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Kinetic independence of the subunits of cytosolic glutathione transferase from the rat

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The steady-state kinetics of the dimeric glutathione transferases deviate from Michaelis–Menten kinetics, but have hyperbolic binding isotherms for substrates and products of the enzymic reaction. The possibility of subunit interactions during catalysis as an explanation for the rate behaviour was investigated by use of rat isoenzymes composed of subunits 1, 2, 3 and 4, which have distinct substrate specificities. The kinetic parameter $k_{cat.}/K_m$ was determined with 1-chloro-2,4-dinitrobenzene, 4-hydroxyalk-2-enals, ethacrynic acid and *trans*-4-phenylbut-3-en-2-one as electrophilic substrates for six isoenzymes: rat glutathione transferases 1–1, 1–2, 2–2, 3–3, 3–4 and 4–4. It was found that the $k_{cat.}/K_m$ values for the heterodimeric transferases 1–2 and 3–4 could be predicted from the $k_{cat.}/K_m$ values of the corresponding homodimers. Likewise, the initial velocities determined with transferases 3–3, 3–4 and 4–4 at different degrees of saturation with glutathione and 1-chloro-2,4-dinitrobenzene demonstrated that the kinetic properties of the subunits are additive. These results show that the subunits of glutathione transferase are kinetically independent.

INTRODUCTION

Since the time of the pioneering studies on the molecular masses of proteins carried out by Svedberg (cf. Svedberg, 1929; Svedberg & Fåhreus, 1926), it has become a well-recognized fact that numerous protein molecules are composed of subunits (cf. Klotz *et al.*, 1975). In some structures the role of the subunits in building an oligomeric or polymeric structure appears obvious in view of the known biological function of the protein. Flagellin in bacterial flagella, capsid proteins of viruses and tubulin of microtubuli are such examples of monomers of polymeric protein ensembles (cf. Stryer, 1981). In allosteric and co-operative proteins, oligomeric subunit structures are instrumental to the regulatory properties (Monod *et al.*, 1965; Koshland *et al.*, 1966; Kurganov, 1982). However, many enzymes are dimeric or tetrameric proteins that lack obvious co-operative effects or regulatory behaviour. In such cases it has been proposed that the oligomer may be energetically more stable than the monomer by maintaining a favourable surface-to-volume ratio, by shielding hydrophobic surfaces, or by affording structural symmetry (Huang *et al.*, 1982).

Glutathione transferases (EC 2.5.1.18) are enzymes occurring as dimeric proteins in the cytosol fraction of various biological species (Jakoby & Habig, 1980; Mannervik, 1985). The kinetics of the enzyme-catalysed reaction deviate from Michaelis–Menten kinetics (Pabst *et al.*, 1974; Askelöf *et al.*, 1975; Jakobson *et al.*, 1977), but in the case of rat glutathione transferase 3–3 (formerly transferase A; for new nomenclature see Jakoby *et al.*, 1984) substrate and product binding at equilibrium can be described by hyperbolic binding isotherms (Jakobson *et al.*, 1979*a*). Thus the rate behaviour of this homodimeric enzyme form has to be sought in kinetic effects. The cytosolic rat transferases afford a suitable system for exploring possible subunit interactions, since the protein subunits naturally occur in both homodimeric and heterodimeric combinations (Mannervik & Jensson, 1982). Furthermore, different subunits display distinct substrate specificities. Thus it is possible to find out if the catalytic activity of a subunit in a dimer is affected by the nature of the neighbouring subunit. In the present investigation two sets of rat isoenzymes were used to study whether any such interactions occur during catalysis. One set comprises rat glutathione transferase 1–1, 1–2 and 2–2, composed of protein subunits 1 and 2; the second set includes transferases 3–3, 3–4 and 4–4, i.e. binary combinations of subunits 3 and 4.

MATERIALS AND METHODS

Chemicals

Glutathione, ethacrynic acid, *trans*-4-phenylbut-3-en-2-one and other chemicals were standard commercial products of highest quality. 4-Hydroxyalk-2-enals were generously provided by Dr. H. Esterbauer, Institut für Biochemie, Universität Graz, Graz, Austria. Stock solutions of glutathione were made up in water and adjusted to pH 5–6 with approximately equimolar amounts of NaOH. The glutathione concentration was determined by means of 5,5′-dithiobis-(2-nitrobenzoate) in 0.2 M-sodium phosphate buffer, pH 7.6 (Ellman, 1959).

Enzyme preparation and assay

Isoenzymes of glutathione transferase were isolated from the rat liver cytosol fraction by affinity chromatography and fast protein liquid chromatofocusing (Ålin *et al.*, 1985*b*). Enzyme activity was measured spectrophotometrically at 30 °C by published methods (Habig & Jakoby, 1981) as detailed by Ålin *et al.* (1985*b*). Conjugation of 4-hydroxyalk-2-enal was measured as described by Ålin *et al.* (1985*a*). Protein concentrations were determined by a modification of the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Kinetic studies

Initial velocities were determined at pH 6.5 and 30 °C on a Varian model 2290 spectrophotometer. Each measurement was replicated at least five times. Determinations of $k_{cat.}/K_m$ were made with 2.5 mM- or 5 mM-glutathione and such low concentrations of the second substrate that the initial velocity was proportional to substrate concentration; measurements were made with 5 μM-*trans*-4-phenylbut-3-en-2-one and -ethacrynic acid or 10 μM-4-hydroxyalk-2-enal and -1-chloro-2,4-dinitrobenzene. Ethanol (5%, v/v) was present except in experiments involving 4-hydroxyalk-2-enals. The $k_{cat.}/K_m$ values were obtained by division of the initial velocity by the product of the substrate and total enzyme concentrations.

RESULTS AND DISCUSSION

For an enzyme obeying a simple rate law, such as the Michaelis–Menten equation, it would be feasible to discern effects of neighbouring subunits by determination of K_m and $V_{max.}$ values. A subunit active with a particular substrate could be studied in a homodimer as well as in a heterodimeric combination with a subunit lacking activity with the same substrate. In the case of the dimeric glutathione transferases, however, the kinetics are more complex and the rate equation may include ten constants (Pabst *et al.*, 1974; Mannervik & Askelöf, 1975; Jakobson *et al.*, 1977):

$$v = \frac{V_1[A][B] + V_2[A]^2[B] + V_3[A][B]^2}{K_1 + K_2[A] + K_3[B] + [A][B] + K_4[A]^2 + K_5[B]^2 + K_6[A]^2[B] + K_7[A][B]^2}$$

Since the estimates of the constants are highly correlated through the variance–co-variance matrix in such multiparameter models, it is not possible to obtain accurate values for individual constants. Therefore it was decided to analyse the kinetics under such experimental conditions that the analysis could be based on the asymptotic properties of the rate equation. Initial velocities were measured at high (2.5 or 5 mM) glutathione concentration and low concentrations of the electrophilic second substrate (A). At sufficiently low substrate concentrations the rate equation degenerates to a first-order expression:

$$\frac{v}{[E]_0} = \frac{k_{cat.}}{K_m^A} \cdot [A]$$

where v is the initial velocity, $[E]_0$ the total enzyme concentration, $k_{cat.}$ the apparent turnover number or molecular activity ($V_{max.}/[E]_0$), and K_m^A the apparent Michaelis constant for A. It can also be shown that the $k_{cat.}/K_m$ values for two or more enzymes or for independent subunits are additive if the first-order conditions apply for each component.

Table 1 shows the $k_{cat.}/K_m$ values determined for the six dimeric rat glutathione transferases composed of protein subunits 1, 2, 3 and 4. Several substrates were used in order to explore a variety of conditions such that each of the two subunits of a heterodimer would be the most active catalyst. For transferases 1–2 and 3–4, $k_{cat.}/K_m$ values were also calculated as the mean of the values for the corresponding homodimers, transferases 1–1 and 2–2 and transferases 3–3 and 4–4 respectively. The data in Table 1 show that the measured and the calculated values for transferases 1–2 and 3–4 agree within the experimental error. The differences were analysed by Student's t test, and in no case did a difference deviate significantly from zero at a probability level $P \leqslant 0.05$. Furthermore, some determinations of $k_{cat.}/K_m$ values were also carried out with equimolar concentrations of homodimeric transferases. For example, a mixture of equimolar amounts of transferases 3–3 and 4–4 gave a $k_{cat.}/K_m$ value of 300 ± 48 mM$^{-1}\cdot$s^{-1} with 4-hydroxydec-2-enal as electrophilic substrate, whereas the true heterodimer, transferase 3–4, gave a value of 320 ± 31 mM$^{-1}\cdot$s^{-1}. Thus the results of the experiments show that each of the four subunits of rat glutathione transferase investigated displays kinetic

Table 1. Determination of $k_{cat.}/K_m$ values for homodimeric and heterodimeric glutathione transferases from rat liver with different substrates

Values are means of replicate determinations made with 2.5 mM-glutathione for 4-hydroxyalk-2-enals and with 5 mM-glutathione for the other substrates. For experimental details see the text. The standard deviations were estimated as 10–20% of the means.

	$k_{cat.}/K_m$ (mM$^{-1}\cdot$s^{-1})				
Isoenzyme	1-Chloro-2,4-di nitrobenzene*	4-Hydroxy non-2-enal†	4-Hydroxy dec-2-enal	Ethacrynic acid	*trans*-4-Phenyl but-3-en-2-one
1–1	560	140	280	63	—
2–2	47	10	55	99	—
1–2 (measured)	310	70	190	86	—
1–2 (calculated)	300	75	168	81	—
3–3	2500	110	250	24	< 2
4–4	190	280	390	150	65
3–4 (measured)	1600	180	320	75	30
3–4 (calculated)	1350	195	320	87	33

* Values in part from Ålin *et al.* (1985*b*).
† Values in part from Ålin *et al.* (1985*a*).

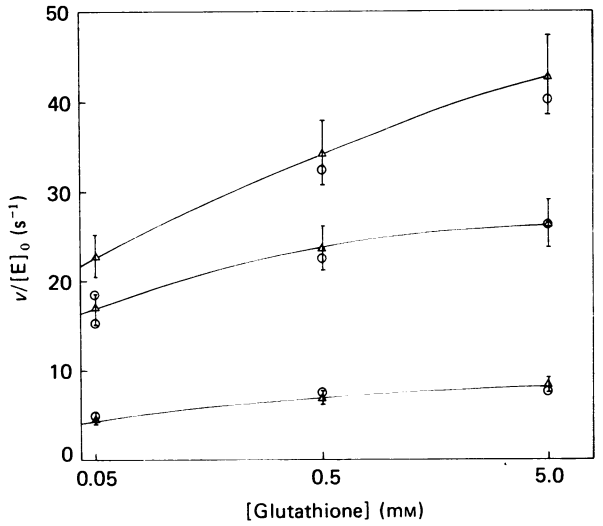

Fig. 1. Comparison of experimental and predicted initial velocities for rat glutathione transferase 3–4

The initial velocities divided by the total enzyme concentration ($v/[E]_0$) are plotted (△) versus glutathione concentration (logarithmic scale) at three fixed concentrations of 1-chloro-2,4-dinitrobenzene (0.01, 0.1 and 1.0 mM, from bottom to top). The predicted values (○) were calculated as the means of corresponding $v/[E]_0$ values determined separately with transferases 3–3 and 4–4 in each experimental point. For experimental details see the text. The measurement was replicated at least five times in each point. The relative experimental error was constant with coefficients of variation of 11.2, 10.3 and 7.2% for transferases 3–3, 3–4 and 4–4 respectively. The error bars show the standard deviations.

properties that are independent of its neighbouring subunit in a native protein dimer.

Even though the data in Table 1 demonstrate kinetic independence of the subunits of glutathione transferase at low concentration of one of the substrates, it cannot be excluded that interaction may occur at other degrees of saturation with the two substrates. In order to investigate this possibility, the initial velocity was determined with the nine possible combinations of low, intermediate and high concentration of glutathione and 1-chloro-2,4-dinitrobenzene. Such determinations were carried out independently with transferases 3–3, 3–4 and 4–4. Fig. 1 shows $v/[E]_0$ of transferase 3–4 as a function of log[glutathione] at three fixed concentrations of 1-chloro-2,4-dinitrobenzene. Also plotted are the predicted $v/[E]_0$ values, calculated as the mean of corresponding values for transferases 3–3 and 4–4 at the same combinations of substrate concentrations. This experiment demonstrated that the experimental and the calculated values agreed within the experimental error. None of the differences between measured and predicted velocities is significantly different from zero as judged by Student's t test (at $P \leqslant 0.05$). Thus we conclude that, irrespective of the degree of saturation with glutathione or the second substrate, the enzymic activities of the subunits are kinetically independent.

An important question is whether the experimental conditions used limit the generality of the above conclusion. Several of the substrates used are added as ethanolic solutions, and ethanol is an inhibitor of glutathione transferase (Aitio & Bend, 1979; Jakobson *et al.*, 1979*b*). However, the experiments involving 4-hydroxyalk-2-enals (Table 1) do not include an organic solvent, and it has been shown that the complex steady-state kinetics are independent of the presence of ethanol (Jakobson *et al.*, 1979*b*). A series of experiments with different substrates have given specific activities for the different isoenzymes of glutathione transferase isolated from rat liver (Ålin *et al.*, 1985*b*). In addition to the substrates used in the present investigation, the following substrates have been assayed at the pH values indicated (Ålin *et al.*, 1985*b*): 1,2-dichloro-4-nitrobenzene (pH 8.0), bromosulphophthalein (pH 7.5), *p*-nitrophenyl acetate (pH 7.0), cumene hydroperoxide (pH 7.0), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (pH 6.5) and androst-5-ene-3,17-dione (pH 8.5). The use of different pH values in examining the additivity of the different subunits is significant in view of the different kinetic effects of bilirubin on glutathione transferase when measured at pH 6.5 and 7.5 (Vander Jagt *et al.*, 1982). Furthermore, leukotriene A_4 methyl ester (pH 7.0) was measured at 25 mM-potassium phosphate concentration (Mannervik *et al.*, 1984), whereas the above substrates were used in 0.1 M- or 0.2 M-phosphate buffer. In all cases the specific activities of the transferase subunits were additive, within experimental error. We therefore conclude that the kinetic independence of the subunits holds under a variety of conditions, including the use of various substrates as well as physiologically relevant ionic strength and pH values.

Finally, it should be added that inhibition studies with compounds showing a high degree of selectivity for one kind of subunit in a heterodimer also suggest independence of the subunits. By use of a substrate specific for subunit 3, it has been demonstrated that the concentration of triethyltin bromide giving 50% inhibition (I_{50}) of this subunit is the same in transferases 3–3 and 3–4. Likewise, the I_{50} value of bromosulphophthalein was found to be the same for subunit 4 in transferases 4–4 and 3–4 (Yalçin *et al.*, 1983).

The results of the present investigation make a significant extension of the previous studies of substrate and product binding at equilibrium (Jakobson *et al.*, 1979*a*). It may now be concluded also that, during catalytic turnover, the subunits in the binary combinations occurring naturally act independently of one another. Furthermore, it may be stated that subunit co-operation as described by Lazdunski's 'flip-flop' model or Boyer's 'alternative-site' mechanism (for a review see Huang *et al.*, 1982) can also be ruled out, since the binding curves are hyperbolic and the binding stoichiometry at saturation is one molecule of ligand per subunit of glutathione transferase (Jakobson *et al.*, 1979*a*). Thus the remaining explanation for the non-Michaelian rate behaviour appears to be limited to an enzyme memory mechanism involving slow conformational changes of the protein (Mannervik, 1985).

The kinetic independence of the subunits of glutathione transferase does not exclude a functional dependence. Grover (1977) has suggested that the active site may be located between the two subunits and that different combinations of subunits would generate the substrate specificities distinctive of the various isoenzymes. An active site built by residues from two adjacent protein subunits has been found for glutathione disulphide in glutathione reductase (Pai & Schulz, 1983), but the

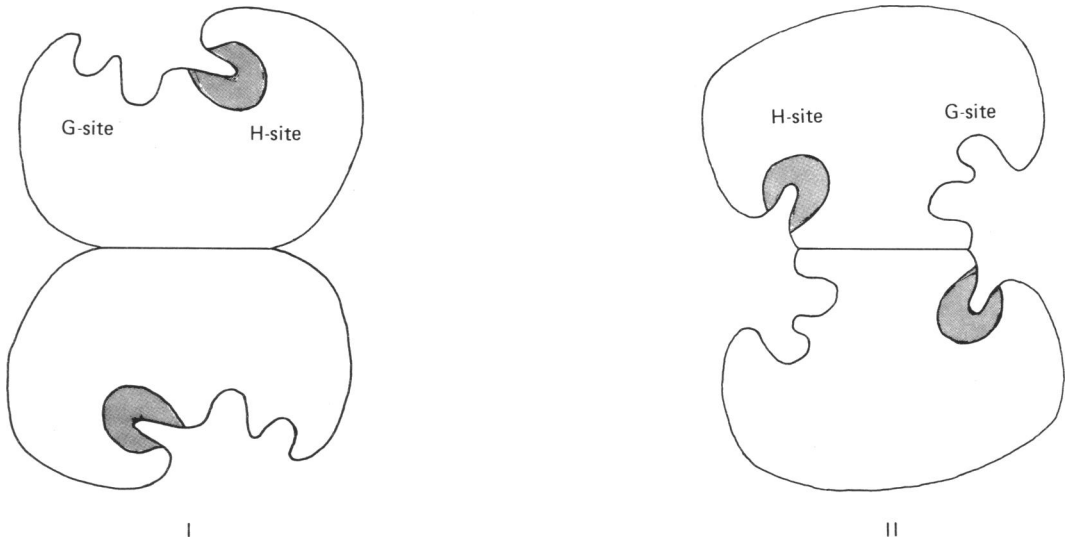

Fig. 2. Alternative models of the topology for the two active sites of a dimeric glutathione transferase molecule

Model I shows a complete active site in each individual subunit, including a subsite for glutathione (G-site) as well as a subsite for the hydrophobic electrophilic substrate (H-site) (Mannervik *et al.*, 1978). In model II, the substrate-binding cavities are localized such that both subunits contribute to the active-site structure (cf. Grover, 1977). Model II appears excluded by the present study and earlier binding experiments (Jakobson *et al.*, 1979*a*).

additivity of $k_{cat.}/K_m$ values now demonstrated and the earlier inhibition studies (Yalçin *et al.*, 1983) seem to exclude Grover's (1977) hypothesis for glutathione transferase. Fig. 2 shows two alternative localizations of the two active sites of a dimeric glutathione transferase molecule. Since each subunit is catalytically competent irrespective of the nature of the neighbouring subunit, we conclude that each polypeptide chain contains the necessary structure for a complete active site, including subsites for glutathione (G-site) and for the hydrophobic electrophilic substrate (H-site) (Mannervik *et al.*, 1978). Nevertheless the subunits of glutathione transferase are probably mutually dependent for maintaining the proper folding of the polypeptide chains and a catalytically active conformation of the enzyme. In this connection it should also be noted that only two of the possible hybrid dimers of subunits 1, 2, 3 and 4 have been demonstrated: transferases 1–2 and 3–4 (Mannervik, 1985). Whether the dimeric structure is of fundamental importance for the function of glutathione transferase or if it is merely a vestige of a haphazard event in the molecular evolution of the isoenzymes cannot be decided on the basis of the informaton available at present.

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