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FACTORS AFFECTING THE INACTIVATION OF HUMAN PLACENTAL GLUTATHIONE *S*-TRANSFERASE π

THE KINETIC MECHANISM AND pH-DEPENDENCE OF SOLVATIONAL AND 1-CHLORO-2,4-DINITROBENZENE-MEDIATED INACTIVATION OF THE ENZYME

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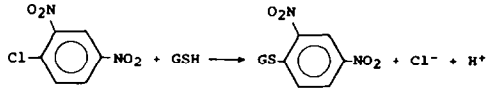
Abstract—The kinetics of the inactivation of human placental GSH *S*-transferase π has been studied at 25° in the pH range $6.5 \leq pH \leq 9$. At pH values ≤ 7.0 the inactivation of GSH *S*-transferase π incubated in the absence of GSH and (i) in the absence or (ii) in the presence of CDNB ($0–1.5 \times 10^{-3}$ mol/dm^3) exhibited pseudo first-order kinetics with k_{obs} for (i) and (ii) approximately equal ($\sim 0.002\ sec^{-1}$). The extent of inactivation in (i) approached a limiting value of 50% at infinite dilution of the enzyme; while in the presence of CDNB the extent of inactivation approached 100%. At any given pH such that $7 < pH \leq 9$ the pseudo first-order inactivation rate constant, k_{obs}, exhibits a linear dependence on [CDNB] (Eqn 1):

$$k_{obs} = k_1 + k_2[CDNB] \quad (1)$$

where k_1 is invariant with pH and approximately equal to $0.002\ sec^{-1}$.

The first-(k_1) and second-(k_2)-order components of k_{obs} suggest at least two mechanisms for the inactivation of GST by CDNB, these are: (i) a pH-invariant facilitation of solvational inactivation and (ii) a pH-dependent nucleophilic reaction of a thiol group ($pK_a = 8.85 \pm 0.08$) at or spatially close to the active site of the enzyme. A mechanistic rationale for the enzyme functioning as a dimer is discussed in detail.

Glutathione *S*-transferases (EC 2.5.1.18) are a family of ubiquitous dimeric enzymes which are involved in the detoxication of xenobiotics. The activity of these enzymes falls into three categories, namely: (i) catalysis of the conjugation of glutathione (GSH)‡ to organic electrophiles such as 1-chloro-2,4-dinitrobenzene (CDNB) to form thioethers (Scheme 1): (ii) non-covalent and reversible binding of non-substrate ligands such as bilirubin; and (iii) covalent binding to the enzyme of highly reactive electrophiles such as CDNB and other alkylating agents.



Scheme 1.

The inactivation of glutathione *S*-transferase isoenzymes by the electrophilic co-substrate CDNB, in the absence of GSH, is well documented [1–7]. In the case of the GSH *S*-transferase (GST) 3-3 and 1-2, it has been proposed that such inactivation results from irreversible alkylation or modification of the enzyme [1, 2]. Studies by Carne *et al.* [8] on the modification of ligandin (isozymes 1-1 and 1-2) by a range of thiol group reagents, demonstrated that modification of a single thiol group out of a total of four was responsible for inactivation of the enzyme. Since CDNB reacts spontaneously with thiol groups, it would be entirely reasonable to expect that were such an -SH group present at the active site of GSH π it would react with CDNB to form a covalent complex. If the -SH group were involved in the catalytic process, or if the covalent complex sterically hindered the reaction it would lead to concomitant irreversible inhibition of the enzyme. The kinetics of such inhibition would be expected to follow a second-order rate law.

Conversely, we have recently speculated [9] on the basis of our own and literature data, that at low pH, solvation of the enzyme may significantly contribute to inactivation. Furthermore, we have noted that the primary effect of substrate/non-substrate ligands on the inactivation of the enzyme at pH values ≤ 7 is merely one of the facilitation of solvational interaction, and not via a covalent inactivated complex.

In this report we present a detailed study of the pH-variation of the kinetics of inactivation of GSH *S*-transferase π; firstly at pH 6.50 in the absence of both GSH and CDNB, and secondly in the presence of CDNB but not GSH in the pH range $6.5 \leq pH \leq 9.00$.

‡ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, GSH *S*-transferase π; DTT, dithiothreitol; DMSO, dimethylsulphoxide; EDTA, ethylenediamine tetraacetate.

The results demonstrate two mechanisms for the inactivation process. The first one is [CDNB]- and pH-independent in the range of pH 6.5 to 9.0 and corresponds to CDNB-facilitation of solvational inactivation of GST π as proposed previously [9], a detailed mechanism for this purpose is discussed. The second mechanism is [CDNB]- and pH-dependent. The pH-dependence of the spontaneous conjugation of GSH with CDNB has been used as a model reaction for the latter inactivation process. Comparison of the pK_a of the group involved in the inactivation of GSH π with that of the thiol group in GSH identifies the inactivation process as a reaction of CDNB with an -SH group which must of necessity be at or in close proximity to the active site.

MATERIALS AND METHODS

Materials were obtained as follows: GSH and human placental GSH *S*-transferase (Sigma Chemical Co., St Louis, MO) or prepared by us as previously described [9]; CDNB, Merck Chemicals, Darmstadt, F.R.G. Human placental GSH *S*-transferase was dialysed overnight, at 4°, against potassium phosphate $(1 \times 10^{-2}\,mol/dm^3)$/DTT $(1 \times 10^{-3}\,mol/dm^3)$ (pH 7.0) to remove GSH and EDTA. SDS–gel electrophoresis was used to demonstrate homogeneity of the GST π preparations. The enzyme was stored at −196° without loss of activity. Deoxygenated aqueous stock solutions of GSH $(6 \times 10^{-2}\,mol/dm^3)$ (pH 6–7) and stock solutions of CDNB $(6 \times 10^{-2}\,mol/dm^3)$ in dimethylsulphoxide (DMSO) were prepared daily and the latter protected from light. Stock solutions of GSH *S*-transferase π $(1.2 \times 10^{-6}\,mol/dm^3)$ were made up by dilution of the stored enzyme solution.

Incubations of GST π were performed without shaking at 25° (±0.1) in potassium phosphate or Tris–HCl buffer ($\mu = 0.1$; $6.5 \leq pH \leq 9.0$) containing CDNB $(0–1.5 \times 10^{-3}\,mol/dm^3)$. In the case of experiments conducted at pH 6.50 and low concentrations of GST, the enzyme was incubated for a fixed time (0–1800 sec) in a cuvette (1 cm path length and 3 cm^3 total volume) containing buffer in the absence or presence of CDNB. When the incubation mixture did not contain CDNB the enzyme was assayed by simultaneous addition of GSH (to $1 \times 10^{-3}\,mol/dm^3$) and CDNB (to $1 \times 10^{-3}\,mol/dm^3$), using a double syringe technique to ensure that no excess CDNB-facilitated inactivation occurred, prior to reaction being initiated with GSH, when solvational inactivation alone was being studied. In the case of experiments conducted at higher GST concentrations, aliquots of the incubated enzyme mixture were assayed directly by addition to cuvettes containing CDNB and GSH at the concentrations previously stated. The concentration of DMSO in all assay mixtures was 2%.

Initial rates were determined by monitoring the assay reaction continuously for 1–5 min at 340 nm using either a Varian 635 or Unicam SP-1800 UV/Visible spectrophotometer. The absorbance/time data was digitized using the spectrophotometer output combined with a Hewlett-Packard 3438 A Digital Multimeter and interfaced with a Hewlett-Packard HP-85 microcomputer. Typically activity decay curves were determined at 4–6 concentrations of CDNB.

Data analysis was carried out as follows: initial rates of reaction were obtained from the digitized Abs/time data by least-squares regression of a quadratic function (Eqn 2) to the data obtained in the first 120 sec of reaction

$$\text{Abs} = A + Bt + Ct^2 \qquad (2)$$

the coefficient B in Eqn 2 being $(d\text{Abs}/dt)_{t=0}$ − the tangent to the activity/time curve at $t = 0$. This procedure allows numerical correction for any slight degree of curvature in the Abs/time plots which may affect 'manual' (i.e. ruler and pencil) estimation of initial rates.

Activity decay curves were in all cases corrected for the spontaneous reaction between GSH and CDNB (see below) and first-order rate constants for the decay were evaluated by non-linear least-squares fitting of data to the generalized first-order kinetic, Eqn 3.

$$\text{Activity} = A + Be^{-k_{obs}t}. \qquad (3)$$

The kinetics of the spontaneous reaction between GSH and CDNB were monitored by following the appearance of the conjugate at 340 nm as in the case of the enzyme assay. At any given pH, the initial rate of conjugate formation is proportional to the product [GSH]*[CDNB]. Thus the variation of the initial rate of the spontaneous reaction with pH at constant [GSH] and [CDNB] will directly reflect the pH dependence of the apparent second-order rate constant for the reaction of GSH and CDNB. As discussed later, pH-variation of this constant implies that the reaction is between GS^- and CDNB; and is furthermore used as a model system for this process.

pK_a values, for the pH variation of the enzymic and spontaneous processes referred to above, were derived from non-linear least-squares regression of second-order rate constant/pH data, the Henderson–Hasselbach equation (Eqn 4).

$$pK_a = \text{pH} - \log\left(\frac{\alpha}{1-\alpha}\right) \qquad (4)$$

$$\alpha = (k_{2(obs)}/k_{2(max)})$$

with $k_{2(max)}$ as the variable parameter.

RESULTS

In Fig. 1 we show the inactivation kinetics of GST π $(4 \times 10^{-9}\,mol/dm^3)$ at pH 6.5 in the presence (i) and absence (ii) of CDNB $(1 \times 10^{-3}\,mol/dm^3)$. In both cases the data are closely approximated by a single exponential pseudo first-order rate law (inset to Fig. 1). In (ii) the relative residual activity (A_t) tends to 50% of the initial activity while in (i) it approaches zero. The relatively rapid $(t_{1/2} \simeq 5\,min)$ pseudo first-order partial inactivation phase observed in the absence of CDNB is followed by a very slow and complete inactivation of the enzyme over a period of 24–48 hr.

When the GST concentration was varied between 1×10^{-9} and $2 \times 10^{-7}\,mol/dm^3$ in the absence of

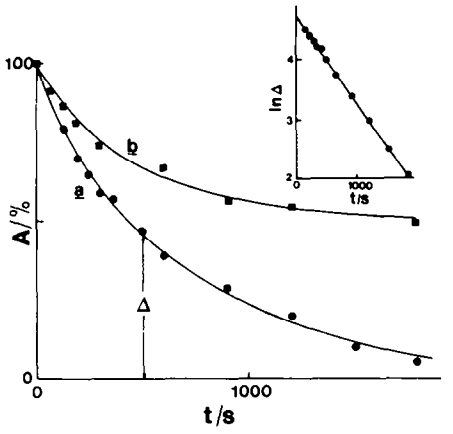


Fig. 1. (a) (-●-) Decay in GST (4×10^{-9} mol/dm^3) activity on incubation at pH 6.5 with CDNB (10^{-3} mol/dm^3) at 25.0°. The inset shows a plot of ln(100 − A) vs t, the straight line indicating close adherence to first-order kinetics to >95% reaction. (b) (-■-) As in (a) but minus CDNB. The solid lines shown are calculated from the non-linear least-squares fit to

$$A\,(\%) = a + Be^{-kt}.$$

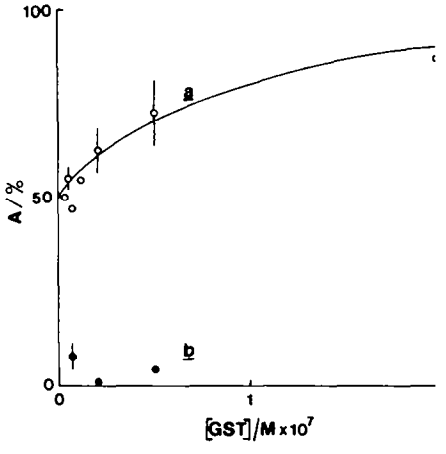


Fig. 2. (a) (-○-) Plot of limiting residual activity from the first-order curve fitting process (parameter a in equation in legend to Fig. 1) vs [GST] in the absence of CDNB. (b) (-●-) As in (a) but in presence of CDNB (10^{-3} mol/dm^3).

CDNB, the family of activity–time curves generated could in all cases be satisfactorily approximated by a first-order rate law. A plot of the limiting relative residual activity (A_∞: obtained from the single exponential fit) vs [GST] shows clearly (Fig. 2a) that at infinite dilution of GST, A_∞ approached 50%. In the presence of CDNB (1×10^{-3} mol/dm^3) and variable concentrations of GST similar pseudo first-order activity decay was observed, but in all cases the limiting extrapolated activity (A_∞) was close to zero (Fig. 2b).

The pseudo first-order rate constant for the inactivation was similar in both the absence and presence

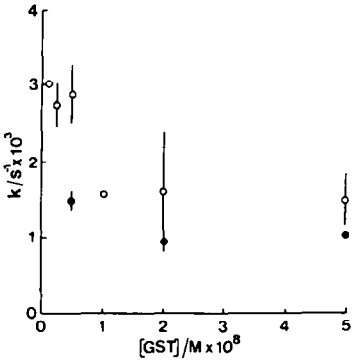


Fig. 3. Plot of pseudo first-order rate constant (k in equation in legend to Fig. 1) vs [GST]. (○) absence of CDNB; (●), in presence of CDNB (10^{-3} mol/dm).

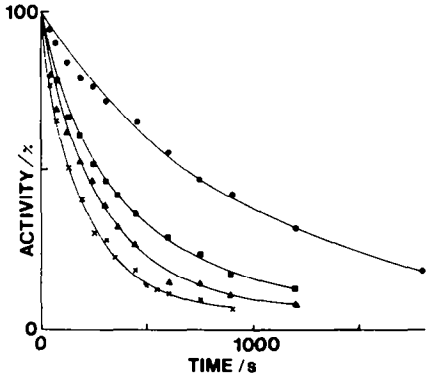


Fig. 4. The effect of [CDNB] variation on the pseudo first-order inactivation of GST π at pH 7.8. The solid lines are calculated from the least-squares fit of Eqn 3 to the data. [CDNB] are (-●-) = 0.4×10^{-4} mol/dm^3; (-■-) = 0.7×10^{-4} mol/dm^3; (-△-) = 1.00×10^{-3} mol/dm^3; and (-×-) = 1.5×10^{-3} mol/dm^3.

of CDNB (Fig. 3). However, a small but significant decrease in the value of k_{obs} was found with increasing [GST].

In Fig. 4 we show an example of typical activity decay profiles for GST π in the presence of varying concentrations of CDNB (legend to Fig. 4) at pH 7.80. In each case the solid line is calculated on the basis of the least-squares regression parameters obtained from regression of the data to the general first-order kinetic equation. At all other pH values studied the activity decay was also found to be closely modelled by a first-order kinetic function.

Figure 5 shows replots of the pseudo first-order rate constants vs the concentration of CDNB at a series of representative pH values. In all cases the lines are straight and pass, not through the origin, but through $k_{obs} \sim 0.002$ sec^{-1} at [CDNB] = 0. Kinetics of this type are well documented in physical organic chemistry (e.g. Ref. 10) and imply that two independent concomitant mechanisms are contributing to the inactivation process. The first of these mechanisms is both pH- and [CDNB]-independent with a characteristic pseudo first-order rate constant

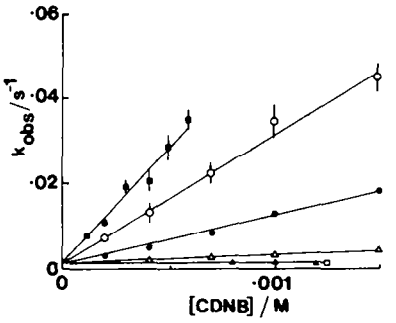


Fig. 5. Variation of k_{obs} with [CDNB] at several representative pH values. (■), pH 9.00; (○), pH 8.6; (●), pH 8.20; (△), pH 7.5; (□), pH 7.0 and (▲), pH 6.5.

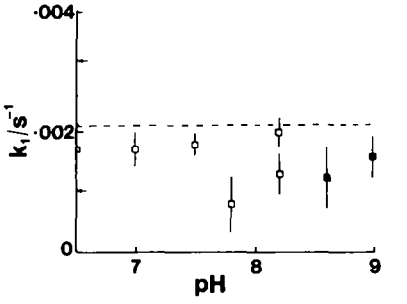


Fig. 6. pH variation of k_{obs} for dilutional inactivation of GST π (i.e. [CDNB] = 0). (□) Values obtained by fitting Eqn 3 to inactivation data at [CDNB] = 0; (■) values exvrapolated to [CDNB] = 0 from a straight-line fit to the data shown in Fig. 5. The dotted line and values of k_{obs} arrowed are the mean and SD calculated for the 19 values of the inactivation rate constant at pH 6.5 given in Table 1 of Ref. 9.

of approximately 0.002 sec^{-1}. The other is a second-order reaction between CDNB and GST π. The pH-dependent second-order rate constants for this process are obtained directly from the slopes of the straight line plots shown in Fig. 5. Figure 6 shows the [CDNB]-invariant rate constant (k_1) in the range $6.5 \leq pH \leq 9$. Values of k_1 were obtained either by measuring the rate constant for the spontaneous dilutional inactivation of GST π at [CDNB] = 0 as previously described, or as the intercept of the least-squares straight-line fit to k_{obs} vs [CDNB] plots. It is clear from the results shown that, as previously stated, k_1 is pH-invariant in the range given, and lies within the range of values reported for dilutional and facilitated GST inactivation of a number of GST isoenzymes discussed previously [9].

Figure 7 shows the pH-variation of the second-order component of k_{obs} derived from the slope of the lines in Fig. 5, and the pH-variation of the kinetics of the spontaneous reaction between GSH and CDNB. The solid lines are those calculated from the best-fit of the data to the Henderson–Hasselbach equation (Eqn 4).

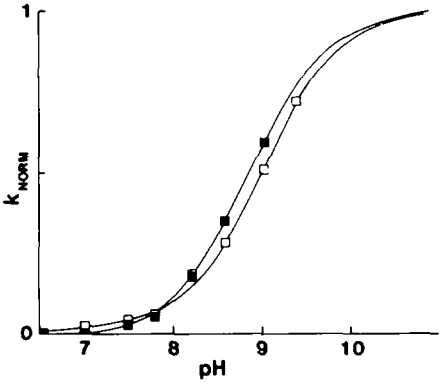


Fig. 7. pH variation of the second-order inactivation rate constant (■) obtained from the slope of the lines in Fig. 5. (□) Shows the pH variation of the spontaneous conjugation reaction between CDNB and GSH. The solid lines are calculated from the "best-fit" Henderson–Hasselbach equation and the data have been normalized to allow direct comparison of the two data sets.

DISCUSSION

Incubation of GST in buffer at pH 6.5 in the absence of GSH and CDNB results in a relatively rapid inactivation of the enzyme, and the kinetics of the inactivation process are satisfactorily modelled by a pseudo first-order rate law. On completion of the first-order phase, the extent of inactivation approaches 50% of the initial value in the limit [GST] = 0 mol/dm^3; the extent of inactivation decreasing with increasing enzyme concentration. This latter observation clearly indicates that dimer–dimer interactions are important for the preservation of catalytic integrity of the GST in aqueous solution. Additionally, this observation requires that the process be reversible, since at each point on the curve there must be an equilibrium between varying proportions of active and 50% active enzyme. If the system were not a reversible process then dilutional inactivation would result in 50% inactivation at *all* enzyme concentrations i.e. no [GST]/activity effect would be observed. It is not possible however, to directly assess the reversibility by reconcentration of the enzyme, since this in itself invariably leads to a degree of irreversible inactivation when attempted at the low concentrations used here.

In the presence of CDNB the enzyme is completely inactivated in a pseudo first-order process independent of GST concentration. The rate constants for the inactivation of GST in the presence and absence of CDNB are approximately equal, suggesting that the same chemical process is responsible for loss of catalytic integrity in each case. Since GST π is a homodimer, our observation implies an inactivation process in which a maximum of one active site per dimer is inactivated in the absence of CDNB, while in the presence of CDNB both sites are inactivated.

Two possible explanations exist for the observed inactivation process. Firstly, it could be argued that a catalytically essential active site thiol group [8], is oxidized by molecular oxygen in solution, alternatively, it could be argued that inactivation is due to active site solvation [9] which increases the polarity

of the catalytic centre with concomitant decrease in catalytic efficiency.

Thiol group oxidation can be discounted for the following reasons:

(i) -SH oxidation involving the coupling of two -SH groups to form a disulphide bond would require two thiol groups at the enzyme active centre. However, there is no evidence for such a configuration in the GSTs.

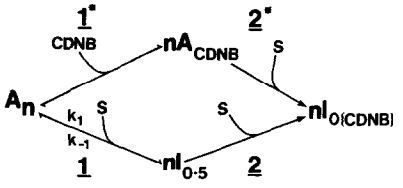
(ii) Were an oxidative inactivation mechanism of the type discussed in (i) possible, then binding of GSH to the enzyme should *facilitate* inactivation by providing the second -SH alluded to in (i) at the catalytic point in the active site. On the contrary, it is well documented that GSH protects against the type of inactivation characterized here [5, 6, 9]. Additionally we note that the purification of a recently characterized rat GST of the μ class is only possible if thiols are present to prevent oxidation of a single -SH (tentatively identified as Cys 184) on the enzyme [11]. This provides an example of an -SH oxidative inactivation not involving intramolecular disulphide formation.

(iii) More conclusively, we note that the observed pseudo first-order inactivation rate constant is unaltered when enzyme inactivation experiments are carried out anaerobically or under conditions of variable oxygen tension [9].

In contrast to the points raised against oxidative inactivation, the possibility that the observed inactivation is solvational in origin is strongly supported by the model system studies of Semenow-Garwood [12]. Solvation of the active centre would result in increased polarity of this centre; Semenow-Garwood has demonstrated clearly that the nucleophilicity of the $-S^-$ anion is enhanced by hydrophobic shielding (i.e. low polarity) of the environment around the anion. Thus, the dilutional inactivation of the enzyme characterized here can be rationalized with mechanistic studies on the nucleophilic reactivity of thiol groups (i.e. the same reactions as that catalysed by the GSTs (Scheme 1)) if it is considered solvational in origin. Our observations and conclusions are also in accord with the views of Kosower [13] who considered desolvation of the active site to be the mechanism by which GSTs achieved catalysis of the conjugation reaction.

Half-sites inactivation in the absence of CDNB indicates either that the sites are not equally accessible to solvent, or alternatively, that solvation of one site on the dimer reduces the solvent accessibility of the second site.

GSH or *S*-methylglutathione when included in the incubation mixture, prevent inactivation in the presence or absence of CDNB [5, 6, 9]. It has been suggested [14] that binding of GSH to GSH *S*-transferase is accompanied by a conformational change. We propose that GSH binding renders the point of catalysis inaccessible to water either by causing such a conformational change or merely by steric hindrance [9]. Small thiol reagents such as L-cysteine and 2-mercaptoethanol have been found not to act as substrates for the enzyme [14]. We propose that such thiols either do not induce a conformation in which the active sites are desolvated or that they are too small to physically hinder access of water to the catalytic point. Furthermore we propose that *S*-methylglutathione—which binds to the enzyme but is not a substrate—prevents inactivation in the same way as GSH. Upon addition of GSH and CDNB, *S*-methylglutathione exchanges with GSH and catalysis occurs. Solvational inactivation can be represented by the simple kinetic scheme (Scheme 2) where S is the solvent; A, the two-site active dimer and A_n, the functional aggregate of homodimers with $2n$-site activity. Solvation by route 1 leads to n single-site active, partially solvated dimers ($I_{0.5}$) which are slowly further solvated via route 2 to the totally inactive enzyme I_o. CDNB binding to the enzyme via route 1* is rapid and gives CDNB-bound dimers in which both sites are accessible to water molecules, allowing solvation and 100% inactivation of the enzyme. The kinetic scheme (Scheme 2) also explains the concentration dependence of the pseudo first-order inactivation rate-constant shown in Fig. 3 (see Appendix).



Scheme 2.

Fitting the second-order rate constants for inactivation of GST π and for the model reaction of GSH with CDNB to the Henderson–Hasselbach equation (Eqn 4) gave derived pK_a values of 8.85 ± 0.08 for the CDNB-mediated inactivation of GST π, and 8.99 ± 0.05 for the spontaneous GSH/CDNB conjugation. The latter reaction was used as a model for the nucleophilic conjugation of a thiol group on a cystyl side chain of GST with CDNB. Both pK_a values are in excellent agreement with the range of 8.5 to 8.8 [15] expected for the pK_a values of -SH groups in proteins; and the value of 8.72 (at 30°) for the pK_a of the $-SH \rightleftharpoons -S^- + H^+$ transition in glutathione [16]. This observation, taken in conjunction with the previously discussed results of Carne *et al.* [8], provides good evidence that a second mechanism of CDNB inactivation of GST π is the conjugation of CDNB with a cystyl -SH group which is either at or proximal to the enzyme catalytic centre.

The pH-independent inactivation route is considered to be due to facilitated solvational inactivation proposed previously [9] and further characterized in the present work. The rate of -SH group oxidation in GSH is strongly pH-dependent over the range studied here [17], k increasing with increasing pH. The pH-invariance of k_1 observed here therefore provides further evidence that dilutional inactivation of the enzyme is not due to -SH group oxidation, and reinforces our suggestion that this type of inactivation is due to active site solvation in GST π.

Concluding remarks

Two mechanisms have been presented for the

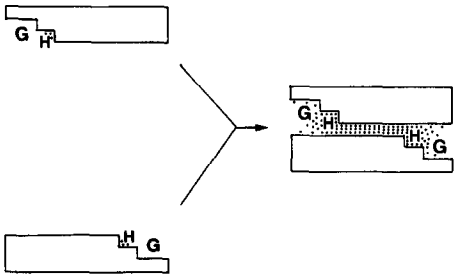


Fig. 8. Schematic dimerization model for GST π illustrating the increased hydrophobicity at the catalytic point attained on dimerization. G and H (after Ref. 18) denote the GSH and CDNB binding loci, respectively, while hydrophobicity density is denoted by the solid points.

inactivation of human placental GST π by CDNB. They provide added insight into the nature of the active site and the mechanism by which GSTs achieve catalysis of the conjugation of GSH to electrophilic substrates.

It is conceivable that for other GSTs only one or both of these mechanisms will be observed. CDNB-facilitated and dilutional inactivation have been observed for other GST isoenzymes [1–7, 9]. CDNB-mediated inactivation of GSTs via the pH-dependent route should be observed for isoenzymes which have a cystyl residue at or proximal to the active site. In the absence of such a cystyl residue, the observed pseudo first-order rate constant for CDNB-facilitated inactivation would be pH-independent and equal to the rate constant for dilutional inactivation alone. It follows therefore that the variation or invariance of the inactivation rate constant (k_{obs}) with pH could provide direct information on the presence or absence of such residues at the active site.

Active site desolvation provides a simple chemically plausible rationale for the hitherto unexplained requirement that, in order to function catalytically, the enzyme must be dimeric, in spite of the fact that each subunit apparently contains both a CDNB and a GSH binding locus [18]. Mannervik and Danielson [18, 19] conclude that the "subunit–subunit interface does not harbour the active site", a major reason for this statement apparently being that the subunit active sites of cytosolic dimeric GSTs are catalytically and topologically independent. We must point out however that the catalytic independence of the subunits in no way excludes the subunit–subunit interface as the region in which the active sites are located. Indeed for sound chemical reasons [12] the most likely location of the active site is precisely at this interface. While we fully agree with Mannervik [20] that the concept of—the GSH *S*-transferases promoting catalysis only by increasing the nucleophilicity of the -SH group—is oversimplistic (the physical aspects of enzymic reaction-rate acceleration, i.e. steric effects, have, after all, been well appreciated for decades), we must stress that the available evidence supports the contention of Keen *et al.* [21] that the main (chemical)* catalytic device of the glutathione *S*-transferases is precisely this nucleophilicity increase. This is attained through hydrophobic shielding resulting from dimerization and is rationalized by the model system studies of Semenow-Garwood [12]. A simple model illustrating this concept is shown in Fig. 8. The model explains the catalytic requirement for dimerization without recourse to more complex explanations involving the maintenance of the proper folding of the polypeptide chains and a catalytically active conformation [18].

* Our insert.

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APPENDIX

Stage 1 of Scheme 2 can be written in detail as

$$A_n \overset{K}{\longleftrightarrow} nA \tag{A1}$$

$$A \underset{k_{-1}}{\overset{k_1[S]}{\longleftrightarrow}} I_{0.5}. \tag{A2}$$

Under the reasonable assumption that the aggregation/disaggregation equilibrium is very rapid compared to the solvation desolvation process we can state that:

(i) as [GST] → 0 the GST will tend to the dimeric form A and the inactivation process is essentially described by Eqn A2 alone. Thus, at low [GST], the inactivation tends toward accurately pseudo first-order kinetics, with an apparent rate constant $k_1[S] + k_{-1}$;

(ii) as [GST] increases, aggregation (Eqn A1) becomes increasingly important and the loss of activity for the system described by Eqns A1 and A2 will *always* occur at a lower rate than for the equivalent pseudo-first order system

$$A \underset{k_{-1}}{\overset{k_1[S]}{\longleftrightarrow}} I_{0.5}.$$

(N.B. By equivalent pseudo first-order system we mean that system which—in the absence of aggregation—would inactivate to the same *relative* activity e.g. 100% at $t = 0$ to say 85% at $t = \infty$. The GST concentrations for the two systems will *not* be the same.)

Thus the fitting of a single exponential function will give an apparent first-order rate constant decreasing with increasing [GST] as is in fact observed (Fig. 3).