (d, H_3, J_{35} = 8·67 Hz, 1H), 8·4 (double d, H_5, J_{35} = 8·67 Hz, J_{56} = 3·34 Hz, 1H), 8·6 (d, H_6, J_{56} = 3·34 Hz, 1H). For purification of compound **5** the *n*-butanol was slowly evaporated. The residual water phase, which contained oily drops, was extracted with ethyl acetate. This ethyl acetate phase was washed five times with a 5 % (w/v) sodium bicarbonate solution to remove excess 2-chloro-5-nitrobenzoic acid, dried over anhydrous sodium sulphate and the ethyl acetate evaporated *in vacuo*. The product **5** appeared as a light brown oil in a yield of 70 %. From hplc analysis (assay described in 'Determination of extinction coefficients of GS-conjugates') it was concluded that the purity of substrate **5** was > 99 %. ^{1}H-nmr (^{2}H-dimethylsulphoxide): δ 0·94 (t, CH_3, J = 8·0 Hz, 3H), 1·43 (m, CH_2, 2H), 1·72 (m, CH_2, 2H), 4·35 (t, CH_2, J = 6·67 Hz, 2H), 7·89 (d, H_3, J_{35} = 8·67 Hz, 1H), 8·38 (double d, H_5, J_{35} = 8·67 Hz, J_{56} = 3·34 Hz, 1H), 8·56 (d, H_6, J_{56} = 3·34 Hz, 1H).

t-Butyl 2-chloro-5-nitrobenzoate (**6**) was synthesized by cooling a mixture of 4 g (20 mmol) 2-chloro-5-nitrobenzoic acid in 25 ml dichloromethane to −20 °C in a flask able to resist 2 atm of pressure. Of isobutylene, 20 ml was condensed with the aid of a CO_2/acetone mixture. The liquid isobutylene was added to the dichloromethane mixture via a calcium chloride tube. After addition of 0·2 ml sulphuric acid (95–98 %) the flask was closed and shaken for 2 days at room temperature. After cooling the reaction mixture it was poured into 300 ml of a saturated sodium bicarbonate solution. This mixture was extracted three times with dichloromethane. The dichloromethane fractions were washed with a 5 % (w/v) sodium bicarbonate solution, water and a saturated sodium chloride solution, respectively. To remove polymers, the dichloromethane phase was eluted over a small silica column, dried over sodium sulphate and the dichloromethane evaporated. The residue was purified over a silica column, eluted with a mixture of petroleum ether and ether (10:1). The yield of the white crystalline product (**6**) was 3·3 g (64 %), m.p. 91 °C. ^{1}H-nmr (^{2}H-chloroform): δ 1·6 (s, CH_3, 9H), 7·59 (d, H_3, J_{35} = 8·67 Hz, 1H), 8·2 (double d, H_5, J_{35} = 8·67 Hz, J_{56} = 3·34 Hz, 1H), 8·55 (d, H_6, J_{56} = 3·34 Hz, 1H).

Synthesis of 1-bromo-2-chloro-5-nitrobenzene (**10**) was accomplished according to Everly and Traynham (1979) with a slight modification. To a mixture of 1 g (6·35 mmol) 1-chloro-4-nitrobenzene, 1 g Ag_2SO_4 and 10 ml sulphuric acid (95–98 %), 0·4 ml bromine was slowly added. This was heated on a water bath of 80 °C for 6 h under constant stirring. After cooling, the reaction mixture was poured into ice and the solid materials were removed by filtration. The filtrate was extracted with diethyl ether. This extract was dried over magnesium sulphate and the diethyl ether evaporated. Recrystallization of the solid residue from ethanol gave white crystals of **10** in a yield of 30 %, m.p. 58 °C. ^{1}H-nmr (^{2}H-chloroform): δ 7·6 (d, H_3, J_{35} = 8·34 Hz, 1H), 8·1 (double d, H_5, J_{35} = 8·34 Hz, J_{56} = 0·67 Hz, 1H), 8·5 (d, H_6, J_{56} = 0·67 Hz, 1H).

Purification of GST-isoenzymes

GST-isoenzymes were purified from rat liver (GST 1-1, 3-3 and 4-4) and kidney (GST 7-7) using affinity chromatography (*S*-hexylglutathione-Sepharose 6B) as described previously (Vos *et al.* 1988). The separation of the different isoenzymes was achieved by chromatofocusing on polybuffer exchangers (Pharmacia, Uppsala, Sweden) with PBE 118 for GST 1-1, 3-3 and 4-4, and PBE 94 for GST 7-7 as described previously (Ploemen *et al.* 1993). Purity was confirmed by SDS gel electrophoresis, isoelectric focusing and hplc analysis as earlier described by Vos *et al.* (1988) and Bogaards *et al.* (1989). Protein concentrations were determined by the method of Lowry *et al.* (1951), with BSA as a standard.

Determination of maximal absorbance wavelengths of GS-conjugates

The maximal absorbance wavelengths (λ_{max}) of the GS-conjugates of the 1-chloro-4-nitrobenzene derivatives were determined spectrophotometrically by performing a reaction between GSH and substrate, catalysed by GST 3-4, followed by repetitive scanning between 250 and 550 nm. The absorbance measured was corrected for the contributions of substrate, enzyme and GSH.

Determination of extinction coefficients of GS-conjugates

A GSH conjugation reaction was performed with known, low substrate concentrations (between 0·01 and 0·05 mM) and 1 mM GSH, catalysed by GST 3-4 (23 μg/ml). At two time intervals (t = 0 and 3 h)

samples were taken and added to a 15 % (w/v) trichloro-acetic acid solution (final concentration 5 %). The samples were analysed for residual amounts of unreacted substrate by hplc. A 15 μm Chromsep C_{18}-column (3 × 200 mm) was used under isocratic conditions (flow rate 0·4 ml/min). Eluent mixtures of methanol and acetic acid buffer (3 ml acetic acid and 1 ml triethylamine/litre water) were used for different substrates: 40:60 (v/v) for substrates **2** and **11**, 50:50 (v/v) for substrates **7** and **10**, 60:40 (v/v) for substrates **8** and **4**, and 70:30 (v/v) for substrates **3**, **5** and **6**. The substrate peaks were detected at the λ_{max} of the substrates (table 1) with a Perkin Elmer UV/VIS LC 75 spectrophotometric detector. Comparison of substrate peak heights at $t = 0$ and 3 h indicated the amount of reacted substrate and consequently of GS-conjugate formed. After measurement of the absorbance of the 3 h sample at the λ_{max} of the GS-conjugate the extinction coefficient of the GS-conjugate was calculated (table 1).

Determination of enzyme kinetic parameters

Enzyme activities towards CDNB (**1**) and its derivatives were measured by a slight modification of the method described by Habig *et al.* (1974). Assays were performed with cuvettes of 1·0-cm path length at a temperature of 37 °C, using a thermostatted cell block. GSH was dissoved in 0·1 M potassium phosphate buffer (pH 6·5, 0·1 mM EDTA), kept on ice and freshly prepared. Substrate stock solutions were made in ethanol and further diluted in ethanol/phosphate buffer mixtures. The final assay medium (0·5 ml) comprised 0·1 M potassium phosphate buffer (pH 6·5, 0·1 mM EDTA), 25 μl 20 mM GSH, 25 μl diluted enzyme (different concentrations depending on substrate tested) and substrate. The final concentration of GSH was 1 mM and that of the substrates varied by experiment. The reaction was started with the addition of substrate. The final concentration of ethanol in the assay mixture did not exceed 5 %. The increase in absorbance at the λ_{max} of the respective GS-conjugates was recorded on a Philips PU 8720 UV/VIS spectrophotometer. A correction was made for the non-enzymatic reaction.

Experimental data were analysed using Lineweaver–Burk plots, giving apparent Michaelis constants (K_m) and maximum velocities (V_{max}). V_{max} was recalculated to k_{cat}, using the following molecular masses of the GST-subunits: GST 1 (25400), GST 3 (25750), GST 4 (25500) and GST 7 (23300) (Mannervik and Danielson 1988). All enzyme kinetic parameters were determined at least in triplicate on independent days and have been expressed as the mean ± SD.

Results and discussion

Determination of extinction coefficients of GS-conjugates

The methods used to determine λ_{max} and the extinction coefficients (ε) of the GS-conjugates of the 1-chloro-4-nitrobenzene derivatives were rapid and accurate (table 1). In this way time-consuming synthesis and purification of each of the 11 different GS-conjugates was avoided. The methodology for the extinction coefficient determination was checked for substrate **7**. The corresponding GS-conjugate (GS-**7**) was synthesized and purified and used to determine the extinction coefficient ε in the conventional way. This led to an ε of 9·5 $mM^{-1}.cm^{-1}$, which is almost identical to the 9·6 shown in table 1. Moreover, from substrate **1** the extinction coefficient was determined (9·8 ± 0·2 $mM^{-1}.cm^{-1}$) and compared with the literature value (9·6 $mM^{-1}.cm^{-1}$) (Habig and Jakoby 1981), which further confirms that the method to determine extinction coefficients described in the present study is accurate.

Theoretically, substrates **9** and **10** could be GSH conjugated at the 2-position, with Cl^- and Br^- acting as leaving group respectively. In nucleophilic aromatic substitution reactions, nitro-substituents are known to be *ortho*- and *para*-directing, implying that attack of GSH at the 2-position is not very likely. Moreover, during repetitive wavelength scanning of GST 3-4 catalysed incubations of these substrates and GSH, only one λ_{max} was found, further confirming that only a single GS-conjugate is formed.

Table 2. Apparent Michaelis constants (K_m) of GSTs towards 2-substituted 1-chloro-4-nitrobenzenes obtained via Lineweaver–Burk plots. K_m's are mean ± SD of at least three measurements.

	K_m (μM)			
Substrate	GST: 1-1	3-3	4-4	7-7
1	101 ± 2	49 ± 11	156 ± 36	118 ± 10
2	71 ± 3	40 ± 9	63 ± 12	118 ± 3
3	90 ± 19	36 ± 6	154 ± 18	[a]
4	629 ± 96	437 ± 107	554 ± 97	1085 ± 133
5	42 ± 4	48 ± 7	341 ± 26	94 ± 11
6	53 ± 5	77 ± 13	[a]	246 ± 38
7	386 ± 62	315 ± 76	292 ± 48	565 ± 36
8	276 ± 5	389 ± 88	435 ± 53	287 ± 36
9	442 ± 4	240 ± 37	855 ± 107	1172 ± 152
10	241 ± 80	175 ± 6	381 ± 70	390 ± 40
11	929 ± 75	1074 ± 143	1267 ± 165	4430 ± 195

[a] No Lineweaver–Burk plots were determined due to the limited solubility of substrates **3** and **6** (< 300 μM).

Apparent K_m *values*

The Michaelis constant K_m is an apparent dissociation constant that may be considered as the overall dissociation constant of enzyme-bound species and reflects the affinity of a substrate for the active site of an enzyme (Fersht 1977). Assuming Michaelis-Menten kinetics, K_m is the substrate concentration at which the reaction rate equals half of the maximal velocity. In table 2 the experimentally determined K_m of 11 2-substituted 1-chloro-4-nitrobenzenes towards four GST-isoenzymes (GST 1-1, 3-3, 4-4 and 7-7) are shown.

Evaluation of the apparent K_m of the different substrates for the four GST-isoenzymes showed interesting differences. Differences as large as 22, 30, 20 and 47 times existed between the lowest and highest K_m measured for GST 1-1, 3-3, 4-4 and 7-7 respectively. With all four isoenzymes substrate **11** had the highest K_m, which is indicative for a relatively low affinity of this substrate for the substrate binding site of all four GST-isoenzymes. Probably the pyridine-ring, which is rather hydrophilic compared with the phenyl-ring in the other substrates, disturbs general lipophilic interactions or a more specific interaction between the *ortho*-substituent and amino acid(s) in the active site.

Substrates **1**, **2**, **3**, **5** and **6** had a relatively high affinity for the substrate binding site (H-site) of all GST-isoenzymes examined in this study. Substrates **1** and **2**, which are sterically very similar, showed a K_m in the same range (high affinity). However, substrate **3** also showed high affinity for the H-site, even though it appeared from minimal energy conformation calculations that the phenyl-ring of the benzophenone-substituent is twisted out of the aromatic plane, due to steric hindrance between the chlorine and phenyl orbitals (data not shown). Also the *n*-butyl and *t*-butyl ester derivatives (**5** and **6**), which are quite bulky, showed high affinity for the GST-isoenzymes, except for GST 4-4. This suggests that there is apparently sufficient space in this area of the active site, possibly with additional aromatic and/or lipophilic interactions. An exception to this suggestion is substrate **4** (the methyl ester derivative), which is relatively small and had low affinity for all four GST-isoenzymes. On steric grounds this substrate should also fit in the active site, but probably misses essential lipophilic interactions at the *ortho*-substituent.

Table 3. Turnover numbers (k_{cat}) of GSTs towards 2-substituted 1-chloro-4-nitrobenzenes obtained via Lineweaver–Burk plots. k_{cat}'s are mean ± SD of at least three measurements.

	k_{cat} (min^{-1})			
Substrate GST:	1-1	3-3	4-4	7-7
1	614 ± 7	377 ± 82	137 ± 14	222 ± 15
2	2·3 ± 0·1	8·5 ± 1·1	1·0 ± 0·1	2·1 ± 0·1
3	2·0 ± 0·2	2·9 ± 0·5	2·2 ± 0·5	[a]
4	21 ± 2	37 ± 7	6·5 ± 1·4	9·5 ± 0·4
5	2·9 ± 0·1	8·7 ± 1·3	12·1 ± 0·8	2·6 ± 0·2
6	1·6 ± 0·1	6·2 ± 0·7	[a]	1·3 ± 0·2
7	98 ± 9	232 ± 15	24 ± 4	56 ± 4
8	7·3 ± 0·2	53 ± 14	8·9 ± 1·5	5·0 ± 0·3
9	4·9 ± 0·3	24 ± 5	5·4 ± 1·5	6·4 ± 0·1
10	4·0 ± 0·7	34 ± 1	6·1 ± 0·8	3·3 ± 0·2
11	32 ± 3	58 ± 6	6·4 ± 0·8	45 ± 2

[a] No Lineweaver–Burk plots were determined due to the limited solubility of substrates **3** and **6** (< 300 μM).

Comparison of substrates **9** and **10** (Cl- and Br-substituents) showed a higher affinity of the bromine-derivative for all four GST-isoenzymes, despite the fact that Br is more bulky. This again suggests that steric factors, positioned *ortho* relative to the site of GSH attack, are not major determinants in the affinity of 2-substituted 1-chloro-4-nitrobenzenes for the GST-isoenzymes.

There was no clear GST-isoenzyme selectivity when K_m's were compared in the present series of 11 substrates. In general, GST 3-3 showed the highest affinity for 2-substituted 1-chloro-4-nitrobenzenes and GST 7-7 the lowest. When GST 3-3 and 4-4, both members of the mu class, were compared, it appeared that GST 4-4 has remarkably lower affinities for large, bulky substrates (**3** and **5**). This might be indicative for a more sterically restricted active site.

The k_{cat} *values*

The first-order rate constant k_{cat} is often called the turnover number of the enzyme, because it represents the maximum number of substrate molecules converted to products per active site per unit time. It refers to the properties and reactions of the enzyme–substrate, enzyme–intermediate, and enzyme–product complexes (Fersht 1977). The experimentally determined k_{cat}'s of substrates **1–11** for all four isoenzymes are given in table 3.

The difference between the highest and lowest k_{cat} with GST 1-1 was more pronounced (384-fold) than with GST 3-3 (130-fold), GST 4-4 (137-fold) and GST 7-7 (171-fold). The highest and second highest k_{cat}'s were found for CDNB (**1**) and the benzonitrile derivative (**7**) respectively in all four GST-isoenzymes. Probably the negative charges in the respective Meisenheimer intermediates are best stabilized with the electron-withdrawing *ortho*-substituents NO_2 and CN.

In a study by Chen *et al.* (1988) a linear correlation was found between log k_{cat}'s of five 4-substituted 1-chloro-2-nitrobenzenes towards GST 4-4 and the Hammett substituent constant (σ_p^-). The physico-chemical parameter σ_p^- gives an indication of

Table 4. Specificity constants (k_{cat}/K_m) of GSTs towards 2-substituted 1-chloro-4-nitrobenzenes obtained via Lineweaver–Burk plots. k_{cat}/K_m's are mean ± SD of at least three measurements.

	k_{cat}/K_m ($\mu M^{-1}.min^{-1}$) × 10^{-2}			
Substrate GST:	1-1	3-3	4-4	7-7
1	608 ± 3	716 ± 234	93 ± 19	189 ± 4
2	3·3 ± 0·1	24 ± 5	1·5 ± 0·4	1·8 ± 0·1
3	2·2 ± 0·2	9·1 ± 1·6	1·5 ± 0·3	[a]
4	3·3 ± 0·3	8·3 ± 0·8	0·9 ± 0·1	0·9 ± 0·1
5	6·8 ± 0·6	18·3 ± 1·6	3·5 ± 0·1	2·8 ± 0·2
6	3·1 ± 0·2	8·3 ± 0·6	[a]	0·5 ± 0·1
7	26 ± 2	75 ± 15	8 ± 1	9·9 ± 0·1
8	2·6 ± 0·01	15 ± 2	2·0 ± 0·03	1·7 ± 0·1
9	1·1 ± 0·05	10 ± 1	0·6 ± 0·2	0·60 ± 0·08
10	1·7 ± 0·3	19·3 ± 0·1	1·6 ± 0·2	0·80 ± 0·03
11	3·4 ± 0·1	5·2 ± 0·5	0·5 ± 0·05	1·0 ± 0·07

[a] No lineweaver–Burk plots were determined due to the limited solubility of substrates **3** and **6** (< 300μM).

the electron-withdrawing capacity of *para*-substituents. In the present study, *ortho*-substituents were varied. However, no Hammett substituent constants are known for substituents at the *ortho*-position. Electronically, *ortho*- and *para*-positions are similar, but sterically there are major differences. In a study by Hess *et al.* (1971) the ^{13}C-H coupling constants of *ortho*-substituted toluenes, anisoles and benzaldehydes were recalculated to σ's at the *ortho*-position (σ_0). It appeared that, depending on the nature of the remainder of the molecule, this σ_0 correlated well with σ_p. Apparently, *para*-parameters can be used for *ortho*-substituents, although it may also lead to discrepancies, as can be seen in the following case. The σ_p^- of NO_2 and CN-substituents (0·78 and 0·66 respectively) differ only slightly from that of, for example, a $CO_2C(CH_3)_3$-group (0·45), whereas the substrate with the latter substituent (**6**) had by far the lowest k_{cat} in, for example, GST 1-1 and 7-7. This suggests that σ_p^- is not always equal to σ_0^- and that other factors, probably with a steric basis, are also involved in stabilization of the Meisenheimer intermediates.

The halogen-substituted substrates **8**, **9** and **10** had a relatively high k_{cat} for GST 3-3 when compared with the other isoenzymes. These substrates are relatively selectively conjugated by GST 3-3, probably because the active site possesses extra or other important amino acids, which can act as interaction site, stabilizing the corresponding Meisenheimer intermediates. Substrate **11** showed a 5–9-fold lower k_{cat} with GST 4-4 than with the other three GST-isoenzymes, indicating that the influence of the hydrophilic pyridine-ring on the turnover is largest in GST 4-4. GST 3-3 showed the highest k_{cat} towards all substrates, except for CDNB (**1**), which is best conjugated by GST 1-1. The lowest catalytic efficiency was found for the conjugation of GSH to substrate **2**, catalysed by GST 4-4.

The k_{cat}/K_m *values*

The k_{cat}/K_m is an apparent second-order rate constant that refers to the properties and the reactions of free enzyme and free substrate (Fersht 1977). In

Table 5. Concentrations of GS-conjugate and time points at which the GST-catalysed reaction (±6·3 μg/ml) is about to become non-linear for 1 mM GSH and 1 mM of substrates **1** and **7**. Values were obtained by drawing lines along the linear part of a plot, representing the formation of GS-conjugate with time (for example, figure 1). The time points where the reaction became non-linear were marked and the GS-conjugate concentrations were calculated from the absorption and ε.

	Substrate **1**		Substrate **7**	
GST	Time (s)	GS-**1** (μM)	Time (s)	GS-**7** (μM)
1-1	20	40	140	50
3-3	15	25	30	45
4-4	45	55	280	45
7-7	60	50	> 600	–

table 4 the k_{cat}/K_m's are given for the GSH conjugation reaction of 2-substituted 1-chloro-4-nitrobenzene derivatives, catalysed by four GST-isoenzymes.

GST 3-3 showed the highest k_{cat}/K_m in the GSH conjugation of all the chloronitrobenzenes. This means that GST 3-3, member of the mu class, catalyses the overall reaction, including binding of substrates, stabilisation of intermediates and release of the product, most efficiently. The alpha class GST 1-1 showed the second highest and the mu class GST 4-4 the lowest k_{cat}/K_m's. In this regard the overall catalytic efficiency does not follow the classification of GST-isoenzymes into four gene classes.

CDNB (**1**) had the highest, and substrate **7** the second highest k_{cat}/K_m with all four GST-isoenzymes. The difference between the highest and lowest k_{cat}/K_m in GST 1-1 was 553-fold, whereas this difference was less pronounced for GST 3-3 (138-fold), GST 4-4 (186-fold) and GST 7-7 (378-fold). Compounds **2**, **8**, **9** and **10** showed selectivity for GST 3-3, when k_{cat}/K_m's were compared. These substrates might be used in the case where a specific substrate would be needed with selectivity towards GST 3-3. No selective substrates were found for GST 1-1, 4-4 and 7-7 within this series of compounds.

Time dependency of GSH conjugation

In figure 1 the time dependency of the GST 1-1 and 4-4 catalysed conjugation of substrates **1** and **7** (1 mM) to GSH (1 mM) is shown. The GSH conjugation reaction of substrate **1** became non-linear after about 20 and 45 s with GST 1-1 and 4-4 respectively. For substrate **7** these figures are approximately 140 and 280 s with GST 1-1 and 4-4 respectively. At these time points, non-linear kinetics became visible. There are two possible explanations for this phenomenon. First, the amount of GSH or substrate might become rate-limiting. However, around the time points where the GSH conjugation rate started to become non-linear with GST 1-1 and GST 4-4, the concentration of 1-(*S*-glutathionyl)-2,4-dinitrobenzene (GS-**1**) was about 40 and 55 μM respectively, and that of 2-(*S*-glutathionyl)-5-nitrobenzonitrile (GS-**7**) was around 50 and 45 μM respectively (based on $\varepsilon = 9{\cdot}6\ mM^{-1}.cm^{-1}$). Therefore, the non-linearity can not be due to a limited availability of GSH or substrate because the starting concentration for both reactants was 1 mM. More likely, product inhibition may play a critical role in the non-linearity of the

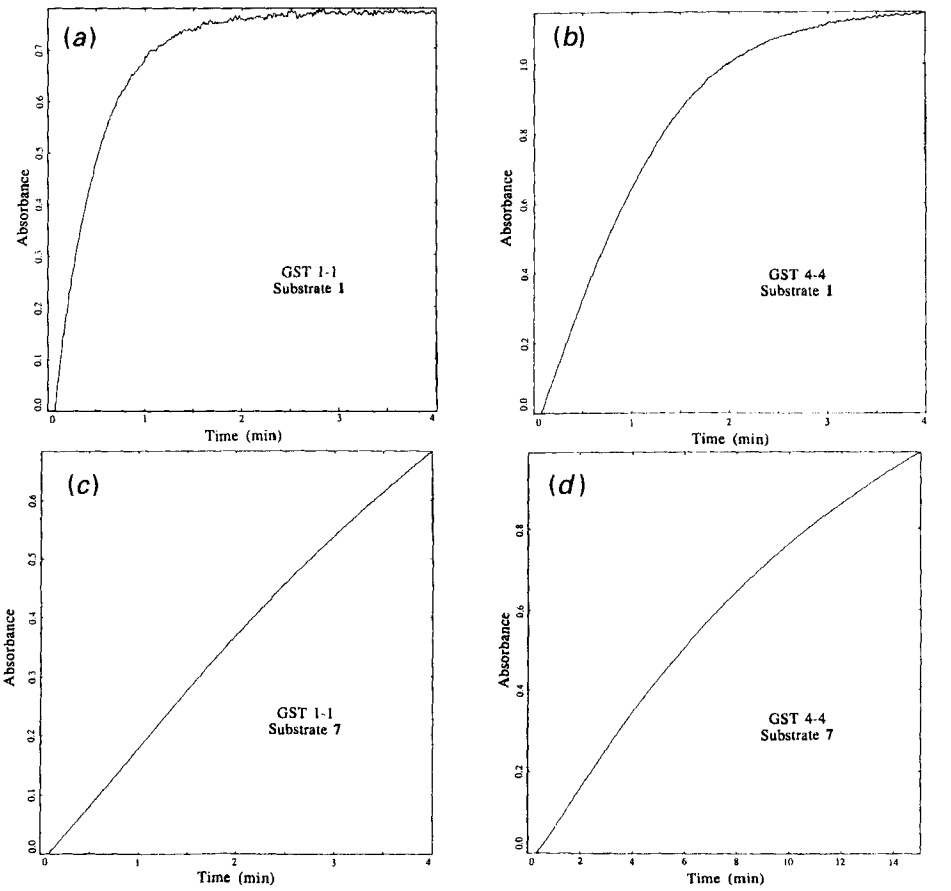


Figure 1. Time-dependent formation of GS-conjugates of substrates **1** and **7**. Substrates (1 mM) were incubated with GSH (1 mM) and GST 1-1 (6·3 μg/ml) and 4-4 (6·2 μg/ml) at 37 °C. Product formation from substrates **1** and **7** was monitored spectrophotometrically at 340 and 338 nm respectively.

respective GSH conjugation reactions. Table 5 shows the time points and concentrations at which the GSH conjugation of 1 mM substrate **1** or **7** with 1 mM GSH, when catalysed by GST 1-1, 3-3, 4-4 and 7-7 (±6·3 μg/ml), was about to become non-linear. The time during which product formation was linear, was much longer for substrate **7** than for **1** in the case of all four GST-isoenzymes. As derived from the k_{cat} for **1** and **7** (table 3), substrate **1** is conjugated much faster to GSH than substrate **7** and, consequently, **1** can reach the inhibitory concentration of corresponding GS-conjugate faster. The concentration of GS-**7** needed to reach non-linearity was around 50 μM in all GST-isoenzymes. With substrate **1**, however, non-linearity occurred at different concentrations of GS-conjugate, 25 μM of GS-**1** already inducing non-linearity in the GSH conjugation by GST 3-3. This concentration is up to 2-fold lower than for the other GSTs. Recently, Johnson *et al.* (1993) proposed a hydrogen bridge between Ser-209 and Tyr-115 in GST 3-3 by which the elimination of GS-**1** from the active site might be restricted. This phenomenon could result in non-linear kinetics at very low concentrations of GS-**1**.

In case of substrate **7** all four GSTs demonstrated product inhibition at a GS-conjugate concentration of $\pm 50\ \mu M$, indicating that release of GS-**7** from GST 3-3 is not rate-limiting. Apparently, a high affinity of GS-conjugates for the active site of the GST-isoenzymes may also cause non-linear kinetics by product inhibition.

In summary, in the present study the GSH conjugation of a new series of substrates (2-substituted 1-chloro-4-nitrobenzenes) was investigated with four different rat GST-isoenzymes of the alpha, mu and pi class. Theta class isoenzymes were not included in the study since it is known that CDNB, and presumably its analogues, are not conjugated by these isoenzymes (Meyer *et al.* 1991). A rapid and accurate method is presented for determination of extinction coefficients of the corresponding GS-conjugates. In addition, K_m, k_{cat} and k_{cat}/K_m's of the substrates were determined. An extremely low affinity (high K_m) was found for substrate **11** towards all four GST-isoenzymes, probably because of the pyridine-ring, which is rather hydrophilic compared with the phenyl-ring in the other substrates. This may disturb general lipophilic interactions or a specific interaction between the *ortho*-substituent and amino acid(s) in the active site. It appeared that GST 3-3 catalysed the reaction between GSH and the substrates most efficiently. There was also a clear isoenzyme selectivity towards GST 3-3 with substrates **2**, **8**, **9** and **10** when k_{cat}/K_m's were compared. Substrates **1** and **7** had an average affinity (K_m) and a high GSH conjugating activity (k_{cat}/K_m) towards all four GST-isoenzymes. It is suggested that substrate **7** might be recommended as model substrate in GST-research. The substrate is commercially available and shows linear enzyme kinetics with all GST-isoenzymes for a considerably longer period of time than CDNB (**1**), a substrate usually used for this purpose.

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