The Mechanism of the Reaction between Glutathione and 1-Menaphthyl Sulphate Catalysed by a Glutathione *S*-Transferase from Rat Liver

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1. The glutathione *S*-transferase that catalyses the reaction of 1-menaphthyl (naphth-1-ylmethyl) sulphate with GSH was purified 76-fold from rat liver. 2. The properties of the purified enzyme were studied by gel filtration and isoelectric focusing. 3. The initial-velocity pattern in the absence of products and the product-inhibition pattern have been determined. These are consistent with an Ordered Bi Bi mechanism in which the GSH adds to the enzyme before 1-menaphthyl sulphate and the products are released in the order SO_4^{2-} followed by *S*-(1-menaphthyl)glutathione. 4. Dead-end-inhibition studies with *p*-aminobenzoic acid, which has been shown to be competitive with GSH and non-competitive with 1-menaphthyl sulphate, support the suggestion that an Ordered Bi Bi mechanism is operative. 5. Values were determined for some of the dissociation and Michaelis constants for the reaction of the substrates and products with the enzyme. 6. It appears that *S*-(1-menaphthyl)glutathione activates the enzyme when the concentration of GSH is saturating and that of 1-menaphthyl sulphate is low (of the order of its Michaelis constant).

Although methods for the partial purification of several glutathione *S*-transferases have been reported (see Boyland & Chasseaud, 1969) no attempt to elucidate the mechanism of this class of enzyme has been described. Indeed few attempts have been made to treat glutathione *S*-transferases kinetically as two-substrate enzymes. A glutathione *S*-transferase was purified from rat liver (Gillham, 1971) and was shown to catalyse the reaction of GSH with acid-sulphate esters of aralkyl alcohols to form SO_4^{2-} and the corresponding *S*-aralkylglutathione. In a preliminary study of the enzyme based on the assumption that the reaction sequence is random, i.e. that the enzyme is capable of forming binary complexes with either substrate, values for substrate constants were determined. This assumption has been examined in the present work by using a more highly purified enzyme preparation.

Materials and Methods

Chemicals

Sodium 1-menaphthyl (naphth-1-ylmethyl) sulphate was prepared as described by Clapp & Young (1970). *S*-(1-Menaphthyl)glutathione was prepared by the method of Hyde & Young (1968). Bacitracin, cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, albumin, γ -globulins and apoferritin were obtained from Schwarz-Mann Laboratories Inc., New York, N.Y., U.S.A. Calcium phosphate gel (3%, w/v) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals

Male barrier-maintained C.F.E. albino rats, weight 200–240g, were used throughout.

Methods

U.v. spectroscopy. The Hilger Uvispek spectrophotometer and the Unicam SP.800 spectrophotometer (fitted with a Honeywell Controls Ltd. external recorder, range -1.0 to $+10\text{mV}$) were used. In experiments with enzymes a cuvette carrier was used through which water from a constant-temperature bath was circulated.

Determination of protein. The biuret method as modified by Cleland & Slater (1953) was used, as described by Gillham (1971), for the determination of protein in the first five stages of the enzyme purification. The protein present in the final two stages of the purification was measured by the extinction at 280nm : $E_{280}^{1\text{cm}}$ 1.0/2.9mg of protein in 3ml (calibrated by comparison with biuret samples).

Determinations of molecular weight. These were carried out by gel filtration on a column ($2.5\text{cm} \times 60\text{cm}$) of Sephadex G-100 calibrated with standard protein preparations.

Isoelectric focusing. Ampholine carrier ampholytes were obtained from LKB Instruments Ltd., Croydon, Surrey, U.K. Isoelectric focusing was carried out according to the manufacturer's instructions, by using an LKB 8101 column (110ml capacity). In early studies 1% Ampholine, pH range 3–10, was used. In

later work a mixture of 1% Ampholine, pH 6–8, and 1% Ampholine, pH 7–9, was used to form the pH gradient in the column. Separations were allowed to run for 40 h, the power input being maintained as near as possible to 3 W. The column was kept at 13°C by circulating water.

At the end of the run the column contents were pumped out at the rate of 45 ml/h and fractions (2.5 ml) were collected. The pH values of the fractions together with E_{280} were determined. No attempt was made to correct the latter measurements for the presence of sucrose in the samples. The activity of the enzyme was measured in the presence of sucrose and Ampholine, as these materials had no effect on the assay.

Assay of 1-menaphthyl sulphate-dependent glutathione S-transferase activity. The assay method was detailed by Gillham (1971). All measurements were made in 50 mM-triethanolammonium chloride – NaOH buffer, pH 8.3, at 37°C.

Enzyme purification. The method of purification of the rat liver enzyme that catalyses the reaction of 1-menaphthyl sulphate with GSH has been modified and extended from the method given by Gillham (1971). The enzyme was separated from the livers of 24 rats instead of eight. As a result of this scaling-up the stage (1) extract was obtained by centrifuging the rat liver homogenate for 1 h at 45 000g. Stages (2)–(4) were carried out as described by Gillham (1971). When stage (5) was reached in this larger-scale preparation it was found that a higher recovery of activity was obtained if the protein which separated between 45 and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ was collected.

Stage (6) was an extension of the method. The precipitate obtained at stage (5) was dissolved in 25 ml of water, which was adjusted to pH 7.0 with 10 mM-NaOH. The solution was dialysed against two changes, each of 2.5 litres, of the same water. The volume of the protein solution obtained after dialysis was adjusted with water, pH 7.0, to give a final protein concentration of 9.8 mg/ml. Calcium phosphate gel (3%, w/v) was added to the diluted solution to give 0.71 g of gel/100 ml of solution. The mixture was stirred for 10 min before centrifuging for 5 min at 2500g. The gel was discarded and after protein was determined in the supernatant a further batch of gel was added to give 6 mg of gel/mg of protein remaining and the mixture was stirred for 10 min. The gel was centrifuged for 5 min at 2500g and the active protein was removed from the gel by stirring with two 50 ml batches of 50 mM- KH_2PO_4 –NaOH buffer, pH 7.6.

In stage (7) the active fractions from stage (6) were concentrated by positive-pressure dialysis to 5 ml. The concentrate was applied to a column (2.5 cm × 60 cm) of Sephadex G-200, which had been equilibrated with 50 mM- KH_2PO_4 –NaOH buffer, pH 7.6. The column was developed with the same buffer and

fractions (5.6 ml) were collected and assayed. Fractions which contained more than 30% of the activity found in the most active fraction were combined (usually tubes 47–57). The purification of the enzyme at this stage was about 76-fold. The fractions were used for kinetic studies and for isoelectric-focusing studies.

Collection and analysis of kinetic data. The studies of product-inhibition reported indicated that the enzyme-catalysed reaction of 1-menaphthyl sulphate with GSH is a sequential one and that GSH adds to the enzyme first. The reaction must therefore obey the initial-rate equation:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

described by Cleland (1963), where A represents GSH and B 1-menaphthyl sulphate.

The values of the kinetic constants K_a , K_{ia} and K_b were determined from slope and intercept replots by the method of Cleland (1970). The initial rates were usually measured at four concentrations of GSH between 1.40 and 6.00 mM for each of four concentrations of 1-menaphthyl sulphate in the range 0.07–0.60 mM. A duplicate set of assays were performed, one from the fastest to the slowest initial rate and one in the reverse direction. In this way any slight decrease in enzyme activity that might have occurred during the determinations was compensated. The velocities (expressed as $\Delta E_{280}^{1\text{cm}}/\text{min}$) were first treated graphically by the linear transformation by Lineweaver & Burk (1934) of the Michaelis–Menten equation. The reciprocals of the concentrations of GSH were plotted against the reciprocals of the velocities for each concentration of 1-menaphthyl sulphate. The slopes and intercepts on the ordinate of these lines were plotted against the reciprocals of the concentrations of 1-menaphthyl sulphate to yield secondary graphs.

When it had been established that the results were graphically linear for both primary and secondary plots the velocities for the primary plots were fitted by the method of Wilkinson (1961) to give values for V and the apparent K_m together with their associated S.E.M. values. From these data the slopes and intercepts on the ordinate, $\pm$ S.E.M., were calculated. These parameters were fitted by the same method, except that the weightings now used were $1/(\text{S.E.M.})^2$ rather than the fourth power of the velocity. This process yielded values for K_a , K_{ia} and $K_b \pm$ S.E.M.

The values for the slopes and intercepts on the ordinate of all lines used to determine the product-inhibition patterns were calculated by the method of Wilkinson (1961) modified, where necessary, as described above. The calculations were performed with the aid of a Diehl Combित्रon S programmable calculator.

Results

Purification of the enzyme

Table 1 outlines the steps in the purification of the enzyme. The specific activity of the enzyme in the extract (stage 1) is low compared with those of many enzymes and suggests the possibility that the enzyme has other more important functions in the cell.

Characteristics of the purified enzyme

Isoelectric focusing. Fig. 1 shows the focusing behaviour of the purified enzyme solution obtained at stage (7) of the preparation. The solution was concentrated to 2ml by positive-pressure dialysis and was introduced into the sucrose gradient in the column at 27% (w/v) sucrose. The preparation showed two major activity peaks which had apparent isoelectric

points at pH 6.9 and 8.1, designated enzymes X and Y respectively. Similar results were obtained if the enzyme was applied to the column without prior concentration. When a 100000g supernatant fraction prepared from 2% (w/v) NaCl homogenate of rat liver was subjected to isoelectric focusing in a gradient of pH 6–9, enzymes X and Y were both present, although the apparent pI values were slightly different, being pH 7.1 and 8.0 respectively. In addition there was relatively more of enzyme X in this last preparation. Enzymes X and Y were taken separately and refocused when it was found that a mixture of the two enzyme species was generated during each focusing run.

Gel filtration. A possible explanation of the presence of the enzyme forms X and Y is that they represent different states of aggregation of a single enzyme species. To test this hypothesis enzymes X and Y were

Table 1. Purification of the 1-menaphthyl sulphate-dependent glutathione S-transferase from rat liver

For experimental details see the text.						
Stage	Vol. (ml)	Total protein (mg)	Total activity (μ mol/min)	Recovery (%)	$10^3 \times$ Specific activity (μ mol/min per mg)	Purification
(1) 45000g Supernatant ('extract')	960	22400	39.2	100	1.7	1.0
(2) 15% (v/v) Ethanol supernatant	1070	10200	36.9	94	3.6	2.1
(3) 2.2mM-ZnSO ₄ supernatant	1000	4400	24.1	62	5.4	3.2
(4) 15mM-ZnSO ₄ precipitate	560	2500	21.0	54	8.4	5.9
(5) (NH ₄) ₂ SO ₄ precipitate (45–60% saturation)	52	910	14.2	36	15.6	9.2
(6) Ca ₃ (PO ₄) ₂ gel treatment	109	132	10.2	26	77.6	45.7
(7) Gel filtration on Sephadex G-200	56	24	3.1	8	129.3	76.1

Fig. 1. Isoelectric focusing of the 1-menaphthyl sulphate-dependent glutathione S-transferase obtained after stage (7) of the purification
—, pH values of the fractions from the column; ○, E₂₈₀; ●, activity (ΔE₂₉₈/min).

Table 2. Kinetic constants at pH 8.3 and 37°C for preparations of the 1-menaphthyl sulphate-dependent glutathione *S*-transferase from rat liver

The kinetic constants were those in the initial-rate equation:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

where A is GSH and B is 1-menaphthyl sulphate, K_a and K_b are Michaelis constants for A and B, K_{ia} is the dissociation constant for the reaction of free GSH with the enzyme, V is the maximum velocity of the reaction and v is the observed velocity. The kinetic constants were estimated from the slopes and intercepts on the ordinate of the secondary plots as described in the text. Values are means $\pm$ S.E.M.

Enzyme	Kinetic constant (mM)		
	K_a	K_{ia}	K_b
Mixture of enzymes X and Y obtained at stage (7) of the purification	2.185 $\pm$ 0.180	3.224 $\pm$ 1.437	0.032 $\pm$ 0.008
Enzyme X separated by isoelectric focusing	2.202 $\pm$ 0.938	1.891 $\pm$ 1.461	0.042 $\pm$ 0.009
Enzyme Y separated by isoelectric focusing	1.941 $\pm$ 0.147	2.724 $\pm$ 1.000	0.026 $\pm$ 0.009

Fig. 2. 1-Menaphthyl sulphate-dependent glutathione *S*-transferase: secondary kinetic plots obtained in the absence of inhibitor

GSH was used as the variable substrate at four fixed concentrations of 1-menaphthyl sulphate to provide the primary plots from which the slopes ($\circ$) and intercepts on the ordinate (Δ) were taken for the secondary plots shown, as detailed in the text.

applied separately to a column (2.5cm $\times$ 60cm) of Sephadex G-100 equilibrated with 50mM-KH₂PO₄-NaOH buffer, pH 7.6, which had been calibrated with proteins of known molecular weight. Each enzyme had a molecular weight of about 40000 when examined in this way. This was also true for a sample of the enzyme obtained at stage (1) of the purification (the 'extract').

Substrate kinetic analysis

The three kinetic constants K_a , K_{ia} and K_b were determined for enzymes X and Y and for a mixture in which the two enzymes had not been resolved. Table 2 shows that the values for individual constants for each enzyme preparation are closely similar. This finding suggests that under the conditions used for assay only one enzyme is present.

The primary plots obtained with a mixture of X and Y intersected in the fourth quadrant and the secondary plots derived from them are shown in Fig. 2. The intersecting pattern of the primary plots indicates that the mechanism is a sequential one, but as the initial-rate equation for such a mechanism is algebraically symmetrical with respect to A and B this result does not allow the order of addition of substrate to the enzyme to be distinguished.

Product-inhibition analysis

In order to characterize further the enzyme mechanism the products of the reaction, namely SO₄²⁻ and *S*-(1-menaphthyl)glutathione, were used as inhibitors.

Inhibition by SO₄²⁻. Na₂SO₄ was found to be a non-competitive inhibitor with respect to both GSH and 1-menaphthyl sulphate and the secondary plots of slopes and intercepts on the ordinate against concentration of SO₄²⁻ were linear (Fig. 3). Table 3 gives the apparent inhibitor constant for the inhibition of SO₄²⁻ against 1-menaphthyl sulphate and the value of the inhibitor constant, K_{ip} , which may be calculated from it.

Inhibition by *S*-(1-menaphthyl)glutathione. This product was a non-competitive inhibitor with respect to 1-menaphthyl sulphate. When GSH was used as the variable substrate and the concentration of 1-menaphthyl sulphate was high (approx. 10–20 times the K_b

Fig. 3. 1-Menaphthyl sulphate-dependent glutathione *S*-transferase: secondary kinetic plots obtained when Na_2SO_4 was present as a product inhibitor

In order to prepare the primary plots, from which the slopes ($\circ$) and intercepts on the ordinate (Δ) were determined, first 1-menaphthyl sulphate (a) and then GSH (b) was taken as the variable substrate at fixed concentrations of Na_2SO_4 . The concentrations of GSH and 1-menaphthyl sulphate when these were held constant were 2.00 mM and 0.60 mM respectively.

value) *S*-(1-menaphthyl)glutathione was found to be a competitive inhibitor of the reaction. If the concentration of 1-menaphthyl sulphate was decreased the point of intersection of the primary plots moved into the first quadrant, i.e. the apparent maximum velocity of the reaction increased. Thus under these conditions *S*-(1-menaphthyl)glutathione appears to act as an activator of the enzyme. The secondary plots of the slopes and intercepts on the ordinate for the non-competitive inhibition and the slopes for the competitive inhibition against the concentration of the inhibitor were linear (Fig. 4). Table 3 lists the apparent inhibitor constants (the intercepts on the abscissa in Fig. 4) and the kinetic constants that may be calculated from them.

The inhibition of the enzyme by SO_4^{2-} is therefore linear non-competitive with respect to both substrates, whereas that by *S*-(1-menaphthyl)glutathione is linear non-competitive against 1-menaphthyl sulphate and linear competitive against GSH provided that the concentration of 1-menaphthyl sulphate is high.

Dead-end-inhibition analysis

To help characterize the mechanism of the enzyme *p*-aminobenzoic acid was used as a dead-end inhibitor.

Fig. 5 shows that this compound is a competitive inhibitor against GSH but is non-competitive against 1-menaphthyl sulphate.

Discussion

Nature of the purified enzyme

The experiments performed in the present work indicate that in terms of molecular weight and kinetic properties the purified preparation from rat liver which catalyses the reaction of 1-menaphthyl sulphate with GSH contains a single enzyme. This conclusion is based on the findings that the enzyme activity is eluted in a single symmetrical peak from Sephadex G-100 and G-200 and that the primary and secondary plots used to determine the three kinetic constants K_a , K_{ia} and K_b were linear.

One result which appears to be in conflict with this conclusion that only one enzyme is present derives from the isoelectric focusing. This experiment suggested the presence of two enzymes (X and Y) with distinct apparent isoelectric points. When enzyme X or Y was taken separately and refocused, however, part of the former enzyme was converted into the latter and vice versa. This observation suggests that it is only the special conditions required for isoelectric

Table 3. Kinetic constants at pH 8.3 and 37°C derived from product-inhibition studies of the 1-menaphthyl sulphate-dependent glutathione S-transferase

The kinetic constants are those in the full rate equation for an Ordered Bi Bi reaction mechanism as given by Cleland (1963):

$$v = \frac{V_1 V_2 ([A][B] - [P][Q]/K_{eq})}{K_{ia} K_b V_2 + K_b V_2 [A] + K_a V_2 [B] + V_2 [A][B] + \frac{K_p V_1 [Q]}{K_{eq}} + \frac{K_q V_1 [A][P]}{K_{eq}} + \frac{K_a V_2 [B][Q]}{K_{eq}} + \frac{V_2 [A][B][P]}{K_{ip}} + \frac{V_1 [B][P][Q]}{K_{ib} K_{eq}}}$$

where A is GSH, B is 1-menaphthyl sulphate, P is SO_4^{2-} and Q is S-(1-menaphthyl)glutathione; K_{eq} is the equilibrium constant for the reaction, K_{ia} and K_{ib} are dissociation constants for the reaction of free A and Q with the enzyme, K_a , K_b , K_p and K_q are Michaelis constants for A, B, P and Q respectively, K_{is} and K_{iq} are inhibitor constants without simple significance, V_1 and V_2 are the maximum velocities in the forward and reverse direction. The apparent inhibitor constants were estimated from the intercepts of the secondary plots (Figs. 3 and 4) on the abscissa as detailed in the text. Values are means $\pm$ S.E.M.

Inhibitor	Varied substrate	Apparent inhibitor constant (mM)		Significance of apparent inhibitor constant	Calculated kinetic constant (mM)
		Slope	Intercept		
SO_4^{2-}	1-menaphthyl sulphate	0.035 ± 0.005	3.876 ± 0.187	$K_{ip} (1 + K_a/[A])$	$K_{ip} = 1.855 \pm 0.183$
S-(1-Menaphthyl)glutathione	GSH	0.022 ± 0.003		K_{iq}	$K_{iq} = 0.035 \pm 0.005$
S-(1-Menaphthyl)glutathione	1-menaphthyl sulphate		0.092 ± 0.014	$K_{ia} (1 + [A]/K_a)$	$K_{ia} = 0.015 \pm 0.002$
				$K_{iq} (1 + [A]/K_a)$	$K_{iq} = 0.055 \pm 0.011$

focusing that lead to the generation of the two species. This conclusion appears to be borne out by the observations of Fjellstedt *et al.* (1973), who have purified to electrophoretic homogeneity a glutathione S-alkyl-epoxidetransferase from rat liver. This enzyme was shown by gel filtration and ultracentrifugation to have a molecular weight of 40000. Fjellstedt *et al.* (1973) have further shown that this enzyme consists of two subunits, which may be dissociated from each other by using guanidine hydrochloride. The enzyme was isoelectric at variable pH values and the position of the apparent isoelectric point depends on the conditions used to prepare the sample for focusing. In several respects, therefore, glutathione S-alkylepoxide-transferase appears to behave in a manner analogous to that observed with the 1-menaphthyl sulphate-dependent enzyme.

It seems reasonable to conclude that under the conditions used for the assays a single 1-menaphthyl sulphate-dependent glutathione S-transferase is present.

Mechanism of the 1-menaphthyl sulphate-dependent glutathione S-transferase

Cleland (1963) has tabulated the pattern of product inhibition that would be expected for ten different types of reaction mechanism. Table 2 of his paper shows that the product-inhibition pattern seen in the present case is inconsistent with Ping Pong and Theorell-Chance mechanisms. However, it is consistent with two other mechanisms, referred to as Ordered Bi Bi and Iso Theorell-Chance. The reaction sequences may be written:

(Ordered Bi Bi)

(Iso Theorell-Chance)

where E^* is a modified (isomeric) form of the enzyme.

In an attempt to distinguish between the Ordered Bi Bi and Iso Theorell-Chance mechanisms, *p*-aminobenzoic acid was used as a dead-end inhibitor of the enzyme. It has been noted that this compound is an inhibitor of the enzyme (Gillham, 1971), and it was suggested that as the carboxyl and amino groups of *p*-aminobenzoic acid and of GSH are superimposable (in models), the former might compete with the latter for the binding site on the enzyme. For an Ordered Bi

Fig. 4. 1-Menaphthyl sulphate-dependent glutathione *S*-transferase: secondary kinetic plots obtained when *S*-(1-menaphthyl)glutathione was present as a product inhibitor

In order to prepare the primary plots from which the slopes ($\circ$) and intercepts on the ordinate (Δ) were determined first 1-menaphthyl sulphate (a) and then GSH (b) was taken as the variable substrate at fixed concentrations of *S*-(1-menaphthyl)glutathione. The concentrations of GSH and 1-menaphthyl sulphate when these were held constant were 1.40 mM and 0.20 mM respectively.

Fig. 5. Dead-end inhibition of the 1-menaphthyl sulphate-dependent glutathione *S*-transferase by *p*-aminobenzoic acid

The fixed concentrations of 1-menaphthyl sulphate (a) and GSH (b) were 0.17 mM and 2.44 mM respectively. The concentrations of *p*-aminobenzoic acid were: $\circ$, none; Δ , 0.23 mM; $\square$, 0.56 mM.

Bi mechanism the initial velocity in the presence of a dead-end inhibitor which is competitive with GSH (A) is given by an equation of the type:

$$v = \frac{V[A][B]}{K_{ia}K_b(1 + [I]/K_i) + K_a[B](1 + [I]/K_i) + K_b[A] + [A][B]} \quad (1)$$

where K_i is the dissociation constant for *p*-aminobenzoic acid (I) from the EI complex. In such a case, double-reciprocal plots made with respect to GSH will intersect at a point on the ordinate. Eqn. (1) also predicts that both the slopes and the intercepts on the ordinate of double-reciprocal plots made with respect to 1-menaphthyl sulphate will be affected by the presence of *p*-aminobenzoic acid, i.e. the inhibition will be non-competitive.

With an Iso Theorell–Chance mechanism the initial velocity in the presence of a dead-end inhibitor which is competitive with GSH (A) is given by an equation of the type:

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B](1 + [I]/K_i) + [A][B]} \quad (2)$$

This equation predicts that if I is competitive with A then the inhibition observed when B is the variable substrate will be uncompetitive, i.e. double-reciprocal plots made with respect to 1-menaphthyl sulphate ought to be parallel. Therefore as the inhibitions found in the present work were competitive and non-competitive the Iso Theorell–Chance mechanism may be eliminated and the Ordered Bi Bi mechanism seems to be appropriate in this case.

The fact that GSH appears to bind to the enzyme before 1-menaphthyl sulphate suggests that a conformational change might be induced in the enzyme by the binding of GSH and that this change permits the

binding of the second substrate. It seems less likely that the effect is purely kinetic, i.e. that GSH is bound first simply because the enzyme has a much greater

affinity for this compound, because the value of K_{ia} (the dissociation constant for the enzyme–GSH complex) is high (about 2.5 mM). Although a measure of the affinity of the enzyme for 1-menaphthyl sulphate is not readily obtainable the low value of K_{ia} (about 0.03 mM) probably reflects a high affinity of the enzyme for the second substrate.

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