4-Hydroxyalk-2-enals are substrates for glutathione transferase

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The 4-hydroxyalk-2-enals are established products of lipid peroxidation that are conjugated with intracellular glutathione. Cytosolic glutathione transferases from rat liver were shown to give high specific activities with 4-hydroxynonenal and 4-hydroxydecenal. The isoenzyme giving the highest specific activity was glutathione transferase 4-4. The rate of the spontaneous conjugation reaction is negligible in comparison with the rate calculated for the cellular concentration of the glutathione transferases. It is proposed that a major biological function of the glutathione transferases is to protect the cell against products of oxidative metabolism, such as epoxides, organic hydroperoxides, and 4-hydroxyalkenals.

4-Hydroxyalk-2-enal *Glutathione* *Glutathione transferase* *Lipid peroxidation*

1. INTRODUCTION

The biological function of the glutathione transferases is far from established [1]. Various electrophilic xenobiotics, such as aromatic halogeno and nitro compounds, have been used as substrates for the enzyme, but substrates of possible intracellular origin have been largely neglected. Recent work emphasizing the importance of toxic aldehyde products of lipid peroxidation (cf. [2]) suggested to us the possibility that 4-hydroxyalk-2-enals and other activated alkenes may be 'natural' substrates for glutathione transferases. It had previously been established [3] that glutathione spontaneously reacts with 4-hydroxyalkenals (fig.1). This paper demonstrates that 4-hydroxyalkenals are good substrates for glutathione transferases purified from rat liver. The different isoenzymes have recently been renamed [4].

2. EXPERIMENTAL

2.1. *Materials*

Isoenzymes of glutathione transferase were isolated from rat liver by use of affinity chromatography and chromatofocusing [5,6]. The purification procedure described in [7] was modified by using a Mono P column and an FPLC chromatograph (Pharmacia) for the chromatofocusing. The 6 major isoenzymes, previously referred to as glutathione transferases L_2, BL, B_2, A_2, AC, and C_2 [6], now named glutathione transferases 1-1, 1-2, 2-2, 3-3, 3-4, and 4-4 [4], were isolated. All forms except transferase 2-2 were homogeneous after the chromatofocusing as judged by dodecyl sulfate-polyacrylamide slab gel electrophoresis. The latter enzyme was further purified to homogeneity by chromatography on CM-Sepharose.

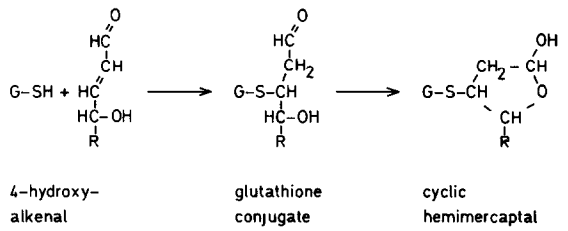

Fig.1. Reaction between glutathione (GSH) and 4-hydroxyalkenals. The primary product is further transformed to a cyclic hemiacetal derivative [3].

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2.2. *Assay of glutathione transferase activity*

The standard assay used during purification and for comparison of isoenzymes was based on the reaction between 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione in a 1 ml reaction system at 30°C containing 0.1 M sodium phosphate (pH 6.5). The activity was measured spectrophotometrically at 340 nm (ϵ = 9600 $M^{-1} \cdot cm^{-1}$) [8]. The conjugation of the 4-hydroxyalkenals with glutathione was monitored spectrophotometrically at 224 nm (ϵ = 13750 $M^{-1} \cdot cm^{-1}$). In the standard assay 0.1 mM 4-hydroxynon-2-enal (or 4-hydroxydec-2-enal) and 0.5 mM glutathione were used in the above reaction system at 30°C. Owing to the high background absorbance at 224 nm (>2 absorbance units) it was necessary to use a spectrophotometer giving a linear response at high absorbance values. The instrument used in this work, a Varian model 2290 spectrophotometer, was linear up to 4 absorbance units at 224 nm; the absorbance normally did not exceed 3 during the measurements. The stock solutions of 4-hydroxyalkenals were kept in $CHCl_3$. Before use, the solvent was removed on a rotary evaporator (at 20–28°C) and the residue dissolved to 5 mM in water. The aqueous solution is stable for approx. 1 week at 4°C according to Dr H. Esterbauer (personal communication).

3. RESULTS

All isoenzymes of glutathione transferase tested were active with 4-hydroxynonenal and 4-hydroxydecenal as substrates. Table 1 shows the results of experiments with 6 major rat isoenzymes. Other isoenzymes from rat tissues as well as mouse liver also gave significant activity with these compounds (to be published). The specific activities given in table 1 refer to standard assay conditions at pH 6.5 and 30°C. It should be noted that the values for the 4-hydroxyalkenals range from 4 to 40% of the corresponding specific activities with 1-chloro-2,4-dinitrobenzene. The latter substrate gives the highest specific activity of the substrates investigated with any of the isoenzymes used here.

The initial velocity of the enzymatic reaction measured with a 4-hydroxyalkenal as electrophilic substrate was proportional to the enzyme concentration. However, the initial phase of the progress curve was not linear, and the deviation from zero

Table 1

Specific activities and k_{cat}/K_m values for 6 major rat glutathione transferases with 4-hydroxyalk-2-enals and 1-chloro-2,4-dinitrobenzene at pH 6.5 and 30°C

Isoenzyme[a]	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)			k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)[b]	
	4-Hydroxy-nonenal	4-Hydroxy-decenal	1-Chloro-2,4-dinitrobenzene[c]	4-Hydroxy-nonenal	1-Chloro-2,4-dinitrobenzene[c]
1-1	2.6	3.5	50	0.14	0.56
1-2	1.6	2.8	25	0.07	0.31
2-2	0.67	0.97	17	0.01	0.05
3-3	2.7	2.7	58	0.11	2.5
3-4	2.9	3.6	45	0.18	1.6
4-4	6.9	7.1	17	0.28	0.19

[a] Rat isoenzymes named according to [4]

[b] The k_{cat}/K_m values were determined at 10 μM electrophilic substrate at which concentration first-order kinetics apply. Under the assumption of Michaelis-Menten kinetics, the initial rate divided by the product of the total enzyme and substrate concentrations equals k_{cat}/K_m. This value was determined at saturating concentrations of glutathione; 2.5 mM glutathione with 4-hydroxynonenal, and 5 mM with 1-chloro-2,4-dinitrobenzene

[c] Values determined by P. Ålin, H. Jensson, C. Guthenberg, U.H. Danielson, M.K. Tahir and B. Mannervik (submitted)

order kinetics was particularly strong at high enzyme concentrations. Since *S*-alkyl derivatives of glutathione are good inhibitors of glutathione transferase [9,10] it was considered possible that the product of the reaction caused progressive inhibition as it was being formed. The product was prepared in larger amounts by reacting 4-hydroxynonenal with a stoichiometric amount of glutathione. The product at 5 μM was shown to effect 50% inhibition of rat glutathione transferase 4-4 when the enzymatic activity was measured with 1-chloro-2,4-dinitrobenzene as electrophilic substrate. The effect of product inhibition thus demonstrated was minimized by using low concentrations of enzyme in the assay system, thereby limiting the formation of the inhibitory product.

The effect of pH on the enzymatic reaction with 4-hydroxynonenal was investigated. The non-enzymatic reaction is strongly pH-dependent [3] and the enzymatic conjugation increased approx. 2-fold when the pH value changed from 5 to 8. A pH value of 6.5 was selected for the standard assay system in order to obtain a favourable ratio between the rates of the enzymatic and the spontaneous reactions.

In comparing the individual isoenzymes of rat glutathione transferase, it was found that transferase 4-4 gave the highest specific activity with 4-hydroxynonenal as well as with 4-hydroxydecenal (table 1). The values for the different enzyme forms fall within a comparatively narrow range. Somewhat more marked differences were obtained in the comparison of the k_{cat}/K_m values for the isoenzymes. The specific activities appear to reflect the catalytic properties of their constituent subunits, as previously found with various other substrates [6]. Thus, subunit 4 has the highest and subunit 2 the lowest specific activity with the two 4-hydroxyalkenals tested. Qualitatively similar, but more pronounced, differences between the various rat isoenzymes were previously noted in the conjugation of leukotriene A_4 methyl ester with glutathione [11].

4. DISCUSSION

Our results show that 4-hydroxynonenal and 4-hydroxydecenal are good substrates for 6 major cytosolic glutathione transferases from the rat. Even though the rate of spontaneous reaction between the 4-hydroxyalkenals and glutathione is significant, it is noteworthy that for the enzymes studied almost no other electrophilic substrate than 1-chloro-2,4-dinitrobenzene gives higher specific activities under standard assay conditions. In this comparison it should be observed that the activity with 1-chloro-2,4-dinitrobenzene is assayed at 1 mM, whereas the 4-hydroxyalkenals are used at 0.1 mM (table 1). The activities of the various isoenzymes with 0.1 mM 1-chloro-2,4-dinitrobenzene are significantly lower (2–7-fold less than at 1 mM, depending on the isoenzyme used), reducing the differences between the alternative substrates. The comparatively high enzymatic velocities with the 4-hydroxyalkenals are further attested by the comparison of the k_{cat}/K_m values for 4-hydroxynonenal with those for 1-chloro-2, 4-dinitrobenzene (table 1). Using this parameter the most active isoenzyme, transferase 4-4, was found to be more efficient with 4-hydroxynonenal.

It has been demonstrated that 4-hydroxynonenal is a major product of lipid peroxidation in rat liver microsomes [12,13]. Furthermore, addition of this compound to a suspension of isolated rat hepatocytes has been found to cause a rapid depletion of intracellular glutathione with concomitant formation of a glutathione conjugate [14]. However, the role of the hepatic glutathione transferases in this conjugation reaction has not been recognized previously.

The enzymatic reaction rate was also measured at higher pH and glutathione concentration than in the standard assay system in order to approach in vivo conditions in the hepatocyte. At pH 7.25 and with 2 mM glutathione the enzymatic activity with transferase 4-4 was approx. 50% higher than at pH 6.5 with 0.5 mM glutathione. The effect of increasing the glutathione concentration at pH 7.25 was less than 10%, demonstrating that the somewhat higher concentration in liver (approx. 10 mM, see [15]) will not change the rate markedly. Since the reaction velocity was found to be strictly proportional to enzyme concentration in vitro, it appears possible to estimate the velocity of the enzyme reaction in the cell by use of the average enzyme concentration in the liver tissue. The abundant rat transferases used in this study each occur at approx. 0.02 mM. At 0.2 μM glutathione transferase 4-4 in the assay system containing 25 μM 4-hydroxynonenal and 2 mM glutathione at

pH 7.25, the measured enzymatic velocity was 6.6-times the spontaneous velocity. Thus, by linear extrapolation to 0.02 mM enzyme, it appears that the enzymatic rate is more than 600-times the spontaneous rate. The corresponding ratio between enzymatic and spontaneous reaction with 100 μM 4-hydroxynonenal was 280. These concentrations of 4-hydroxynonenal are in the range considered relevant in connection with lipid peroxidation of biological membranes [12]. Even if the extrapolation from the in vitro system to possible cellular conditions is uncertain, it should be noted that the calculation involves only one of several isoenzymes and that the sum of the activities of all enzyme forms is larger by approximately an order of magnitude than the activity of a single isoenzyme. We therefore conclude that conjugation of 4-hydroxyalkenals, such as 4-hydroxynonenal and 4-hydroxydecenal, with glutathione is effected by glutathione transferases in vivo. Thus, the 4-hydroxyalkenals constitute a new important class of substrates of cellular origin for this group of enzymes. Epoxides and organic hydroperoxides are other groups of substrates for the glutathione transferases (cf. [1]). Like the 4-hydroxyalkenals, these compounds may arise intracellularly as products of oxidative processes. Consequently, a major biological function of the glutathione transferases may be to protect the cell from toxic compounds originating in oxygen metabolism.

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