Structure–activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases

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The substrate specificities of 15 cytosolic glutathione transferases from rat, mouse and man have been explored by use of a homologous series of 4-hydroxyalkenals, extending from 4-hydroxypentenal to 4-hydroxypentadecenal. Rat glutathione transferase 8-8 is exceptionally active with the whole range of 4-hydroxyalkenals, from C₅ to C₁₅. Rat transferase 1-1, although more than 10-fold less efficient than transferase 8-8, is the second most active transferase with the longest chain length substrates. Other enzyme forms showing high activities with these substrates are rat transferase 4-4 and human transferase μ . The specificity constants, k_{cat}/K_m , for the various enzymes have been determined with the 4-hydroxyalkenals. From these constants the incremental Gibbs free energy of binding to the enzyme has been calculated for the homologous substrates. The enzymes responded differently to changes in the length of the hydrocarbon side chain and could be divided into three groups. All glutathione transferases displayed increased binding energy in response to increased hydrophobicity of the substrate. For some of the enzymes, steric limitations of the active site appear to counteract the increase in binding strength afforded by increased chain length of the substrate. Comparison of the activities with 4-hydroxyalkenals and other activated alkenes provides information about the active-site properties of certain glutathione transferases. The results show that the ensemble of glutathione transferases in a given species may serve an important physiological role in the conjugation of the whole range of 4-hydroxyalkenals. In view of its high catalytic efficiency with all the homologues, rat glutathione transferase 8-8 appears to have evolved specifically to serve in the detoxication of these reactive compounds of oxidative metabolism.

INTRODUCTION

Multiple forms of glutathione transferase have been isolated from a variety of sources (for a review, see Mannervik, 1985). The enzymes have been characterized with respect to molecular and enzymatic properties. These include apparent molecular mass, isoelectric point, immunological reactivity, substrate specificities and inhibition characteristics. In addition, amino acid sequence information has given a solid structural basis for the three classes of cytosolic glutathione transferases recently described (Mannervik *et al.*, 1985). Each class includes enzymes of similar characteristics, isolated from different sources. Within a class the characteristic properties, such as the activities with different substrates and the effect of different inhibitors, vary within a certain range. This indicates that although there are overall similarities in structure and function of the enzymes within a class, there are, nevertheless, significant differences in their active-site structures.

4-Hydroxy-2,3-*trans*-alk-2-enals are an important group of toxic compounds recently found to be substrates for glutathione transferase (Ålin *et al.*, 1985a). A range of 4-hydroxyalkenals occur in the cell as a result of oxidative metabolism of endogenous as well as foreign compounds. It has been shown that relatively large amounts of 4-hydroxynonenal are produced during stimulated lipid peroxidation by rat liver microsomes (Benedetti *et al.*, 1980; Esterbauer *et al.*, 1982) and

isolated hepatocytes (Poli *et al.*, 1985). The precursors for this aldehyde are most likely phospholipid-bound (ω -6)-polyunsaturated fatty acids, such as arachidonic acid and linoleic acid (Esterbauer *et al.*, 1986). Smaller amounts of 4-hydroxyhexenal, 4-hydroxyundecenal and 4,5-dihydroxydecenal have also been detected (for a review, see Esterbauer, 1985). 4-Hydroxynonenal was a major cytotoxic compound linked to the toxic-oil syndrome in Spain 1981/82 (Turner *et al.*, 1985). Further, the toxic pyrrolizidine alkaloid, senecionine, is converted into 4-hydroxyhexenal in the liver (Segall *et al.*, 1985), and this metabolite plays a major role in the hepatotoxicity of the alkaloid. The importance of glutathione conjugation of 4-hydroxyalkenals *in vivo* is further indicated by experiments involving perfused rat heart and the finding that the glutathione conjugate is efficiently exported by a carrier (Ishikawa *et al.*, 1986). 4-Hydroxyalkenals are electrophilic molecules that readily react with thiol groups. Since these compounds are amphipathic, they can occur in membranes as well as in cytoplasm. Many targets in the cell have been shown to be affected and extensive cell damage can be envisioned, unless the 4-hydroxyalkenals are efficiently inactivated. Their strong mutagenic and cytotoxic effects are of particular interest (for a review, see Esterbauer, 1985).

The efficiency of glutathione transferases in the conjugation of glutathione with 4-hydroxyalkenals (Ålin *et al.*, 1985a) is dependent on the homologue used. In an early study by Chasseaud (1967), activity with the

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one.

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analogous *trans*-alk-2-enals was shown to be dependent on the chain length. A preparation of glutathione transferase, containing an unresolved mixture of iso-enzymes, showed increased reaction velocity when the chain length of the substrate molecule was increased from butenal to nonenal. A further extension of the chain led to a diminished catalytic rate.

In the present investigation, the activity of different mammalian glutathione transferases with a homologous series of 4-hydroxyalkenals was measured in order to estimate the relative importance of the various enzyme forms in detoxication and to probe the properties of their active sites.

MATERIALS AND METHODS

Chemicals and enzymes

4-Hydroxyalkenals with chain lengths from five to 12 carbon atoms were prepared as described (Esterbauer & Weger, 1967; Esterbauer, 1971), and stored as chloroform solutions at -20°C . Essentially the same procedure was used for the synthesis of the homologous compounds with 14 and 15 carbon atoms, except that in this case the starting aldehydes were undecanal (for C_{14}) and dodecanal (for C_{15}). The resulting 4-hydroxyalkenals were solid substances. They were purified by re-precipitation from cold (-20°C) petroleum ether. 4-Hydroxytetradecenal has a melting point of 52°C and a solubility in water (25°C) of 5.4 mg/l . The melting point of 4-hydroxypentadecenal is 55°C and the solubility in water is 1.8 mg/l . The chloroform solutions of the aldehydes (C_5 to C_{12}) were evaporated under N_2 gas and the residue was dissolved in acetonitrile. The solid preparations of 4-hydroxytetradecenal and 4-hydroxypentadecenal were dissolved directly in the same solvent. The concentrations of the resulting solutions were determined spectrophotometrically at 224 nm by assuming an extinction coefficient of $13.75\text{ mm}^{-1}\cdot\text{cm}^{-1}$. The small deviations in the extinction coefficients for different analogues (Esterbauer & Weger, 1967) were ignored. Other chemicals were standard commercial products.

Glutathione transferases 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 8-8 were purified from rat liver (Alin *et al.*, 1985b; Jensson *et al.*, 1986), 6-6 from rat testis (Guthenberg *et al.*, 1983), 7-7 from rat intestine (Mannervik *et al.*, 1986), MI, MII and MIII from mouse liver (Warholm *et al.*, 1986), ϵ and μ from human liver (Warholm *et al.*, 1985) and π from human placenta (Mannervik & Guthenberg, 1981) according to standard procedures. In the recent nomenclature for mouse transferases (Warholm *et al.*, 1986), the present forms correspond to N 1-1 (MIII), N 3-3 (MII) and N 4-4 (MI). The form referred to as ' ϵ ' was the most basic of the major isoenzymes in the human liver (Kamisaka *et al.*, 1975). This enzyme was characterized as being a homodimer of the class Alpha type, and is strongly inhibited by haematin. It is presumably identical to transferase B_1B_1 (Hussey *et al.*, 1986).

Methods

Initial rates were measured spectrophotometrically at 224 nm under such conditions (first-order) that the rates were proportional to 4-hydroxyalkenal concentration (Alin *et al.*, 1985a). The initial portions of the progress curves did not deviate significantly from linearity. The assay system (at 30°C) contained $10\text{ }\mu\text{M}$ 4-hydroxyalkenal and 2.5 mM glutathione in 0.2 M sodium phosphate

buffer, pH 6.5 and a maximum of 1% (v/v) acetonitrile. The activity was expressed as the specificity constant, $k_{\text{cat.}}/K_m$ (cf. Danielson & Mannervik, 1985). The specific activity for each enzyme preparation with 1-chloro-2,4-dinitrobenzene (CDNB) was measured in the standard assay system (Habig & Jakoby, 1981).

The computer program SIMCA 3B (Sepanova AB, Sweden) was used for the multivariate analysis (Wold *et al.*, 1983).

RESULTS

Activity measurements

Specificity constants, $k_{\text{cat.}}/K_m$, were determined as a measure of catalytic efficiency for glutathione transferase from rat, mouse and man. A homologous series of 4-hydroxyalkenals, ranging from 4-hydroxypentenal to 4-hydroxydecapentenal, were tested as substrates. The $k_{\text{cat.}}/K_m$ values were estimated from initial rates at low substrate concentrations; the $k_{\text{cat.}}$ and K_m values cannot be accurately determined individually, because of limited substrate solubility and because of the deviations from Michaelis-Menten kinetics (Mannervik, 1985). The apparent K_m value estimated for rat transferase 4-4 with 4-hydroxynonenal as substrate is in the range of $30\text{--}60\text{ }\mu\text{M}$; a value of $50\text{ }\mu\text{M}$ has been reported for the same isoenzyme (Ishikawa *et al.*, 1986). Table 1 presents the $k_{\text{cat.}}/K_m$ values obtained and their standard deviations, based on a minimum of five replicate measurements. Both the relative activities with different substrates and the absolute values of the activities serve to characterize the various enzymes. The relative activities illustrate the general trend of increased activity with extended chain length of the substrate. However, with most enzymes, a decrease in activity was noticed for the longest compounds, probably resulting from steric hindrance. A comparison of the absolute activities of different enzyme forms reveals large differences in the maximal activity in the series of homologues when measured under first-order conditions. These values are not correlated with their specific activities with CDNB (cf. the legend to Table 1 or Table 2).

Binding energies

The change in binding energy between a glutathione transferase and 4-hydroxyalkenals with the extension of the carbon chain can be calculated from the specificity constants for two substrates by using eqn. 1 (Fersht, 1985).

$$\Delta\Delta G = RT \ln \frac{k_{\text{cat.}}/K_{m(\text{R-S})}}{k_{\text{cat.}}/K_{m(\text{H-S})}} \quad (1)$$

The incremental Gibbs free energy represents the difference in binding energy for two homologous substrates and an enzyme. The index H-S represents the reference compound and R-S denotes the compound with the substituent group R.

Fig. 1 shows the incremental Gibbs free energy of binding of the substrate to the enzyme. Positive values represent increased affinity in comparison with the reference compound 4-hydroxynonenal. This substrate gives an intermediate activity with all enzyme forms and normalization to this 4-hydroxyalkenal facilitates comparisons between different enzymes.

In order to establish the type of binding interactions, the hydrophobic nature of the substrates was considered.

Table 1. Specificity constants ($k_{\text{cat.}}/K_m$) for glutathione transferases with a series of 4-hydroxyalkenals

4-Hydroxyalkenals with a chain length of five to 15 carbon atoms were used as substrates for glutathione transferases from rat, mouse and man. Values for $k_{\text{cat.}}/K_m$ ($\text{mm}^{-1}\cdot\text{s}^{-1}$) are presented with their S.D. values, based on a minimum of five replicates. No standard deviations are reported when less than five replicates were measured. The specific activities with CDNB ($\mu\text{mol}/\text{min}\cdot\text{mg}$) for the enzyme preparations used were for glutathione transferases 1-1, 65; 1-2, 29; 2-2, 8; 3-3, 57; 3-4, 36 (calculated from corresponding homodimers); 4-4, 15; 6-6, 190; 7-7, 9; 8-8, 20; MI, 19; MII, 138; MIII, 316; μ , 64; π , 275; ϵ , 91.

Transferase	4-Hydroxyalkenal chain length...	Specificity constants ($\text{mm}^{-1}\cdot\text{s}^{-1}$)												
		5	6	7	8	9	10	11	12	14	15			
Rat	1-1	1±2	12±9	7±6	43±17	120±22	256±51	274±55	602±85	1090±85	1040±401			
	1-2	1±1	6±7	2±2	24±8	30±11	82±27	79±18	203±22	422±59	290±47			
	2-2	<0.1	3±4	4±4	0.1±0.3	18±6	25±3	49±6	63±16	153±15	158±19			
	3-3	124±16	25±4	22±6	48±7	94±12	162±12	168±11	184±12	210±17	154±25			
	3-4	32±6	24±4	43±5	89±8	104±8	123±14	132±8	133±14	128±8	89±14			
	4-4	16±7	26±5	105±16	195±18	258±12	275±27	221±13	187±11	178±3	100±17			
	6-6	<0.1	1±2	0.2±0.5	19±5	28±7	75±11	91±1	96±17	245±27	207			
	7-7	8±2	10±7	43	6±1	23±4	17±14	32±9	29±5	73±39	82±50			
	8-8	1400±280	560±40	3040±740	5120±370	7850±720	8760±1730	16100±2630	15600±2330	16800±1100	14600±1930			
	Mouse	MI	1±2	3±4	13±9	31±8	36±11	46±21	44±3	67±11	94±18	96±8		
Human	II	<0.1	4±8	23±14	34±21	36±32	99±42	112±8	147±45	199±28	154±26			
	MIII	33±43	33±31	12±22	38±33	93±33	108±54	178±25	151±96	239±96	209±40			
	π	34±15	19±3	10±3	26±2	39±4	54±7	93±5	93±24	87±8	48±11			
	μ	49±14	120±6	401±50	740±13	708±58	839±100	915±125	901±61	786±110	430±79			
	ϵ	0.4±0.9	6±5	26±3	76±11	111±21	166±21	227±15	258±20	295±19	197±25

Fig. 2. Pattern recognition analysis of incremental Gibbs free energy data of 4-hydroxyalkenals binding to different glutathione transferases

The data presented in Fig. 1 were analysed and the positions of the various enzymes were plotted in the plane of the first two principal components, P(1) and P(2). The shape of the symbols represent the three structural classes Alpha (□), Mu (△) and Pi (○), (Mannervik *et al.*, 1985).

In general, the n-octanol/water partition coefficients of a series of compounds containing homologous alkyl groups show that the incremental Gibbs energy of transfer between the phases is virtually constant for straight chains (Hansch & Coats, 1970). Corresponding values for the incremental Gibbs free energy were calculated for the series (C_5 – C_{10}) of 4-hydroxyalkenals by using their solubility in water (Esterbauer & Weger, 1967; Esterbauer, 1971; the solubilities of the C_{14} and C_{15} homologues are given in the Materials and methods section). The average Gibbs free energy contribution for a $-CH_2-$ group of the homologues in the series of 4-hydroxyalkenals was not significantly different from the standard value of 2.9 kJ/mol at 30 °C (Fersht, 1985), which is represented by the dashed line in Fig. 1. A section of each graph is almost parallel to the dashed line. The hydrophobicity of the active site of the glutathione transferases therefore appears to be similar to that of n-octanol.

In Fig. 1 the isoenzymes are presented in three groups (panels a, b and c) on the basis of their responses in

Fig. 1. Incremental Gibbs free energy for the binding of 4-hydroxyalkenals to glutathione transferases as a function of chain length

Values are calculated from eqn. 1 with 4-hydroxynonanal as the reference compound. The values for different enzymes forms are separated into panels (a), (b) and (c) as described in the text. The shape of the symbols represent enzymes from different biological species: rat (○), mouse (□) and human (△). Key to the individual glutathione transferases: ●, 1-1; ⊗, 2-2; ○, 3-3; ○, 4-4; ○, 6-6; ●, 7-7; ●, 8-8; □, MI; □, MII; □, MIII; △, π; △, μ; △, ε. (The symbols for 1-1 and 2-2 overlap for C_{14} and C_{15} .)

Table 2. Specific activities of glutathione transferases with CDNB and substrates related to 4-hydroxyalkenals

Data compiled from the present study (CDNB) and from Ålin *et al.* (1985a,b); C. Andersson & B. Mannervik (unpublished work); Guthenberg *et al.* (1985); Jensson *et al.* (1986); H. Jensson & B. Mannervik (unpublished work); Mannervik *et al.* (1984a,b); Meyer *et al.* (1985); Söderström *et al.* (1985); Warholm *et al.* (1983, 1986). Abbreviations: AraOOH, arachidonic acid hydroperoxide; EA, ethacrynic acid; HNE, 4-hydroxynonenal; LTA₄Me, leukotriene A₄ methyl ester.

Transferase	Substrate ...	Specific activities (μmol/min · mg)					
		CDNB	tPBO	EA	LTA ₄ Me × 10 ⁻³	AraOOH	HNE
1-1		65	< 0.004	0.08	7.6	2.6	2.6
2-2		8	< 0.004	1.24	0.83	1.7	0.67
3-3		57	0.05	0.08	2.5	0.2	2.7
4-4		15	1.18	0.62	102	0.2	6.9
7-7		9	0.22	3.8	—	—	—
8-8		20	0.1	7	8	—	170
MII		138	0.013	1.4	—	—	2.6
μ		275	0.36	0.081	210	—	—

activity to increases in the carbon chain length of the substrate. These characteristics can be identified in the shape of the curve, especially in the region of the lower homologues in the series. In one group (Fig. 1a), the binding energy decreases for the first two homologues succeeding 4-hydroxypentenal, and a clear minimum in the curve is observed. In the second group (Fig. 1b), the binding energy of the shortest homologues are clearly lower than that for the reference compound, 4-hydroxynonenal, whereas the higher homologues do not give markedly higher binding energies (i.e. $k_{\text{cat.}}/K_m$ values) than the latter compound. In the third group (Fig. 1c), the binding energy increases consistently over the whole range of homologues tested. In this group the most prominent feature is the large difference between 4-hydroxypentenal and 4-hydroxyhexenal representing a $\Delta\Delta G$ significantly > 2.9 kJ/mol (cf. the dashed line); only transferases 1-1 and ϵ display any measurable activity with the lowest homologue, 4-hydroxypentenal (Table 1). However, the other enzymes (transferases 2-2, 6-6 and MII) gave reasonable activities with the highest homologues.

The grouping of the enzymes on the basis of the $\Delta\Delta G$ values was supported by multivariate analysis. Enzymes mapping close to one another display similar responses in binding energy to variations of the substrate chain length: the left half of Fig. 2 includes the enzymes in Fig. 1(c), the upper right quarter includes those in Fig. 1(b), and the lower right quarter includes those in Fig. 1(a).

DISCUSSION

Catalytic efficiency

According to transition state theory, an efficient substrate has a structure which resembles that of the activated complex of the transition state (Fersht, 1985). The catalytic efficiency can be described by the specificity constant, $k_{\text{cat.}}/K_m$, which is the rate constant for the reaction between free enzyme and free substrate, including the activation and binding energies. The most efficient enzymes have specificity constants of the order of $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. 4-Hydroxyalkenals have been shown to be

very good substrates for several glutathione transferases (Ålin *et al.*, 1985a; Jensson *et al.*, 1986). The results presented here show that transferase 8-8 has specificity constants of approx. $0.15 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the highest homologues (Table 1). Transferase 8-8 is between one and two orders of magnitude better than most other enzyme forms with the whole range of 4-hydroxyalkenals (Table 1).

Substrate specificity

The active site of a subunit of glutathione transferase has been proposed to be comprised of two subsites (Mannervik *et al.*, 1978), one for glutathione and one for the electrophilic second substrate. Certain glutathione analogues also serve as substrates (Sugimoto *et al.*, 1985). On the other hand, the structure and nature of the electrophilic substrate can be varied to a great extent. Inhibition studies with a series of *S*-substituted glutathione derivatives indicated that the site for the electrophilic substrate adjacent to the glutathione-binding site is hydrophobic (Askelöf *et al.*, 1975). The results of the present investigation support this general conclusion. However, the hydrophobicity of the active site for most of the glutathione transferases appears to be weak in comparison to many other enzymes, which have been shown to have hydrophobic binding energies that are five times higher than those estimated by partition of hydrocarbons in *n*-octanol and water (cf. Fersht, 1985).

In addition to the hydrophobicity, two major structural factors influence the $k_{\text{cat.}}/K_m$ values. The chemical nature of the reactive group of a substrate is one determinant, which governs the maximal activity obtainable in a reaction. The second determinant concerns the overall structure of the substrates with the same reactive group. Comparison of the relative activities obtained with substrates of homologous structure may give detailed information about the active site topography. The highest activity is obtained when both the group involved in the chemical reaction and the rest of the substrate molecule acquire optimal fit to the enzyme in the transition state.

The catalytic activity of a selection of alternative

substrates were examined in order to distinguish the importance of the various structural features that determine the efficiency of 4-hydroxyalkenals as substrates of glutathione transferases. Specificity constants give information about the efficiency of the catalysis that can be used directly for comparison of substrates with different structures and properties. However, these constants are generally not available for the substrates of the glutathione transferases. The following discussion of substrates related to the 4-hydroxyalkenals is therefore primarily based on available specific activities (Table 2). Since the assay conditions are usually not such that the specific activities correspond to maximal velocity (V) or first-order conditions (V/K_m), the discussion is limited to semiquantitative comparisons.

Substrates with functional groups similar to the reactive group of 4-hydroxyalkenals (1) include *trans*-4-phenyl-3-buten-2-one (tPBO) (2) and ethacrynic acid (3).

The reactive group of these compounds is essentially the double bond to which the thiol group of glutathione adds. (The arrow indicates the position of nucleophilic attack by glutathione.) The flanking aldehyde and hydroxyl groups of 4-hydroxyalkenals and the carbonyl group of tPBO enhance the electrophilicity of the alkenes. Enhanced electrophilicity has previously been found to increase the efficiency of the nucleophilic attack by glutathione (cf. Keen *et al.*, 1976).

Substrates with a hydrocarbon skeleton which resembles that of the 4-hydroxyalkenals include leukotriene A_4 derivatives (4) and fatty acid hydroperoxides. These compounds are distinguished from the alkenes by having epoxy and hydroperoxy functional groups.

The active site of transferase 1-1 is clearly one of the most hydrophobic, as indicated by its activity with the series of 4-hydroxyalkenals (Fig. 1c). The binding site for the electrophilic substrate appears to be long but narrow, as judged by the low activities with bulky substrates (e.g. tPBO, ethacrynic acid and leukotriene A_4 methyl ester) in comparison to the significantly higher activities with elongated straight-chain structures (e.g. 4-hydroxyalkenals and arachidonic acid hydroperoxides) (Table 2). This enzyme form does not appear to have a strong preference for a certain type of functional group. Another activity of importance for this enzyme is the Δ^5 - Δ^4

isomerization of androstene-3,17-dione (Ålin *et al.*, 1985b), in which transferase 1-1 is the most efficient of the enzymes tested so far. It also has high affinity for hydrophobic non-substrate ligands, e.g. bilirubin and haematin (cf. Sugiyama *et al.*, 1984).

Transferases 4-4, 8-8 and μ all have a high, or even an exceptionally high, activity with substrates possessing a double bond conjugated to a carbonyl group. The hydrophobic character, or the shape of the molecule is not of critical importance for these enzymes. Nevertheless, the activity of transferase 8-8 with leukotriene A_4 methyl ester and tPBO are moderate (Table 2), which suggests steric hindrance in the binding of bulky substrates to the active site of this enzyme form. Transferases 4-4 and μ show significant activities with bulky substrates (e.g. tPBO, ethacrynic acid and leukotriene A_4 methyl ester). Fatty acid hydroperoxides have been shown to be poor substrates for transferase 4-4. The specific activity with arachidonic acid hydroperoxide for transferase 4-4 is $0.2 \mu\text{mol}/\text{min} \cdot \text{mg}$ and that for transferase 1-1 is $2.6 \mu\text{mol}/\text{min} \cdot \text{mg}$ (Meyer *et al.*, 1985). Similar values are obtained for the reaction with linoleic acid hydroperoxide. The activity of transferase 4-4 is low with arachidonic acid hydroperoxide whereas that with leukotriene A_4 is high. Since the activity with other hydroperoxides (e.g. cumene hydroperoxide) is also low, the difference may be attributed to the chemical nature of the electrophilic group of the substrate rather than to a steric effect. The exceptionally high activity of transferase 8-8 with the whole range of 4-hydroxyalkenals (Table 1) indicates that the active site of this enzyme is particularly well suited to catalyse glutathione conjugation of this reactive alkene group conjugated with an aldehyde group.

A striking finding is the strong apparent similarity between the active-site properties of transferases 3-3, MIII and π . The common denominator is the high activity with the short-chain 4-hydroxypentenal and the decreased activity for the two higher analogues. Since the activity increases again with longer chains it is not clear what causes this effect. The active sites for MIII and 3-3 are expected to be similar since they are the only two enzyme forms which show significant activity with bromosulphthalein. The activity with 1,2-dichloro-4-nitrobenzene is also high with both of these enzyme forms (Ålin *et al.*, 1985b; Warholm *et al.*, 1986).

Physiological aspects

The high values of the specificity constants for glutathione transferase with 4-hydroxyalkenals indicate that they play an important physiological role in the detoxication of these, or similar, reactive compounds. The relative importance of the different enzyme forms may be estimated from the activities and the amounts of enzymes present in the cell.

The high activity of rat transferase 4-4 with 4-hydroxyalkenals has previously been noted (Ålin *et al.*, 1985a; Ishikawa *et al.*, 1986). This enzyme is present in 4- to 5-fold molar excess of that of transferase 8-8 in rat liver (Jensson *et al.*, 1986), but the specificity constant for transferase 8-8 with 4-hydroxyalkenals is between 10- and 100-fold higher than that for transferase 4-4, depending on the chain length. The activity of transferase 8-8 is therefore expected to be of major importance *in vivo*, despite the relatively smaller amounts present. The high activity of transferase 4-4 with intermediate chain length

4-hydroxyalkenals (e.g. 4-hydroxynonenal), suggests that this enzyme form has an especially important role for these compounds.

Of the enzymes tested from human tissues, only transferase μ shows high activities with 4-hydroxyalkenals. Transferase ϵ can be of great significance only for the higher homologues. None of the mouse glutathione transferases tested so far shows high activities with 4-hydroxyalkenals.

Classification and evolutionary aspects

The activities of glutathione transferase with 4-hydroxyalkenals do not correlate with the three structural classes Alpha, Mu and Pi (Mannervik *et al.*, 1985). However, functional similarities in the active-site region are indicated by multivariate analysis of the incremental Gibbs free energies, calculated from the activities with the homologous series of compounds used in this study. In this characterization there are no distinct groups but a gradual differentiation of the enzymes. It is based on the properties and the overall structure of the active site, in particular its hydrophobicity and steric limitations. The three mammalian species investigated have at least one enzyme form in each of the three groups.

In an evolutionary perspective, obtaining a high efficiency of catalysis and a broad substrate specificity are two essential factors in the development of active-site structure and properties of these enzymes. The first of these is ensured by high k_{cat}/K_m values, and the second by having several different forms of glutathione transferase with different types of active sites.

We thank Ms. Kerstin Larson for skilful drawing of the Figures. This work was supported by grants from the Swedish Natural Science Research Council (to B.M.), the Association for International Cancer Research, U.K. and by the Fonds zur Förderung der Wissenschaftlichen Forschung, project no. P6176B, Austria (to H.E.).

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