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1-CHLORO-2,4-DINITROBENZENE-MEDIATED IRREVERSIBLE INACTIVATION OF ACIDIC GLUTATHIONE *S*-TRANSFERASES

INACTIVATION MECHANISM—A SATURATION-TYPE OR SIMPLE SECOND-ORDER KINETIC PROCESS?

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Abstract—A critical analysis of the inactivation kinetics exhibited by the acidic human glutathione *S*-transferase (GST) enzymes is presented. Data on the 1-chloro-2,4-dinitrobenzene (CDNB)-facilitated inactivation of human placental GST π have been utilized in conjunction with published inactivation data from the literature to answer the following two questions: (a) do the inactivation kinetics deviate significantly from a simple pseudo first-order model? (b) What is the kinetic mechanism of irreversible electrophilic co-substrate-mediated inactivation of human acidic GSTs? Inactivation of human placental GST π in the presence of 7-aminocephalosporanic acid, a non-electrophilic non-substrate ligand, is characterized and shown to occur via a process analogous to the second mechanism proposed for CDNB inactivation of the enzyme, namely: pH- and [ligand]-independent solvational inactivation.

The cytosolic glutathione *S*-transferases (GSTs§) (EC 2.5.1.18) are a widely distributed group of dimeric enzymes, a major function of which appears to be the conjugation of glutathione (GSH) with electrophilic xenobiotic co-substrates with concomitant chemical inactivation of these compounds and their subsequent excretion in aqueous solution. Concurrently with their enzymic function, GSTs also bind a wide range of non-substrate ligands at hydrophobic sites which may be spatially distinct from the catalytic region of the protein [1, 2]. It has thus been hypothesized that a second function of the proteins could be the *in vivo* transport of the biologically relevant non-substrate ligands such as haemin (Ferriprotoporphyrin IX) and bilirubin.

A further property of the enzymes is that they may inactivate to varying extents under three clearly defined conditions [3–10]: (a) on dilution in buffer alone; (b) on interaction with non-substrate ligands; and (c) on incubation with electrophilic co-substrates in the absence of glutathione. Two central questions arise from the observations (a–c): firstly, what kinetics are exhibited by the inactivation process and secondly, what mechanism for the inactivation process can be inferred from the observed kinetics?

Although a number of literature sources report simple pseudo first-order kinetics for the inactivation processes for (a–c) [3–5; 10–12], observations claiming significant deviation from pseudo first-order kinetics exist [7, 13]. Furthermore, two divergent mechanisms have recently been inferred for the inactivation of human acidic GSTs by the electrophilic co-substrate 1-chloro-2,4-dinitrobenzene (CDNB) [4, 11]. The first of these reports proposes that site-directed CDNB-promoted inactivation occurs from an intermediate enzyme–CDNB complex and not directly via a second-order conjugation reaction [11], while the second proposes the converse, i.e. inactivation results from just such a second-order process [4]. The question as to which of these two kinetic mechanisms the inactivation proceeds by is not trivial, since if inactivation is from an enzyme–CDNB complex this could be the Michaelis or a closely related complex, thus allowing kinetic characterization of the catalytic CDNB binding step by a non-catalytic route [14].

In the main part of this paper it is demonstrated that the reaction of CDNB with acidic human placental GST π at pH > 7 is a second-order process. It is suggested that the apparent saturation-type inhibition reported for site-directed inactivation of the acidic human transferases by CDNB [11] is a spurious consequence of fixed-time sampling of exponential activity decay curves which exhibit second-order variability of a pseudo first-order rate constant. The latter point is demonstrated both experimentally and theoretically and a caveat is recorded, accordingly, concerning the importance of complete characterization of the variance of the pseudo first-order rate constant in mechanistic discrimination studies. The inactivation of human placental GST π by the non-electrophilic non-substrate ligand 7-aminocephalosporanic acid (7-ACA) is characterized and discussed in relation to previously proposed models for the inactivation of GSTs [3, 4].

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§ Abbreviations: 7-ACA, 7-aminocephalosporanic acid; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, GSH *S*-transferase.

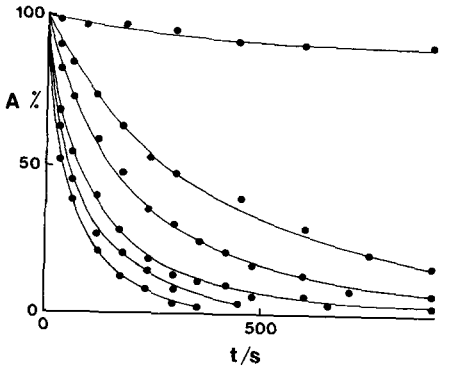

Fig. 1. Inactivation at pH 8.2, 25° of GST π (1.7×10^{-7} mol/dm^3) by CDNB at concentrations of 0, 0.2, 0.4, 0.7, 1.0 and 1.5×10^{-3} mol/dm^3. The solid lines are those obtained by non-linear least-squares fitting of the data to the general first-order integrated rate equation:

$$\text{Activity}\ (\%) = a + (100 - a)e^{-kt}.$$

For all [CDNB] > 0, the activity at $t = \infty$ was within 0 to 5%, i.e. GST π is completely inactivated by CDNB.

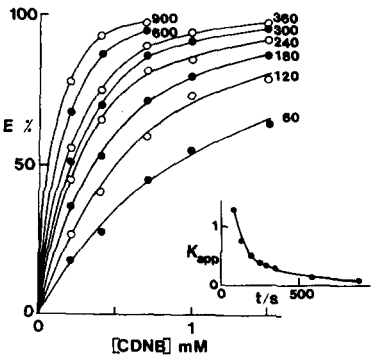

Fig. 2. Plot of extent of GST π inactivation E (%) vs [CDNB] measured from the data shown in Fig. 1 at various fixed times (sec) shown adjacent to the curves. The solid lines are the fit of the data to a Michaelis–Menten type saturation kinetic model:

$$E = \frac{E_{max}[\ ]}{K_{app} + [\ ]}$$

in all cases E_{max} lay between 95 and 100%. The inset shows how the apparent inactivation constant (K_{app} units are mol/dm$^3 \times 10^3$) varies with the time at which the inactivation extent was sectioned.

MATERIALS AND METHODS

GSH, human placental GST π and CDNB were obtained as described previously [3, 4]; 7-ACA was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and used without further purification. Experimental procedures for inactivation reactions have been reported in detail elsewhere [3, 4]; all experiments reported here were carried out at 25 (±0.1)°. Hyperbolic and exponential functions were fitted to our enzyme inactivation data using an iterative non-linear least-squares procedure. Normally distributed random numbers utilized in the simulation experiments reported here were generated directly from a rectangular pseudo-random number generator using the central limits theorem with N = 30.

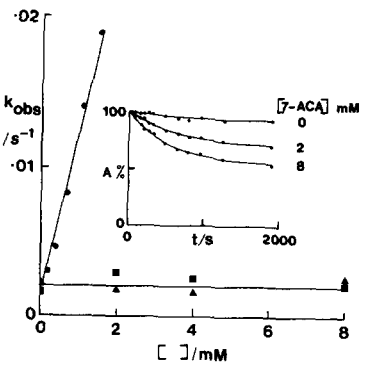

Fig. 3. Concentration dependence of the pseudo first-order rate constant for 7-ACA-mediated inactivation of GST π at pH 6.5 (▲) and 8.2 (■). Also included is the concentration dependence of k_{obs} for CDNB-mediated inactivation data of Fig. 1. The inset demonstrates that 7-ACA affects only the extent of GST π inactivation. The solid lines shown on the inset are the best fit to the integrated pseudo first-order rate equation. Data at 4×10^{-3} mol/dm^3 7-ACA have been omitted to avoid overcrowding the figure.

RESULTS

In Fig. 1 we show the family of inactivation curves obtained at pH 8.2 at CDNB concentrations in the range $0 \leqslant [\text{CDNB}] \leqslant 1.5 \times 10^{-3}$ mol/dm^3. In all cases the curves closely adhere to a pseudo first-order rate law.

As found previously, the final activity was in the range of 0–5% of the zero time activity, taken as 100%, at all [CDNB] > 0 [4].

In Fig. 2 we replot the activity decay data as a family of curves obtained by measuring the extent of inactivation as a function of [CDNB] at the various fixed times of incubation shown in Fig. 1. The solid lines in Fig. 2 are the best non-linear least-squares fit of each data set to the hyperbolic form of the Michaelis–Menten equation, while the inset shows a plot of $K_{i(app)}$ vs the time at which the data cross-section was taken in Fig. 1.

In Fig. 3 we show the pH and [7-ACA] dependence of the first-order rate constant for the inactivation of GST π facilitated by 7-ACA, a non-electrophilic competitive inhibitor of the enzyme-catalysed GSH–CDNB conjugation reaction. 7-ACA affects only the extent of inactivation (i.e. at [7-ACA] = 0, activity at $t = \infty$ is 88% while at [7-ACA] = 8×10^{-3} mol/dm^3 activity at $t = \infty$ is 60%, see inset to Fig. 3); k_{obs} is invariant. Included in Fig. 3 for comparison are the inactivation rate constant vs [CDNB] data obtained from Fig. 1.

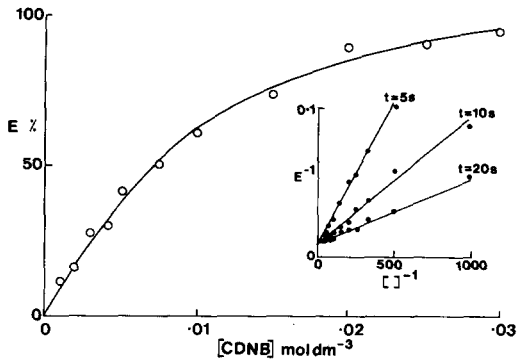

Fig. 4. Plot of simulated (see Results) extent of inactivation E (%) vs [CDNB] at time of inactivation 10 sec. Inset shows double reciprocal plot of data at 10 sec plus other sets of data obtained at $t = 20$ and 5 sec. From the inset it could be inferred that at a particular time section the inactivation was following a saturation mechanism.

Figure 4 shows the results of a simulation experiment to demonstrate that fixed-time sampling of a pseudo first-order inactivation process, where $k_{obs} = k_1 + k_2$ [inactivator], will give rise to an apparent saturation phenomenon. For the simulation experiment GST inactivation is assumed to occur via a reaction of the type:

$$\begin{array}{ll} \text{GST} \xrightarrow{k_2[\text{CDNB}]} & \text{GST–CDNB} \\ \downarrow k_1 & \text{(inactive covalent complex)} \\ \text{GST (inactive).} & \end{array}$$

For simplicity we assume that the extent of the spontaneous [CDNB]-independent GST inactivation is either very small at the [GST] studied (as is the case for both the placental and lung enzyme studies [4, 11]) and can consequently be ignored or if not insignificant that this background inactivation (characterized by k_1) is corrected for by subtraction. For the purposes of the simulation "exact" inactivation percentages were generated at various times according to the pseudo first-order equation:

$$\text{Inactivation extent (\%)} = 100\,(1 - e^{-k_{obs}t}) \quad (1)$$

where $k_{obs} = 10$ [inactivator] (i.e. a simple second-order process carried out under pseudo first-order conditions where [inactivator] remains essentially constant). These values were then perturbed by a generated "error", normally distributed about 0% with a constant deviation of ±2%. As shown in Fig. 4 plots of the resultant inactivation extent vs [CDNB] at various fixed times can in all cases be closely approximated by a hyperbolic function which, since the data were generated according to a simple second-order model Eqn 1 gives a totally meaningless $K_{i(app)}$.

DISCUSSION

In all the cases that we have studied so far we have found the inactivation of human placental GST π by several ligands to be accurately modelled by a pseudo first-order rate law [3–5]. Similar results have also been observed by other authors with a range of GST enzymes and ligands [10–15]. Reports of major deviation from pseudo first-order inactivation kinetics have appeared in the literature [7, 13], but have recently been questioned [12].

7-ACA has been shown here to facilitate inactivation of GST π. However, in contrast to CDNB the pseudo first-order rate constant is both [7-ACA]- and pH-invariant. This clearly demonstrates that a non-electrophilic competitive inhibitor (with respect to CDNB) does not exhibit the second-order reactivity toward GST π which, on the basis of the pH dependence of the second-order component of k_{obs}, we have previously attributed to a reaction between CDNB and an SH group at or proximal to the active centre of the enzyme [4]. We have demonstrated here and in previous publications that the CDNB-mediated inactivation of human placental GST π, an acidic form of the transferase, proceeds via a two-component mechanism: firstly, a [CDNB]- and pH-independent first-order process which has, on the basis of Occams Razor, been attributed to active site solvation; and, secondly, a pH-dependent second-order reaction between CDNB and the enzyme, specifically a reactive -SH group on the enzyme [4]. This highly reactive -SH group has recently been assigned as that of Cys-47 for human placental GST π [16, 17].

Our conclusions concerning the mechanism of the CDNB irreversible inactivation are in direct contrast to those of Corrigall *et al.* [11] who, on the basis of the variation in the extent of inactivation with [CDNB] determined at a fixed time, $t = 900$ sec, claim that the CDNB-mediated irreversible inactivation of human acidic lung GST is "saturable with respect to the CDNB concentration . . . suggesting that the inactivation is occurring from an enzyme–CDNB complex, *not* via a bimolecular reaction". Firstly, we note that the value of $k_{obs} = 0.0053$ sec^{-1} quoted by Corrigall *et al.* [11] for pH 8.2 and [CDNB] = 0.6×10^{-3} mol/dm^3 is in excellent agreement with the value of 0.0067 sec^{-1} interpolated from our data under the same conditions. The GST iso-enzymes would thus appear to be kinetically equivalent. Secondly, we note that fixed time measurement of inactivation extent at various [CDNB] for the GST π studied here can be shown to give rise to curves which closely approximate those expected from saturation type phenomena and can be analysed to give an apparent (but meaningless) K_i. Depending on the fixed time at which these inactivation extent data were obtained, $K_{i(app)}$ for our data on human GST π varied between 0.08×10^{-3} and 1.1×10^{-3} mol/dm^3 (inset to Fig. 2) with the apparent value for $t = 900$ sec, 0.084×10^{-3} mol/dm^3, comparing satisfactorily with the value of $K_i = 0.14 \times 10^{-3}$ mol/dm^3 suggested by Corrigall *et al.* for the human lung acidic GST. These conclusions are also supported by simulation studies which demonstrate that fixed time inactivation extent measurements for a second-order process under pseudo first-order conditions give rise to an apparent saturation-type phenomenon. The spurious nature of such saturation phenomena can only be detected by carrying out a complete inactivation time-course

run at *each* [CDNB] used. Inactivation vs [CDNB] studies carried out at a single fixed time can lead to an incorrect conclusion. Furthermore, we note that a "saturation-type" mechanism involving an enzyme–CDNB complex must be of the type shown below where I is the "inactive covalent complex":

$$\text{GST} + \text{CDNB} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{GST–CDNB} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{I}.$$

Two cases can be considered: firstly, if $k_{-2} = 0$ then inactivation will go to completion at *all* [CDNB] > 0 and there is in consequence no validity in sampling at any one time in preference to another. Furthermore, it is well documented [18, 19] that in this case under pseudo first-order conditions a saturation-type mechanism is consistent with a rate constant k_{obs} which varies hyperbolically with [inhibitor] and not, as observed by ourselves and other authors [4, 15], in a linear fashion. Secondly, if $k_{-2} = 0$ the system will show apparent saturability, the extent of inactivation at $t = \infty$ depending on [CDNB]. However, the inactivation is then by definition *reversible* which is experimentally not found to be the case for the acidic human GSTs considered here.

In conclusion, therefore, it can be stated that there is at present little evidence to suggest that the pH-dependent inactivation of the GSTs by the electrophilic co-substrate CDNB follows other than simple second-order kinetics. In contrast to other workers we have shown that the pH-dependent second-order inactivation reaction between CDNB and GST π can demonstrably give rise to a spurious saturation-type inactivation phenomenon which can lead to incorrect mechanistic interpretation for the inactivation process. Non-electrophilic inhibitors of the CDNB-mediated conjugation reaction do not exhibit the pH-dependent second-order component found for CDNB-mediated inactivation, reinforcing further the suggestion that this component arises via a second-order, covalent interaction between such substrates and the enzyme.

REFERENCES

1. Mannervik B, Isoenzymes of glutathione transferase. *Adv Enzymol* **57**: 357–417, 1985.
2. Boyer TD and Kenney WC, Preparation, characterization and properties of glutathione *S*-transferases. In: *Biochemical Pharmacology and Toxicology* (Eds. Zakim D and Vessey DA), Vol. 1, pp. 292–364. John Wiley, New York, 1985.
3. Adams PA, Goold RD and Sikakana CNT, Active site solvation contributes significantly to inactivation of the glutathione *S*-transferases (GST). *Biochem Pharmacol* **38**: 3124–3126, 1989.
4. Adams PA and Sikakana CNT, Factors affecting the inactivation of human placental glutathione *S*-transferases π: the kinetic mechanism and pH-dependence of solvational and 1-chloro-2,4-dinitrobenzene-mediated inactivation of the enzyme. *Biochem Pharmacol* **39**: 1883–1889, 1990.
5. Adams PA and Goold RD, Haem peptide–protein interactions: Part 4—The microperoxidase-8-mediated inhibition of the human placental glutathione *S*-transferase π catalysed conjugation of glutathione and 1-chloro-2,4-dinitrobenzene. *J Chem Soc Faraday Trans* **86**: 1803–1806, 1990.
6. Ketley JN, Habig WH and Jacoby WB, Binding of non-substrate ligands to the glutathione *S*-transferases. *J Biol Chem* **250**: 8670–8673, 1975.
7. Simons PC and Vander Jagt DL, Bilirubin binding to human liver ligandin (glutathione *S*-transferase). *J Biol Chem* **255**: 4740–4744, 1980.
8. Pabst MJ, Habig WH and Jakoby WB, Glutathione *S*-transferases. A: A novel linear mechanism in which the major reaction pathway depends on substrate concentration. *J Biol Chem* **249**: 7140–7150, 1974.
9. Vander Jagt DL, Wilson SP and Heidrich JE, Purification and bilirubin binding properties of glutathione *S*-transferase from human placenta. *FEBS Lett* **136**: 319–321, 1981.
10. Harvey JW and Beutler E, Binding of heme by glutathione *S*-transferase: a possible role of the erythrocyte enzyme. *Blood* **60**: 1227–1230, 1982.
11. Corrigall AV, Bhargava MM, Ivanetich KM, Ehlers MRW and Kirsch RE, Site-directed inactivation of human lung acidic glutathione *S*-transferase by 1-chloro-2,4-dinitrobenzene in the absence of glutathione. *Biochim Biophys Acta* **991**: 399–404, 1989.
12. Ivanetich KM, Thumser AEA, Phillips SE and Sikakana CNT, Reversible inhibition of rat hepatic glutathione *S*-transferase 1-2 by bilirubin. *Biochem Pharmacol* **40**: 1563–1568, 1990.
13. Vander Jagt DL, Wilson SP, Dean VL and Simons PC, Bilirubin binding to rat liver ligandins (glutathione *S*-transferases A and B). Relationship between bilirubin binding and transferase activity. *J Biol Chem* **257**: 1997–2001, 1982.
14. Phillips MF and Mantle TJ, The initial-rate kinetics of mouse glutathione *S*-transferase Y Y: evidence for an allosteric site for ethacrynic acid. *Biochem J* **275**: 703–709, 1991.
15. Nishihara T, Maeda H, Okamoto K, Oshida T, Mizoguchi T and Terada T, Inactivation of human placenta glutathione *S*-transferase by SH/SS exchange reaction with biological disulfides. *Biochem Biophys Res Commun* **174**: 580–585, 1990.
16. Lo Bello M, Petruzzelli R, De Stefano E, Tenedini C, Barra D and Federici G, Identification of a highly reactive sulphydryl group in human placental glutathione transferase by a site-directed fluorescent reagent. *FEBS Lett* **263**: 389–391, 1990.
17. Tamai K, Satoh K, Tsuchida S, Hatamaya I, Maki T and Sato K, Specific inactivation of glutathione *S*-transferases in class pi by SH-modifiers. *Biochem Biophys Res Commun* **167**: 331–338, 1990.
18. Jencks WP, *Catalysis in Chemistry and Enzymology*, pp. 571–574. McGraw-Hill, New York, 1969.
19. Walsh C, *Enzymatic Reaction Mechanisms*, pp. 86–92. W. H. Freeman and Company, San Francisco, 1979.