ABSENCE OF A PING-PONG PATHWAY IN THE KINETIC MECHANISM OF GLUTATHIONE *S*-TRANSFERASE A FROM RAT LIVER. EVIDENCE BASED ON QUANTITATIVE COMPARISON OF THE ASYMPTOTIC PROPERTIES OF EXPERIMENTAL DATA AND ALTERNATIVE RATE EQUATIONS

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1. Introduction

Glutathione *S*-transferase A is a member of a group of enzymes in rat liver [1] involved in the detoxification of electrophilic substances by conjugation with the thiol group of glutathione (GSH). This enzyme has higher specific activity with 3,4-dichloro-1-nitrobenzene (DCNB) than any of the other GSH *S*-transferases [1] and is apparently responsible for a major part of the GSH *S*-aryltransferase activity discovered in rat liver [2]. Independent of the work of Jakoby et al [1], we have found [3] and purified [4] two GSH *S*-transferases active with DCNB which were designated as forms I and II of GSH *S*-aryltransferase [4]. It seems evident that forms I and II correspond to *S*-transferases C and A, respectively, [1] and we will adopt the latter nomenclature.

The steady-state kinetics of GSH *S*-transferase A have been studied by both groups of investigators and been found to yield non linear graphs in double reciprocal or v versus v/A plots [4,5]. However the interpretations of the kinetic data are basically different: one being based on a random sequential mechanism [4] and the second on a scheme involving a ping-pong branch and an ordered sequential branch [5]. The present paper provides evidence to permit the conclusion that the second reaction scheme is not applicable.

2. Materials and methods

The initial velocities of *S*-(2-chloro-4-nitrophenyl)-glutathione formation were determined at 344 nm ($\epsilon = 10\ mM^{-1}\ cm^{-1}$) with an Aminco-Chance DW-2 UV/VIS Spectrophotometer. The reaction was run in 0.1 M sodium phosphate (pH 8.0) at 30°C. The enzyme (form II of GSH *S*-aryltransferase) was prepared as previously described [4]. The specific activity of batches used in the kinetic studies was > 2.5 units/mg. The enzyme was preincubated in the cuvette for 5 min with the desired GSH concentration before DCNB was added to start the reaction. DCNB was dissolved in ethanol but the final concentration of ethanol was always constant, 3.3% (v/v), in the complete reaction system to avoid the influence of any inhibitory action of the solvent on the algebraic form of the rate law of the reaction. The stock solutions of GSH were kept under N_2 in an ice bath to prevent oxidation. Alternative mathematical models of the kinetics were fitted to the experimental data by a Gauss–Newton non-linear regression program (BMDP3R, University of California, Los Angeles). The comparison of alternative models was based on examination of parameter values, residuals, and the residual sum of squares:

$\sum_{i=1}^{n} (v_i - \hat{v}_i)^2$, where v_i and $\hat{v}_i$ are experimental and predicted velocities in the i-th experimental point. Criteria for discrimination have been detailed previously [6,7]. A good model is expected to have low standard errors of the parameters, normally distributed residuals, which lack correlation, and have a small residual sum of squares.

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3. Theory and results

A random sequential mechanism of a two-substrate enzymatic reaction normally is expected to obey rate law (I) under steady-state conditions

$$v = \frac{V_1 AB + V_2 A^2 B + V_3 AB^2}{K_1 + K_2 A + K_3 B + AB + K_4 A^2 + K_5 B^2 + K_6 A^2 B + K_7 AB^2} \quad \text{(model I)}$$

where v is the initial velocity, V_i ($i = 1-3$) are constants proportional to the total enzyme concentration, K_j ($j = 1-7$) are constants (like V_i composed of rate constants of the elementary steps in the reaction scheme), and A and B denote the concentrations of the two substrates. The reaction scheme proposed by Pabst et al. [5] results in a rate equation (model II), which is identical with model I except for the absence of the constant term (K_1) in the denominator. This difference between the rate laws is fundamental from a mechanistic point of view because demonstration of the presence of K_1 is direct evidence against the mechanism including the ping-pong branch. In fact, it can easily be verified by the topological reasoning of Wong and Hanes [8] that any reaction scheme involving a ping-pong branch will yield a rate equation lacking a constant term in the denominator.

The first attempt to discriminate between the two kinetic models (models I and II) was to fit the two rate laws to experimental data covering wide ranges of substrate concentrations. The results showed that model I was better than model II on the basis of the residual sum of squares (which was lower for model I), the distribution of residuals, and the serial correlation coefficient of the residuals (table 1). Fig.1 demonstrates the fit of model I to the data set. Examination of the standard deviations indicated that V_1 and most certainly K_2 (s.e.$(K_2) \approx 4K_2$) were redundant in model II. Model I shows only the degeneracy that K_7 assumes the lowest limit allowed in the regression, whereas the remaining parameter values have low standard errors considering the large number of parameters. However, this superiority of model I cannot be taken as solid evidence for the conclusion that model I is the true rate law, because all parameters of the rate equation were not necessary to fit the data by this model (or by model II). However, the constant term, K_1, seemed to be significant as judged by its standard error. Even this finding was not considered as conclusive proof for the presence of a constant term in the denominator in the true rate equation, because the possibility exists that a mathematical model containing redundant parameters may degenerate to a form lacking parameters which appear reliable in the original model.

Table 1
Kinetic constants estimated for models I and II by non-linear regression

Constant	Parameter value (± s.e.)	
	Model I	Model II
V_1	1.38 ± 0.52	0.151 ± 0.155
V_2	5.92 ± 1.70	5.74 ± 2.10
V_3	9.53 ± 0.49	14.9 ± 1.00
K_1	0.00106 ± 0.00032	not existent
K_2	0.0416 ± 0.0251	0.0020 ± 0.0080
K_3	0.0075 ± 0.0029	0.0119 ± 0.0024
K_4	0.283 ± 0.082	0.275 ± 0.101
K_5	0.0105 ± 0.0053	0.0129 ± 0.0042
K_6	0.116 ± 0.053	0.0990 ± 0.0691
K_7	10^{-8} (lower limit of this parameter value)	0.649 ± 0.150
Residual sum of squares	0.2598	0.3025
Serial correlation coefficient of residuals	0.220	0.260

The two models were fitted to the experimental data set (number of experimental points = 91), which is presented in fig.1. Units of the constants are expressed in their appropriate dimensions by using nmoles/min and mM as basic units of velocity and concentration, respectively. GSH and DCNB correspond to A and B, respectively, in the equations given in the text.

Thus, two different ways of analysis were used. First, pairs of degenerate forms of models I and II were generated by elimination of corresponding terms (e.g. $V_3 AB^2$) in the two equations and fitted to a complete set of experimental data. The pairs consisted of equations which were identical except for the presence of the constant term, K_1, in the equation deriving from model I. With all pairs tested, the rate law containing the constant was always better than the corresponding rate law lacking the constant. The

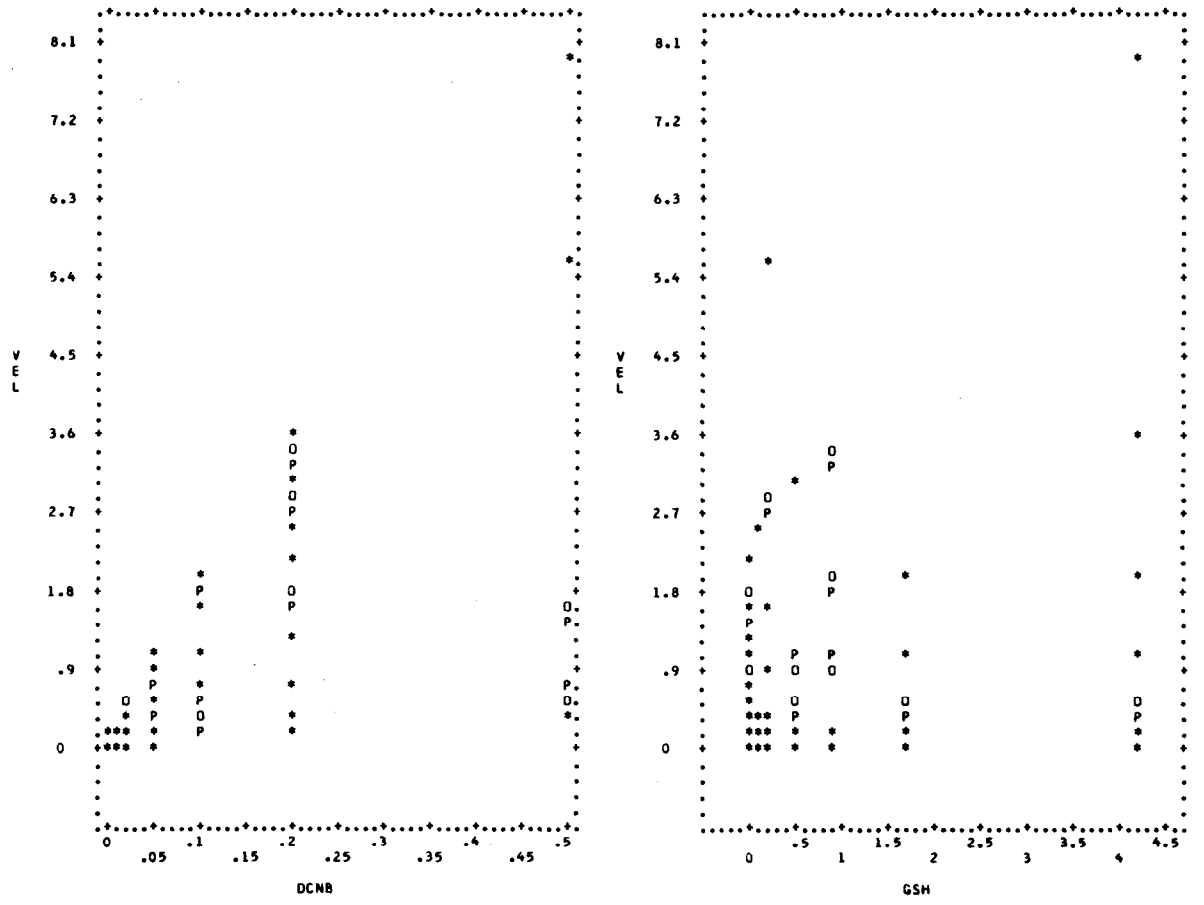

Fig.1. Fit of the rate law of model I to data set consisting of 91 experimental points (cf. table 1). The velocity is expressed in nmol/min and the concentrations in mM. Data at the lowest substrate concentrations are not resolved in the plots owing to the wide concentration range covered. (O) Observed velocities (P) Predicted velocities after regression (*) Coinciding observed and predicted values.

goodness of fit was judged by comparing the residual sum of squares, the residual plots, and the serial correlation of the residuals. All three tests demonstrated the superiority of degenerate models containing a constant term in the denominator.

The second way used to circumvent the difficulty that models I and II have more parameters than required for fitting the data by regression analysis was to use the asymptotic properties of models I and II. It is evident that at sufficiently low concentrations of A and B the terms containing second-power concentrations factors can be neglected, and model I will degenerate to

$$v = \frac{V_1 AB}{K_1 + K_2 A + K_3 B + AB} \qquad \text{(model III)}$$

whereas model II will degenerate to

$$v = \frac{V_1 AB}{K_2 A + K_3 B + AB} \qquad \text{(model IV)}$$

(Also the AB-term will be negligible when both A and B are small, but this condition only makes the parameter values in the denominator proportional to the estimate of V_1 in models III and IV and has no effect

Table 2
Comparison of the fitting of two alternative steady-state-kinetic models to a data set and to subsets generated by successive elimination of data corresponding to the highest substrate concentration levels (see text)

Data set (no.)	Number of data	Residual sum of squares	
		Model III	Model IV
1	48	6.486	9.044
2	24	1.396	2.809
3	20	0.340	0.898
4	16	0.063	0.499

The data set was different from that analyzed in table 1.

on the residual sum of squares). A data set (no.1) based on 8 GSH (5 μM–2 mM) and 6 DCNB concentration levels (10 μM–1 mM) was analyzed according to models III and IV. Next only the data obtained at the four lowest GSH levels ($\leqslant$ 50 μM) were used (data set no. 2). Then the two highest concentrations of DCNB (1 mM and 0.5 mM) were eliminated from the data in two stages (data sets nos. 3 and 4, respectively). Table 2 shows the residual sums of squares obtained by fitting the two models to the original and the restricted data sets. It is clear that the advantage of model III, demonstrated by its lower residual sum of squares, increases in comparison with model IV in the domain of low substrate concentrations. Furthermore, the standard error of K_1 in model III is less than 30% of the parameter value for each of data sets nos. 1–3, which shows that K_1 is not redundant. With data set no. 4 all parameters have low precision owing to the small number of data.

4. Discussion

The analysis presented in this communication shows clearly by objective numerical methods (within the limitations of non linear least-squares regression analysis) that the true rate equation of the reaction between GSH and DCNB catalyzed by GSH *S*-transferase A has to include a constant term in the denominator. This finding is strong evidence against the reaction scheme presented by Pabst et al. [5] and against any kinetic mechanism including a branch containing a ping-pong reaction pattern, i.e. involving release of the first product before both substrates have been bound to the enzyme. The product inhibition pattern supporting the mechanism of Pabst et al. [5] is also consistent with other reaction schemes such as the random sequential mechanism. The most direct evidence for the suggested ping-pong branch are results which demonstrate binding of radioactivity after incubation of the enzyme with labeled 1-chloro-2,4-dinitrobenzene or benzyl chloride [5]. However, 1-chloro-2,4-dinitrobenzene was not released completely by GSH and no data were presented to show that the binding was specific and kinetically relevant.

The random sequential mechanism gives rise to a rate law (model I) which contains the required constant term and which provides a good fit to the steady-state kinetic data. This mechanism seems compatible with all relevant information on the enzyme, but further analysis of the kinetics will be required to definitely assign the mechanism to GSH *S*-transferase A.

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