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Isothiocyanates as substrates for human glutathione transferases: structure–activity studies

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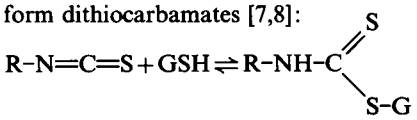
The catalytic properties of four human glutathione transferases (GSTs), A1–1, M1–1, M4–4 and P1–1, were examined with 14 isothiocyanate (R-NCS) substrates. The compounds include aliphatic and aromatic homologues, some of which are natural constituents of human food, namely sulphoraphane [1-isothiocyanato-4-(methylsulphinyl)butane], erucin [1-isothiocyanato-4-(methylthio)butane], erysolin [1-isothiocyanato-4-(methylsulphonyl)butane], benzyl-NCS, phenethyl-NCS and allyl-NCS. All isothiocyanates investigated were substrates for the four GSTs. The enzymes promote addition of the thiol group of GSH to the electrophilic central carbon of the isothiocyanate group to form dithiocarbamates [R–NH–C(=S)-SG] which have high UV absorption at 274 nm. Molar absorption coefficients and non-enzymic rate constants as well as standardized enzyme assay conditions for all compounds were established. Of the four isoenzymes investigated, GSTs M1–1 and P1–1 were generally the most efficient catalysts, whereas GST M4–4 was the least efficient. Isothiocyanates are among the GST substrates that are most rapidly conjugated. On the basis of rate-enhancement data and binding energies, the isothiocyanates were compared with 4-hydroxyalkenals, another class of natural GST substrates previously subjected to systematic kinetic analysis. The incremental transition-state stabilization attributable to an increased number of methylene groups in homologous alkyl isothiocyanates is similar to that previously noted for homologous 4-hydroxyalkenals.

INTRODUCTION

Glutathione transferases (GSTs) make up a family of isoenzymes that accomplish the conjugation of a wide variety of structurally unrelated substrates with the tripeptide GSH (γ-glutamylcysteinylglycine) (see reviews [1–3]). The common feature of the enzymic conjugation reaction is the attachment of the sulphydryl group of GSH to an organic electrophile. The detoxication of the majority of electrophilic xenobiotics has been attributed to GSH conjugation, and a multitude of compounds have been shown to undergo this reaction *in vitro*. However, most of the substrates so far identified are synthetic compounds that are not present in biological systems to provide a selection pressure influencing the evolution of the GSTs. The identification of 'natural substrates' for this family of enzymes has been a challenge which has led to much speculation. Substrates that might have played a role in GST evolution include alkenals in general [4] and particularly the (*E*)-4-hydroxyalk-2-enals (4-hydroxyalkenals) which are products of cellular lipid peroxidation and have previously been shown to be efficiently conjugated by GSTs [5]. Extensive structure–activity studies of GSTs with these compounds have led to insights about structural determinants affecting the substrate preferences of the enzymes [6].

We and others have recently shown that organic isothiocyanates, which are abundant in edible plants, undergo conjugation with GSH enzymically as well as non-enzymically to form dithiocarbamates [7,8]:



These dithiocarbamates are degraded *in vivo* to the corresponding *S*-(*N*-acetyl)cysteinyl conjugates (mercapturates), which undergo rapid renal excretion, and have been shown to be major products of isothiocyanate metabolism in rats and humans, thus providing evidence that isothiocyanates are subject to GSH conjugation *in vivo* [9–12]. A number of isothiocyanates have also been shown to be inducers of GSTs in animal tissues and to display pronounced anticarcinogenic activity (see [13] for a review). Isothiocyanates are abundant in many edible plants, especially crucifers, such as cauliflower, broccoli and cabbage [14,15].

In the present investigation, the kinetic properties of four distinct human GSTs, namely GSTs A1–1, M1–1, M4–4 and P1–1, with 14 structurally related isothiocyanates were determined. The compounds chosen make up a set of aliphatic and aromatic analogues. This work thus extends the previous demonstration that isothiocyanates are efficient substrates for human GSTs [7], with the particular aim of investigating structure–activity relationships and making a comparison with 4-hydroxyalkenals, another group of naturally occurring GST substrates.

MATERIALS AND METHODS

Materials

Propyl-, butyl-, pentyl-, hexyl-, (±)-2-hexyl-, cyclohexyl-, cyclopropyl- and cyclo-octyl-NCS were purchased from Trans World Chemicals, Rockville, MD, U.S.A. Benzyl-, phenethyl- and allyl-NCS were bought from Aldrich, Milwaukee, WI, U.S.A. Erucin [1-isothiocyanato-4-(methylthio)butane], (±)-sulphoraphane [(±)-1-isothiocyanato-4-(methylsulphinyl)-butane] and erysolin [1-isothiocyanato-4-(methylsulphonyl)-

Abbreviations used: GST, glutathione transferase (EC 2.5.1.18); H-site, hydrophobic substrate-binding site; R-NCS, isothiocyanate.

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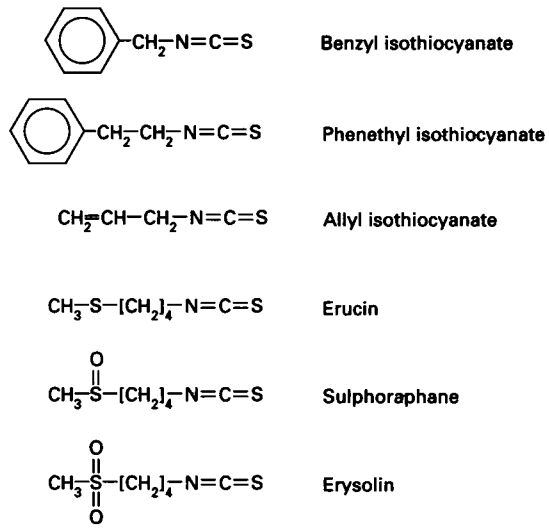

Figure 1 Structures of isothiocyanates occurring in human food

butane] (Figure 1) were synthesized and kindly provided by C.-G. Cho and G. H. Posner, Department of Chemistry, The Johns Hopkins University, Baltimore, MD, U.S.A. All other chemicals were from commercial suppliers and of highest purity available.

4-Hydroxy-pentenal, -hexenal, -heptenal, -octenal, -nonenal, -decenal, -undecenal and -dodecenal dissolved in chloroform were obtained from Professor H. Esterbauer, Institut für Biochemie, University of Graz, Graz, Austria.

Preparation of recombinant human GST

GSTs A1–1 [16], M1–1 (allelic variant b) [17], M4–4 [18] and P1–1 [19] were obtained by heterologous expression in the *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA, U.S.A.). A single bacterial colony was inoculated into 40 ml of an overnight culture of 2TY medium (10 g of yeast extract, 16 g of tryptone, 5 g of NaCl and 50 mg of ampicillin per litre). Typically 3 litres of expression medium (20 g of yeast extract, 30 g of tryptone, 5 g of NaCl, 10 g of glycerol and 50 mg of ampicillin per litre) was inoculated with this preculture (1:100 volume ratio) and agitated in a rotary shaker at 37 °C. When the absorbance at 600 nm reached 0.2–0.3, the inducer isopropyl β-D-thiogalactopyranoside (Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 0.2 mM. The cultures were allowed to grow for an additional 12–16 h. The bacteria were harvested by centrifugation, resuspended in an equal volume of 50 mM Tris/HCl, pH 7.0, and sonicated (Vibra Cell VC 600 Sonifier; Sonics & Materials Inc., Danbury, CT, U.S.A.). The cell lysate was obtained by centrifugation for 2 h at 25000 *g* and combined with 50 ml of an *S*-hexylglutathione-affinity matrix [20] based on epoxy-activated Sepharose 6B (Pharmacia Biotech AB, Uppsala, Sweden). After 2 h of careful agitation, the affinity matrix was washed on a sintered glass funnel with 2 litres of 10 mM Tris/HCl, pH 7.0, containing 0.2 M NaCl, and packed into a column. The enzyme was eluted with the same buffer fortified with 5 mM *S*-hexylglutathione. The eluate was subsequently passed over a Sephadex G-25 gel-filtration matrix (Pharmacia Biotech AB), which rendered the enzyme free of *S*-hexylglutathione, as verified by amino acid analysis.

Absorption coefficients

Each isothiocyanate (20 μM) was allowed to react with 1 mM GSH in 40 mM sodium phosphate, pH 8.5, containing 2% (v/v) acetonitrile. The absorbance at 274 nm was measured after completion of the reaction (4–6 min) and the molar absorption coefficients (ϵ_{274}) were calculated. The contributions of GSH, acetonitrile and the isothiocyanates to the absorbance were found to be insignificant. The millimolar absorption coefficients ($mM^{-1} \cdot cm^{-1}$) of the GSH conjugates derived from the following isothiocyanates were: propyl-NCS, 8.35; butyl-NCS, 7.75; pentyl-NCS, 7.53; hexyl-NCS, 6.55; allyl-NCS, 7.45; cyclopropyl-NCS, 9.46; cyclohexyl-NCS, 8.52; cyclo-octyl-NCS, 6.88; benzyl-NCS, 9.25; phenethyl-NCS, 8.89; sulphoraphane, 8.00; erysolin, 8.45; erucin, 8.60.

4-Hydroxyalkenal solutions were prepared as described by Danielson et al. [6]. The millimolar absorption coefficient for the conjugation reaction was taken as the disappearance of free 4-hydroxyalkenal ($\epsilon_{224} = 13.74\ mM^{-1} \cdot cm^{-1}$), since the absorption coefficients for GSH and the conjugate between GSH and 4-hydroxyalkenal were similar and insignificant (0.7–0.9 $mM^{-1} \cdot cm^{-1}$).

Non-enzymic reaction rates

The formation of dithiocarbamates from isothiocyanates and GSH was determined in 100 mM sodium phosphate, pH 6.5, containing 2% (v/v) acetonitrile at 30 °C. The concentrations of both the isothiocyanate and GSH were varied. The reactions were monitored spectrophotometrically at 274 nm and shown to be pseudo-first-order with respect to each reactant within the range of concentrations investigated. The second-order rate constants were determined by linear regression analysis of the experimental data.

The rate of the non-enzymic reaction between 4-hydroxyalkenals and GSH was determined in 0.2 M sodium phosphate, pH 6.5, using 10 μM 4-hydroxyalkenal and 2.5 mM GSH in a total volume of 1 ml.

Standardization of enzyme concentrations

Exact dilutions of the enzyme stock solutions were prepared and aliquots were analysed for activity under the conditions reported previously [21], in a solution containing 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene, 100 mM sodium phosphate, pH 6.5, and 5% (v/v) ethanol at 30 °C. Product formation was followed by measuring the increase in $A_{340}(\epsilon = 9.6\ mM^{-1} \cdot cm^{-1})$. The values obtained were corrected for the non-enzymic reaction rates. The molar concentration of active enzyme was estimated by use of the specific activities for the different isoenzymes. Values for reference enzymes, obtained from the same expression systems, were: 90 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for A1–1, 61 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for M1–1, 1.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for M4–4 and 140 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for P1–1 ([18]; R. H. Kolm, unpublished work).

Kinetics of enzymic reactions

Initial-rate measurements with isothiocyanates and GSH were carried out for each GST isoenzyme at 30 °C in 100 mM sodium phosphate, pH 6.5, containing 2% (v/v) acetonitrile. The data were corrected for the non-enzymic reaction rates and analysed by non-linear regression with use of the program package SIMFIT [22]. Because of the limited aqueous solubility of many isothiocyanates, concentrations approaching substrate satu-

ration of the enzyme could usually not be attained. A function of the following form was fitted to the experimental data:

$$\frac{v}{[E]_{tot.}} = \frac{k_{cat.}/K_m[\text{isothiocyanate}]}{1+\frac{[\text{isothiocyanate}]}{K_m}} \quad (1)$$

This function, which represents a reparameterized Michaelis–Menten rate equation, allowed the accurate calculation of $k_{cat.}/K_m$, even when $k_{cat.}$ and K_m could not be determined separately [23]. The kinetic constants of the enzyme-catalysed conjugation of 4-hydroxyalkenals have previously been reported [6].

RESULTS AND DISCUSSION

Establishment of assay conditions for isothiocyanates

Conjugation of isothiocyanates with GSH gives rise to dithiocarbamates, which have spectroscopic properties distinct from those of the parent substrates. It was therefore necessary to define quantitative assay conditions. Isothiocyanates have low-intensity ($\epsilon \sim 1000\ M^{-1}\cdot cm^{-1}$) absorbance maxima at 240 nm. In contrast, dithiocarbamates have high (ϵ 6500–9500 $M^{-1}\cdot cm^{-1}$) absorbances in the 270–300 nm range. Difference spectra of the dithiocarbamates with respect to their parent isothiocyanates showed a common peak close to 274 nm which was used for all assays of the velocities of the conjugation reaction.

The rate of the non-enzymic reactions of GSH with isothiocyanates was found to be linearly dependent on the concentrations of both reactants, thus obeying second-order kinetics. Absorption coefficients (see the Materials and methods section) and second-order rate constants (Table 1) at pH 6.5 and 30 °C for 14 isothiocyanates were determined. The absorption coefficients (ϵ_{274}) showed slight differences even between structurally closely related isothiocyanates, and may reflect the purities of these compounds. The isothiocyanates were all dissolved in acetonitrile. For comparison with earlier results the final concentration of solvent was 2% (v/v), which did not inhibit the reaction.

It has previously been noted that isothiocyanates used as substrates irreversibly inhibit GSTs [7]. The inhibitory action was most pronounced with GST P1–1. In the present investigation this effect was minimized by initiating the enzyme reaction by addition of the isothiocyanate substrate.

Catalytic efficiencies of isothiocyanates as substrates

Initial rates of the enzymic reaction were measured for each isoenzyme–isothiocyanate combination. Several of the substrates are hydrophobic and have poor solubility in the assay buffer, and could therefore not be used at sufficiently high concentrations for accurate direct determinations of $k_{cat.}$ values. Nevertheless, the ratio $k_{cat.}/K_m$, i.e. the derivative of the saturation function at the origin, could be determined accurately from the modified Michaelis–Menten equation (eqn. 1), which yields $k_{cat.}/K_m$ directly as a parameter of the equation fitted to experimental data (Table 1). The $k_{cat.}/K_m$ value is a measure of the catalytic efficiency and represents the potential for catalysis at low substrate concentrations, which presumably reflect conditions that prevail *in vivo*.

In general, GST M1–1 appears to be the most efficient catalyst of the four isoenzymes, closely followed by GST P1–1, whereas GST A1–1 is less active by one and GST M4–4 by even two orders of magnitude for all substrates tested. All four enzymes gain in catalytic rate with increasing aliphatic chain length, and, of all the aliphatic substrates with linear carbon chains tested, hexyl-NCS gives the highest rates for all of them. The aromatic substrates, benzyl-NCS and phenethyl-NCS, tend to be the best ones. Moreover, GST M4–4 has a preference for allyl-NCS among the aliphatic compounds. In contrast, sulphoraphane was the poorest substrate for all of the enzymes. All four enzymes displayed an identical pattern of catalytic efficiency for the series of related isothiocyanates that contain a sulphide, sulphoxide or sulphone group in the side chain (Figure 1). The sulphide (erucin) was the best and the sulphoxide (sulphoraphane) was the

Table 1 Non-enzymic reaction rate constants, $k_{cat.}/K_m$ values and rate-enhancement factors (r.e.f.) for human GSTs with isothiocyanates as substrates

Measurements were performed at 30 °C in 100 mM sodium phosphate, pH 6.5, containing 1 mM GSH and 2% (v/v) acetonitrile. Values (means ± S.E.M., obtained by regression analysis) were calculated per GST subunit.

Isothiocyanate substrate	Non-enzymic rate constant ($M^{-1}\cdot s^{-1}$)	A1–1 $k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	A1–1 $10^{-3}\times$ R.e.f.	M1–1 $k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	M1–1 $10^{-3}\times$ R.e.f.	M4–4 $k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	M4–4 $10^{-3}\times$ R.e.f.	P1–1 $k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	P1–1 $10^{-3}\times$ R.e.f.
Propyl-NCS	0.47	6.0 ± 0.7	13	19.5 ± 0.5	41	0.49 ± 0.02	1.0	16.5 ± 0.4	35
Butyl-NCS	0.40	4.7 ± 0.4	12	56 ± 1	140	0.45 ± 0.05	1.1	41 ± 1	100
Pentyl-NCS	0.50	6.4 ± 0.1	13	140 ± 1	280	1.03 ± 0.07	2.1	96 ± 4	190
Hexyl-NCS	0.39	20 ± 4	51	310 ± 30	790	1.31 ± 0.002	3.4	173 ± 6	450
Allyl-NCS	0.61	15 ± 2	25	131 ± 4	210	3.4 ± 0.4	5.6	114 ± 5	190
Cyclopropyl-NCS	2.00	20 ± 2	10	164 ± 6	83	1.26 ± 0.04	0.63	91 ± 9	46
Cyclohexyl-NCS	0.29	6.3 ± 0.4	22	110 ± 10	380	2.0 ± 0.2	6.9	80 ± 2	280
Cyclo-octyl-NCS	0.65	7.9 ± 0.2	12	330 ± 10	510	0.83 ± 0.01	1.3	115 ± 3	180
2-Hexyl-NCS	0.39	5.4 ± 0.2	14	97 ± 2	250	1.42 ± 0.01	3.6	42 ± 3	110
Benzyl-NCS	2.55	10.2 ± 0.1	4.0	250 ± 30	98	4.5 ± 0.4	1.8	340 ± 20	130
Phenethyl-NCS	0.85	14 ± 2	16	239 ± 9	280	4.36 ± 0.03	5.1	200 ± 20	230
Sulphoraphane	0.83	3.4 ± 0.4	4.1	3.9 ± 0.3	4.7	0.16 ± 0.02	0.20	39 ± 2	47
Erysolin	0.47	9.0 ± 0.8	19	18 ± 4	38	0.39 ± 0.01	0.83	74 ± 3	160
Erucin	0.84	10.5 ± 0.4	13	164 ± 4	200	0.64 ± 0.02	0.76	198 ± 6	240

Table 2 Apparent K_m values of human GSTs with isothiocyanates as substrates

Reactions were carried out at 30 °C in 100 mM sodium phosphate, pH 6.5, containing 1 mM GSH and 2% (v/v) acetonitrile. Values (means ± S.E.M.) were obtained by regression analysis.

	K_m (μM)			
Substrate	A1–1	M1–1	M4–4	P1–1
Propyl-NCS	290 ± 70	> 2000	> 2000	1200 ± 760
Butyl-NCS	270 ± 50	510 ± 20	> 2000	620 ± 60
Pentyl-NCS	120 ± 5	170 ± 2	500 ± 100	> 2000
Hexyl-NCS	22 ± 5	43 ± 6	78 ± 3	200 ± 20
Allyl-NCS	220 ± 60	170 ± 10	250 ± 50	190 ± 20
Cyclopropyl-NCS	140 ± 20	63 ± 4	> 2000	110 ± 10
Cyclohexyl-NCS	39 ± 4	100 ± 30	45 ± 5	130 ± 9
Cyclo-octyl-NCS	8.4 ± 0.4	13 ± 6	360 ± 8	100 ± 10
2-Hexyl-NCS	40 ± 2	160 ± 6	280 ± 50	300 ± 80
Benzyl-NCS	200 ± 10	150 ± 30	400 ± 70	87 ± 6
Phenethyl-NCS	60 ± 10	100 ± 6	180 ± 6	120 ± 20
Sulphoraphane	600 ± 20	350 ± 60	> 2000	750 ± 120
Erysolin	530 ± 120	760 ± 530	> 2000	640 ± 70
Erucin	120 ± 6	130 ± 5	700 ± 80	130 ± 6

Table 3 Specific activities of human GSTs with isothiocyanates as substrates

Reactions were carried out at 30 °C in 100 mM sodium phosphate, pH 6.5, containing 1 mM GSH and 2% (v/v) acetonitrile.

		Specific activity (μmol · min^{-1} · mg^{-1})			
	Substrate concn. (μM)	A1–1	M1–1	M4–4	P1–1
Propyl-NCS	400	2.4	16	0.41	15
Butyl-NCS	400	1.8	29	0.38	25
Pentyl-NCS	200	1.1	31	0.35	49
Hexyl-NCS	200	1.0	23	0.18	44
Allyl-NCS	400	5.0	36	1.2	38
Cyclopropyl-NCS	400	4.5	21	1.1	20
Cyclohexyl-NCS	100	0.4	13	0.14	11
Cyclo-octyl-NCS	50	0.13	7.9	0.035	9.8
2-Hexyl-NCS	100	0.36	14	0.26	8.1
Benzyl-NCS	400	3.2	62	2.1	63
Phenethyl-NCS	400	1.7	46	1.3	46
Sulphoraphane	400	1.9	1.6	0.15	26
Erysolin	400	4.9	11	0.31	47
Erucin	400	2.3	37	0.39	49

poorest substrate. Thus sulphoraphane is the slowest to be conjugated of all the compounds investigated, in spite of the fact that its non-enzymic reaction rate with GSH is among the higher velocities determined (Table 1).

In some cases, such as benzyl-NCS and phenethyl-NCS, a high enzyme activity is accompanied by a high non-enzymic reaction rate. In order to account for this expression of inherent reactivity of the isothiocyanates, rate-enhancement factors were calculated, i.e. the ratios of the $k_{cat.}/K_m$ values to the non-enzymic second-order rate constants (Table 1). It is noteworthy that the rate-enhancement factors for each of the enzymes vary greatly with the substrate used. The ratio between the lowest and the highest rate enhancement amounts to only 12 and 13 times for GST A1–1 and GST P1–1 respectively, 35 times for GST M4–4 and, notably, 170 times for the most active enzyme, GST M1–1.

Reverse reaction

For kinetic studies of the catalytic mechanism of GSTs, it is noteworthy that the enzyme reactions between GSH and organic isothiocyanates are readily reversible. We have recently determined kinetic parameters for the reverse reaction using dithiocarbamates derived from some of the isothiocyanates studied in the present investigation [7]. The practically irreversible nature of almost all GST-catalysed reactions studied so far has prevented kinetic studies of the reverse reaction. A recent study by Meyer et al. [8] has also demonstrated the reversibility of the enzyme reaction.

Michaelis constants for isothiocyanates

Michaelis constants were obtained directly by non-linear regression analysis of the experimental data by use of the standard Michaelis–Menten equation (Table 2). In the series of homologous aliphatic substrates, which ranges from propyl-NCS to hexyl-NCS, K_m values decrease as the alkyl chain length increases. The values are approximately inversely proportional to the $k_{cat.}/K_m$ values, indicating that the $k_{cat.}$ values are essentially independent of the size of the alkyl group. As expected, propyl-NCS, being the smallest alkyl-NCS tested, displays the weakest binding, as indicated by its high K_m value. In contrast, cyclopropyl-NCS (except for GST M4–4) and allyl-NCS show relatively low K_m values, indicating high affinity. Furthermore, it is noteworthy that the K_m values for all cyclo-compounds are generally low and do not decrease markedly with increasing molecular size. The behaviour of the straight-chain aliphatic isothiocyanates is thus not mirrored by that of the corresponding cyclic substrates. With benzyl-NCS and phenethyl-NCS, a similar tendency to that with the aliphatic substrates is observed. For all enzymes except GST P1–1, the K_m values for phenethyl-NCS are lower than for benzyl-NCS. Sulphoraphane, erysolin and erucin form a series of naturally occurring substances that differ only in the oxidation state of the sulphur atom that is inserted into the carbon chain. All of them have a chain length corresponding to hexyl-NCS. It is therefore interesting that their K_m values are higher by factors of 3–30 times than that of hexyl-NCS. The thioether erucin is least affected, whereas sulphoraphane and erysolin display substantially weakened binding. Obviously, hydrophobicity of the substrate promotes binding to the hydrophobic substrate-binding site (H-site), and the polar sulphinyl and sulphonyl groups of sulphoraphane and erysolin respectively are not as readily accommodated in the active sites of any of the four enzymes. However, variations in K_m values may also reflect differences in rate-contributing steps in the catalytic mechanism in addition to differences in binding affinities, especially in cases when catalytic efficiencies vary widely.

Specific activities

On the basis of activity measurements at different isothiocyanate concentrations, it was possible to design standard assays for measurement of the specific activity with the different substrates. The principle adopted was to measure activity with the highest substrate concentration that could reproducibly and reliably be used (Table 3).

Table 4 Incremental Gibbs free-energy values for transition-state stabilization of the GST-catalysed conjugation of isothiocyanate substrates with GSH

Values were calculated according to eqn. (2) from $k_{cat.}/K_m$ values presented in Table 1 taking the value for propyl-NCS as reference for each GST.

	$\Delta\Delta G$ (kJ·mol^{-1})			
Substrate	A1–1	M1–1	M4–4	P1–1
Propyl-NCS	0.00	0.00	0.00	0.00
Butyl-NCS	−0.59	2.66	−0.21	2.29
Pentyl-NCS	0.16	4.97	1.85	4.43
Hexyl-NCS	3.09	6.94	2.46	5.93
Allyl-NCS	2.28	4.80	4.82	4.88
Cyclopropyl-NCS	2.97	5.37	2.35	4.31
Cyclohexyl-NCS	0.12	4.34	3.56	3.98
Cyclo-octyl-NCS	0.70	7.12	1.31	4.90
2-Hexyl-NCS	−0.28	4.04	2.66	2.33
Benzyl-NCS	1.33	6.41	5.54	7.64
Phenethyl-NCS	2.20	6.31	5.48	6.22
Sulphoraphane	−1.44	−4.07	−2.80	2.15
Erysolin	1.03	−0.19	−0.61	3.77
Erucin	1.41	5.36	0.67	6.26

Transition-state stabilization

The incremental Gibbs free energy of transition-state stabilization can be calculated [24]:

$$\Delta\Delta G = RT[\ln (k_{cat.}/K_m)_A - \ln(k_{cat.}/K_m)_{Ref}] \quad (2)$$

where A is a given substrate compared with a reference. Analysis of the $\Delta\Delta G$ values for the series of aliphatic isothiocyanates from propyl-NCS to hexyl-NCS shows (Table 4) that the maximal incremental transition-state stabilization contributed by a methylene group is about 2.7 kJ·mol^{-1} in the case of GSTs M1–1 and P1–1 (Figure 2). This value is in good agreement with the maximal value of 2.9 kJ·mol^{-1} per methylene group for GSTs acting on 4-hydroxyalkenals [6]. Also GSTs A1–1 and M4–4 display an increase in $\Delta\Delta G$ as the alkyl chain is extended from pentyl- to hexyl-NCS, but, remarkably, both enzymes show a decrease in $\Delta\Delta G$ as the alkyl chain is extended from propyl- to butyl-NCS. The two arylalkyl substrates examined, benzyl-NCS and phenethyl-NCS, showed relatively minor alterations in $\Delta\Delta G$ values on addition of one methylene group (Table 4). The contributions of the planar phenyl groups in both compounds obviously dominate the interactions in the transition state and place these two compounds among the most efficient substrates tested. Only GST A1–1 shows a gain in the calculated free energy as benzyl-NCS is replaced by phenethyl-NCS; GSTs M1–1 and M4–4 appear to be unaffected by this substitution, and P1–1 shows a marked loss. It can be speculated that these two substrates are close to the optimal size of a molecule fitting into the H-sites of the three last-mentioned enzymes.

Both allyl-NCS and cyclopropyl-NCS, which have the same number of carbon atoms as the reference molecule propyl-NCS, have remarkably high $\Delta\Delta G$ values. Obviously, the restricted flexibility of the cyclic compounds results in a smaller entropy loss on binding to the enzyme than that of the completely flexible n-alkyl chains. For the cycloalkyl derivatives, increasing molecular size does not in general contribute to increased stabilization of the transition state.

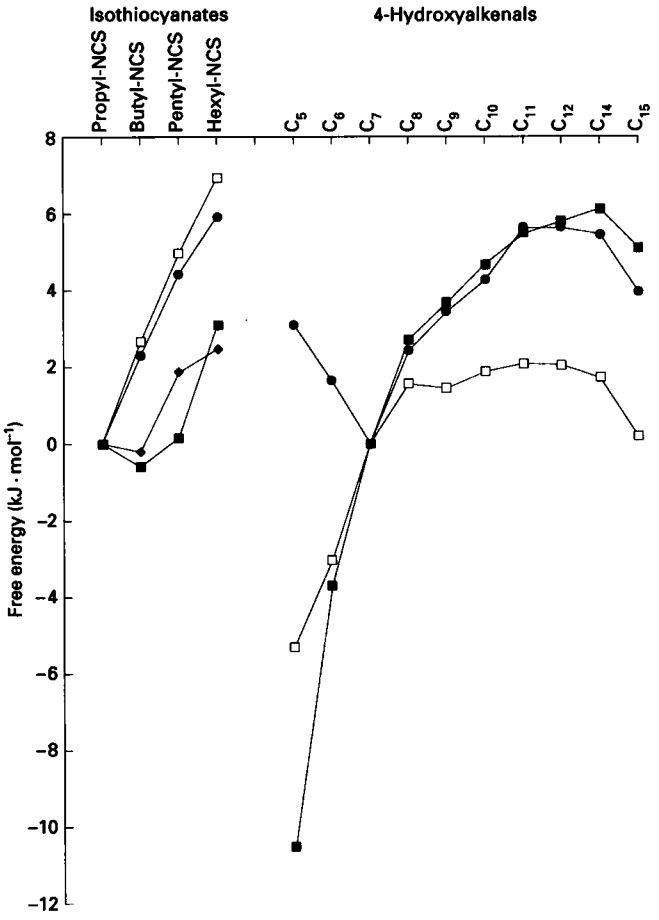

Figure 2 Relative Gibbs free energies for transition-state stabilization of isothiocyanates and 4-hydroxyalkenals binding to human GSTs

Free-energy values were calculated (eqn. 2) from $k_{cat.}/K_m$ values (Table 1) using propyl-NCS and 4-hydroxyheptanal as reference compounds for each enzyme. ■, GST A1–1; □, GST M1–1; ◆, GST M4–4; ●, GST P1–1. 4-Hydroxyalkenals are denoted by the number of carbon atoms per molecule, e.g. C_5 for 4-hydroxypentenal. Values for 4-hydroxyalkenals are from Danielson et al. [6].

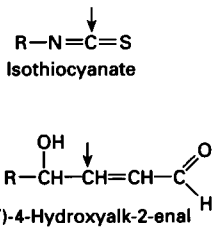

Figure 3 Structural comparison of 4-hydroxyalkenals and isothiocyanates used as GST substrates

Arrows indicate the point of nucleophilic attack by the sulphur atom of GSH.

Comparison of isothiocyanates and 4-hydroxyalkenals as substrates

The $\Delta\Delta G$ values for isothiocyanates as substrates for GSTs can be directly compared (Figure 2) with data obtained for the 4-hydroxyalkenal substrates [6]. Ignoring other structural differences, 4-hydroxyalkenals and isothiocyanates can be aligned

Table 5 Non-enzymic reaction rates, $k_{cat.}/K_m$ values and rate-enhancement factors (r.e.f.) for human GSTs with 4-hydroxyalkenals as substrates

Reactions were performed at 30 °C in 0.2 M sodium phosphate, pH 6.5, containing 2.5 mM GSH and 1% (v/v) acetonitrile. Values were calculated [6] using a molecular mass of 50 kDa for all GSTs.

		A1–1		M1–1		P1–1	
4-Hydroxyalkenals	Non-enzymic ($M^{-1}\cdot s^{-1}$)	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	$10^{-3}\times$ R.e.f.	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	$10^{-3\times}$ R.e.f.	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	$10^{-3}\times$ R.e.f.
C_5	1.8	0.4	0.22	49	27	34	19
C_6	1.1	6.0	5.4	120	110	19	17
C_7	1.7	26	15	401	240	10	5.9
C_8	1.0	76	76	740	740	26	26
C_9	0.9	111	120	708	780	39	43
C_{10}	1.5	166	110	839	560	54	36
C_{11}	1.1	227	200	915	830	93	84
C_{12}	1.2	258	220	901	750	93	77

relative to the point of nucleophilic attack by GSH (Figure 3), which is the carbon atom of the –N=C=S function and the β-carbon (C-3) in the 4-hydroxyalkenal respectively. Thus propyl-NCS corresponds to 4-hydroxyheptenal (C_7 in Figure 2) and, accordingly, the other isothiocyanates to the higher 4-hydroxyalkenal homologues. The isothiocyanate data refer to an arbitrary zero point on the ordinate, which is given by the value of propyl-NCS, and likewise, for the 4-hydroxyalkenals the value for 4-hydroxyheptenal is used as a reference. Increased aliphatic chain length of the isothiocyanate as well as the 4-hydroxyalkenal substrates generally results in an increase in binding energy. For substrates with carbon chains longer than 9 atoms, the incremental energy gain levels off, probably because of steric crowding of the H-site of the GSTs. The isothiocyanates show similar behaviour in terms of the increase in binding energy with increased aliphatic chain length (Table 4), but a clear difference can be seen with respect to the aliphatic chain length which achieves 'steric saturation' of the H-site. Hexyl-NCS is the aliphatic substrate with the highest $k_{cat.}/K_m$ value for all the GSTs investigated. Moreover, benzyl-NCS and phenethyl-NCS, which are even bulkier molecules, bind with an energy that is even higher than that of hexyl-NCS, for all enzymes except GST A1–1.

Table 5 shows non-enzymic rate constants, $k_{cat.}/K_m$ and rate-enhancement factors for the C_5–C_{12} 4-hydroxyalkenals for comparison with the corresponding values obtained with the aliphatic isothiocyanates. It was previously noted [6] that the $k_{cat.}/K_m$ values for 4-hydroxyalkenals are among the highest of all GST substrates tested, approaching 10^6 $M^{-1}\cdot s^{-1}$ for GST M1–1 (see Table 5) and exceeding 10^7 $M^{-1}\cdot s^{-1}$ for rat GST 8–8. However, for the human GSTs M1–1 and P1–1 the 4-hydroxyalkenals gave only about 3-fold higher $k_{cat.}/K_m$ values than did the most active isothiocyanates. Furthermore, in terms of rate enhancement, GST M1–1 is even more efficient with hexyl-NCS (7.9×10^5) than with the optimal 4-hydroxyalkenal (C_{11}) (4.5×10^5). By this criterion, some isothiocyanates rank among the very best substrates for certain GSTs.

Physiological relevance of isothiocyanate conjugation

It has been known for a long time that isothiocyanates are abundant in a variety of vegetables that are part of the human diet [14,15]. Although the –N=C=S function is rather reactive and therefore short-lived, a continuous supply of isothiocyanates may derive from cleavage of plant glucosinolates. Interest in isothiocyanates has recently been stimulated by the finding that these compounds offer chemoprotection against tumour formation in a variety of animal models [13,25,26].

Although only four of more than ten known GST isoenzymes have been tested in this investigation, it is clear that the ensemble of GSTs plays a very significant role in the metabolism of isothiocyanates in man. In terms of individual isoenzymes, GST M1–1 and GST P1–1 appear to be the main contributors. Organic isothiocyanates do react non-enzymically with GSH, but the rate enhancements afforded by GSTs are generally in the range 10^3–10^5-fold (Table 1). Furthermore, the intracellular concentrations of GST A1–1 and GST M1–1 may approach 0.1 mM in tissues such as the liver, and these high enzyme concentrations add to the importance of GSTs in the metabolism of isothiocyanates. Physiological pH is generally somewhat higher than that used in the standard assay (pH 6.5), and the non-enzymic reaction rates will consequently be higher than given in Table 1. However, the enzymic rates also increase with pH (results not shown), and their contribution to the conjugation of isothiocyanates will still be of the same order of magnitude.

In view of its high catalytic efficiency with isothiocyanates and its abundance in liver, GST M1–1, when present, will play a major role in the conjugation of this group of compounds. However, only approx. 50% of the human population expresses this enzyme form [27–29]. GST M1–1-deficient individuals may therefore have a markedly lower capacity for inactivating isothiocyanates than GST M1–1-positive subjects. This is worth investigating by metabolic studies in combination with phenotyping of GST M1–1 expression.

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