THE EFFECT OF ETHANOL ON THE STEADY-STATE KINETICS OF GLUTATHIONE *S*-TRANSFERASE A FROM RAT LIVER

Inga JAKOBSSON, Margareta WARHOLM and Bengt MANNERVIK
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm and
Unit of Occupational Toxicology, National Board of Occupational Safety and Health, S-100 26 Stockholm 34, Sweden

Received 5 April 1979

1. Introduction

Glutathione *S*-transferase A belongs to a family of enzymes in rat liver capable of detoxifying various electrophilic xenobiotics by conjugation with glutathione (GSH) [1]. It was purified extensively [2], and independently under the name form II of glutathione *S*-aryltransferase [3]. This enzyme is the only homogeneous GSH *S*-transferase which has been studied in detail by steady-state kinetic methods. The kinetics were found to be non-Michaelian [3,4] and the highly complex rate behaviour was interpreted by our group in terms of a steady-state random-order mechanism [5,6]. However, deviations from Michaelian kinetics may have many causes, and it is therefore essential to eliminate as many as possible of the alternative explanations. Studies of the binding of GSH and the GSH conjugate, *S*-(2-chloro-4-nitrophenyl)glutathione, formed from the substrates of the enzyme, showed that two binding sites exist per enzyme molecule but did not indicate deviations from a simple hyperbolic binding curve (unpublished experiments and [5,7]). Therefore, cooperative binding of substrates appears not to be the reason for nonhyperbolic kinetics. Likewise, the measured velocity was proportional to enzyme concentration [8], excluding association–dissociation of the enzyme as an alternative explanation. A remaining possible cause of nonhyperbolic kinetics is the inhibition exerted by ethanol [9] which is used as a solvent for the substrate 3,4-dichloro-1-nitrobenzene. If the inhibition were partial, alternative reaction pathways in the mechanism would be introduced, which may be expected to increase the degree of substrate-containing terms in the rate equation. Since ethanol has been included in all kinetic investigations involving 3,4-dichloro-1-nitrobenzene, it was crucial for the interpretation of the rate behaviour of the enzyme to investigate the effect of ethanol on the kinetics. The results reported here show that even if the concentration of ethanol is extrapolated to zero, the nonhyperbolic rate behaviour of both substrates as well as that of the product remains, and a trivial explanation of the complex kinetics is thus eliminated.

2. Materials and methods

GSH and 3,4-dichloro-1-nitrobenzene were obtained as in [3]; *S*-(2-chloro-4-nitrophenyl)glutathione was prepared enzymatically [5]. The enzyme was prepared and assayed as in [3,5]. The enzymatic reactions were started by addition of 3,4-dichloro-1-nitrobenzene dissolved in ethanol. Nonlinear regression analysis of initial velocity data was as in [5]. Weighting factors were based on the analysis of the experimental error structure, which was estimated by replicate velocity determinations [8]. The factors used were proportional to $v^{-\alpha}$, where $\alpha = 1.6$ was used in the absence of product [8] and $\alpha = 2.0$ in the presence of added product inhibitor. The discrimination between alternative rate equations and the judgement of goodness-of-fit were based on the criteria described in [10,11].

3. Results

3.1. *Qualitative examination of the inhibition*

The effect of ethanol on the kinetics of GSH *S*-transferase A was studied by keeping the concen-

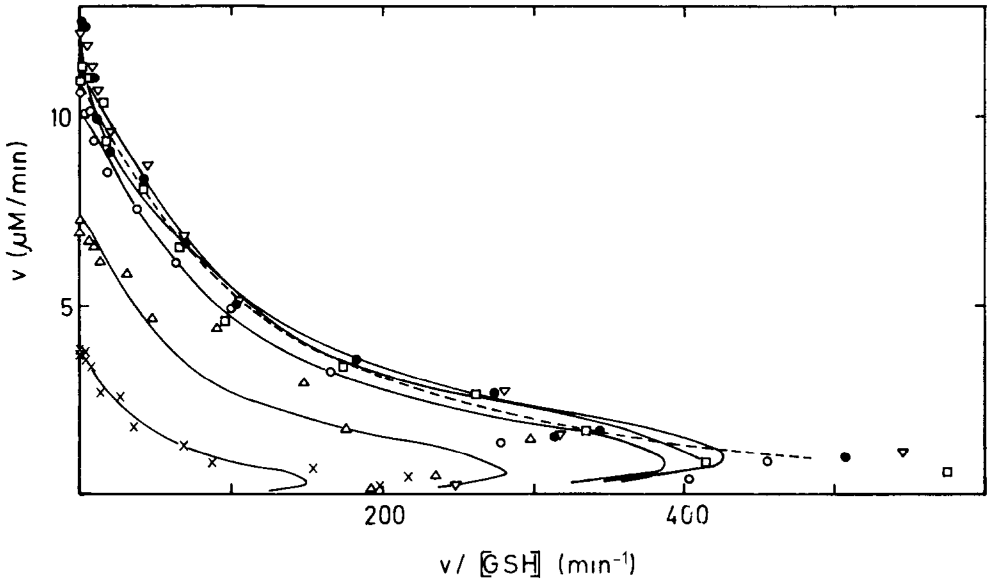

Fig. 1. Effect of ethanol on initial velocity at variable GSH concentrations. The concentrations of ethanol were 0.5% v/v (▽), 1% (□), 2% (○), 5% (△), 10% (X). 3,4-Dichloro-1-nitrobenzene was fixed at 0.1 mM and GSH varied from 1 μM–10 mM. The limit velocities obtained by extrapolation to zero ethanol concentration (●) were fitted separately (dashed curve) by a 4:4 function (cf. table 1).

tration of ethanol at various constant levels and varying the concentration of one of the substrates or of the product *S*-(2-chloro-4-nitrophenyl)glutathione. Ethanol could not be omitted (without addition of another organic solvent) owing to the low solubility of 3,4-dichloro-1-nitrobenzene in the buffer system. It was found that ethanol gave a reversible inhibition of the enzymatic reaction. Figure 1 shows the curves obtained by varying the concentration of GSH at different fixed levels (0.5–10%, v/v) of ethanol. Corresponding experiments involving varying concentrations of 3,4-dichloro-1-nitrobenzene and product, respectively, were carried out. In no case did the deviations from Michaelian kinetics appear to vanish by lowering the ethanol concentration. Figure 2 demonstrates that the inhibi-

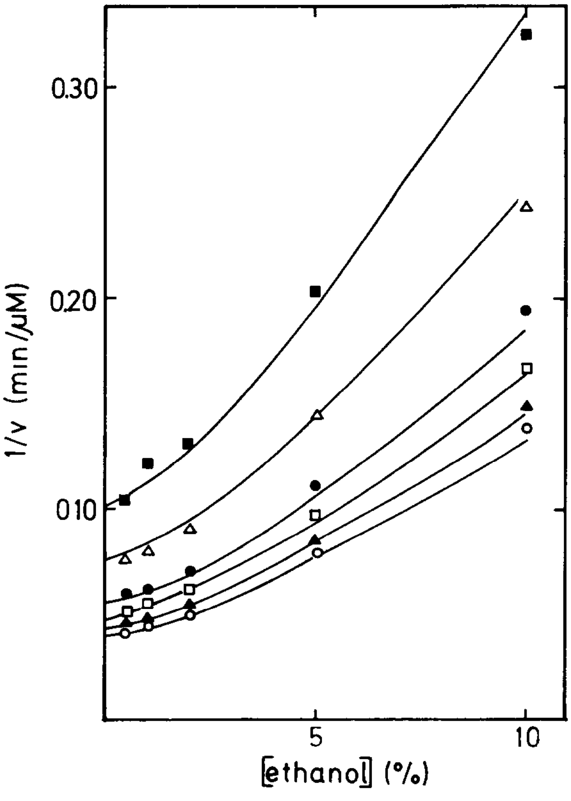

Fig. 2. Effect of ethanol on initial velocity at variable concentrations of the product *S*-(2-chloro-4-nitrophenyl)-glutathione (Dixon plot). The ethanol concentration was varied at the following levels of product: zero (○), 20 μM (▲), 60 μM (□), 100 μM (●), 160 μM (△), 200 μM (■). The substrates were fixed at 8.9 mM GSH and 0.1 mM 3,4-dichloro-1-nitrobenzene.

tion is nonlinear with respect to ethanol concentration when the product is kept at different constant levels. The velocity extrapolates to zero by increasing the ethanol concentration and the inhibition is accordingly not partial. The dependence of velocity on ethanol concentration in the experiments involving GSH or 3,4-dichloro-1-nitrobenzene as the second variable was similar.

3.2. *Regression analysis*

The kinetic data sets were examined by nonlinear regression analysis by the approach in [5] for GSH *S*-transferase A. Several alternative rate equations, based on reaction schemes involving ethanol-containing enzyme forms, were tested. The 'best' fits [10,11] were selected from rate equations involving the concentrations of ethanol and the varying reactants as independent variables. The results indicated that the full rate equation must be second or higher degree in 3,4-dichloro-1-nitrobenzene and GSH concentration; with respect to the concentrations of ethanol and product the full rate equation appeared to be a 0:2 and 2:4 function, respectively. These results are consistent with our earlier conclusions about the kinetics [5,6].

It was also attempted to establish the form of the rate equation applicable in the absence of ethanol (which could not be realized experimentally). To achieve this, the individual curves of the Dixon plots corresponding to the three kinds of data sets (involving one of the two substrates or the product as second variable) were extrapolated to zero ethanol concentration. The limit-velocity values obtained by this procedure were then replotted and analyzed by regression analysis. These velocities also displayed the nonhyperbolic behaviour. The corresponding rate equations for the limit velocities appeared not to be of lower degree with respect to reactant concentrations than the equations applicable in the presence of ethanol (table 1). However, it should be noted that equations of high degree often contain redundant parameters [12,13] and the selection of the 'best' models in table 1 is not unambiguous in view of such redundancies. It was nevertheless well established that the kinetics are non-Michaelian also in the absence of ethanol even if the degree of the rate equation cannot be established with absolute certainty.

Table 1

Fitting of alternative rate equations to limit-velocity values obtained by extrapolation to zero ethanol concentration[a]

Substrate varied	Residual sum of squares (arbitrary units)			
	Rate equation[b] 1:1	2:2	3:3	4:4
3,4-dichloro-1-nitrobenzene ($n = 13$)	4.0381	1.9363	1.6267	1.3207
GSH ($n = 12$)	6.000	0.8590	0.4089	0.3665

[a] The initial velocities analyzed when the concentration of GSH was varied are plotted in fig.1. The regression analysis was made on the limit values obtained by extrapolation to zero ethanol concentration (dashed line in fig.1); n is the number of limit values of the data set analyzed

[b] The rate equations used in the fitting were k:m functions ($k \leqslant m$) of the general form:

$$v = \left(\sum_{i=1}^{k} a_i[\mathrm{R}]^i\right) \Bigg/ \left(\sum_{j=0}^{m} b_j[\mathrm{R}]^j\right)$$

in which a_i and b_j are coefficients composed of rate constants and nonvaried reactant concentrations and R the varied reactant (cf. [5]). One of the coefficients of the denominator (b_j) was made equal to unity in the regression

4. Conclusion

The inhibition of GSH *S*-transferase A by ethanol is of the generalized noncompetitive type [14] with respect to both substrates, and it is furthermore not a case of partial inhibition. Thus, the substrates do not prevent the binding of ethanol and the inhibition appears to be unspecific. More importantly, the present study shows unambiguously that the complex kinetic pattern [5,6] is not an effect of the presence of ethanol. This conclusion was reached by mathematical modelling of the authentic kinetic data obtained in the presence of ethanol as well as of the limit velocities obtained by extrapolation to zero ethanol concentration. These results eliminate a trivial explanation of the non-Michaelian kinetics and lend further support to the steady-state kinetic random mechanism proposed for the enzyme [5,6].

Acknowledgement

This work was supported by a grant (to B M) from the Swedish Natural Science Research Council

References

[1] Jakoby, W B (1978) Adv Enzymol 46, 383–414

[2] Habig, W H , Pabst, M J and Jakoby, W B (1974) J Biol Chem 249, 7130–7139

[3] Askelof P , Guthenberg, C , Jakobson, I and Mannervik, B (1975) Biochem J 147, 513–522

[4] Pabst, M J , Habig, W H and Jakoby, W B (1974) J Biol Chem 249, 7140–7148

[5] Jakobson, I , Askelof, P , Warholm, M and Mannervik, B (1977) Eur J Biochem 77, 253–262

[6] Jakobson, I , Warholm, M and Mannervik, B (1979) Biochem J 177, 861–868

[7] Mannervik, B , Guthenberg, C , Jakobson, I and Warholm, M (1978) in Conjugation Reactions in Drug Biotransformation (Aitio, A ed) pp 101–110, Elsevier/North-Holland, Amsterdam, New York

[8] Askelof, P , Korsfeldt, M and Mannervik, B (1976) Eur J Biochem 69, 61–67

[9] Mannervik, B and Askelof, P (1975) FEBS Lett 56, 218–221

[10] Bartfai, T and Mannervik, B (1972) FEBS Lett 26, 252–256

[11] Mannervik, B and Bartfai, T (1973) Acta Biol Med Ger 31, 203–215

[12] Endrenyi, L and Kwong, F H F (1973) Acta Biol Med Ger 31, 495–505

[13] Reich, J G and Zinke, I (1974) Stud Biophys 43, 91–107

[14] Mannervik, B (1978) FEBS Lett 93, 225–227