EVALUATION OF THE TWO-SUBSTRATE PATHWAY OF GLYOXALASE I FROM YEAST BY USE OF CARBONIC ANHYDRASE AND RAPID-KINETIC STUDIES
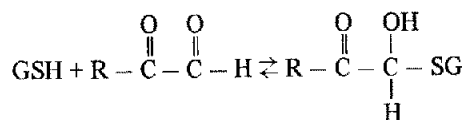
Ewa MARMSTÅL and Bengt MANNERVIK

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

1. Introduction

Glyoxalase I catalyzes the formation of S-D-lactoylglutathione from methylglyoxal and glutathione [1] and of S-D-mandeloylglutathione from phenylglyoxal and glutathione [2]. The formulation of a reaction scheme for the transformation of the reactants to products has to account for the rapid reaction between α-ketoaldehyde and glutathione, which nonenzymatically and reversibly produces a hemimercaptal adduct:

$$GSH + R-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}-H \rightleftarrows R-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}-\overset{\displaystyle OH}{\underset{\displaystyle H}{\overset{\displaystyle |}{C}}}-SG$$

Extensive studies of the steady-state kinetics of the enzyme acting on the equilibrium mixture of glutathione, methylglyoxal, and the adduct led to the proposal of a reaction scheme involving parallel one-substrate and two-substrate branches [3,4]. This model implies that methylglyoxal and glutathione on the one hand, and their hemimercaptal adduct on the other hand can serve as the substrates being bound and transformed to S-D-lactoylglutathione by the enzyme. In an evaluation of this reaction scheme the kinetics of the reaction between free glutathione and α-ketoaldehyde was investigated in the presence and absence of glyoxalase I and compared with the kinetics of formation of product from the equilibrium mixture of glutathione and α-ketoaldehyde [2]. It was found that the rate of dehydration of the hydrated α-ketoaldehyde was limiting for product formation. Therefore, no firm conclusion about the importance of the one- and two-substrate branches of the suggested reaction scheme could be drawn. The present investigation extends the kinetic studies to the rapid initial phase of the reaction by use of the stopped-flow technique. In these studies carbonic anhydrase was used in order to increase the rate of dehydration of the α-ketoaldehydes. The results obtained support the view that the reaction catalyzed by glyoxalase I is primarily a one-substrate reaction involving the hemimercaptal adduct as substrate.

2. Materials and methods

Glyoxalase I from yeast was obtained from Boehringer Mannheim and was purified to homogeneity as earlier described [5]. Homogeneous carbonic anhydrase from bovine erythrocytes (form B) was kindly provided by professor Sven Lindskog, Department of Biochemistry, University of Umeå, Sweden. The carbonic anhydrase preparation was demonstrated to lack glyoxalase I activity with both methylglyoxal and phenylglyoxal.

The stopped-flow experiments were carried out at 30°C on a Durrum Stopped Flow Spectrophotometer Model D 110. The reactions were monitored at 240 nm for methylglyoxal and at 263 nm for phenylglyoxal as substrate and recorded on an Omnigraphic Model 2000 X-Y-recorder. The extinction coefficients used were $3.37 \text{ mM}^{-1} \text{ cm}^{-1}$ for S-lactoylglutathione, $0.44 \text{ mM}^{-1} \text{ cm}^{-1}$ for the hemimercaptal of methylglyoxal, $1.1 \text{ mM}^{-1} \text{ cm}^{-1}$ for S-mandeloylglutathione, and $0.97 \text{ mM}^{-1} \text{ cm}^{-1}$ for the hemimercaptal of phenylglyoxal [2]. After mixing in the stopped-flow spectrophotometer the reaction system contained 50 mM Tris–HCl buffer pH 7.0, 5 mM glutathione, and 0.53 mM methylglyoxal or 0.13 mM phenylglyoxal. Glyoxalase I, when present, had a concentration of about 0.1 mg ml^{-1} in the reaction mixture.

The steady-state kinetics of glyoxalase I were investigated as described previously [3,4]. The equilibrium (dissociation) constant used for the system of glutathione, phenylglyoxal and their hemimercaptal adduct was 0.6 mM [2].

3. Results

3.1. The nonenzymatic reaction

The first experiments were designed to show whether or not the non-enzymatic formation of the hemimercaptal adduct from glutathione and α-ketoaldehyde was rate-limiting for the enzymatic formation of product. For this purpose the kinetics of the reaction between glutathione and α-ketoaldehyde were investigated in the absence of enzyme. The rate of formation of the hemimercaptal adduct has previously been shown to be independent of the concentration of glutathione, but directly proportional to
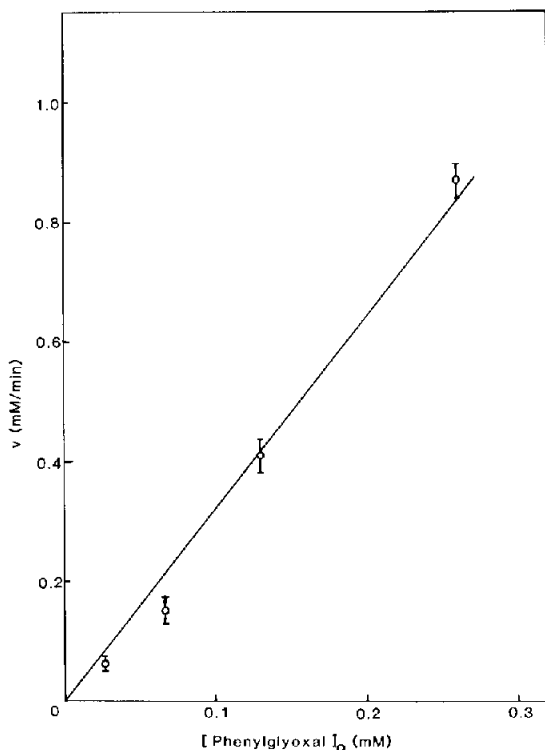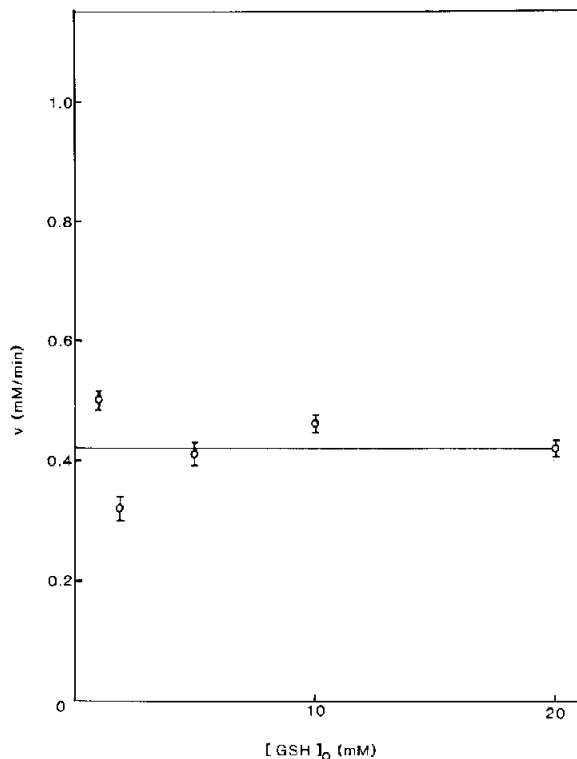

Fig.2. Rate of nonenzymatic hemimercaptal adduct formation as a function of glutathione concentration. After mixing in a stopped-flow spectrophotometer the assay system (30°C) contained 50 mM Tris–HCl (pH 7.0), 0.13 mM phenylglyoxal, and glutathione (1–20 mM).

the concentration of α-ketoaldehyde [2,6,7]. These findings were confirmed using various concentrations of glutathione and the two α-ketoaldehydes (figs. 1 and 2). The rate of formation of adduct using 5 mM glutathione was 0.49 ± 0.03 mM min^{-1} ($n = 4$) for 0.53 mM methylglyoxal and 0.35 ± 0.05 mM min^{-1} ($n = 4$) for 0.13 mM phenylglyoxal, corresponding to rate constants of 0.92 min^{-1} and 2.7 min^{-1}, respectively.

The rate-determining step in the hemimercaptal adduct formation is probably dehydration of the hydrated α-ketoaldehyde [2], which most likely takes place prior to the reaction with glutathione. In an attempt to accelerate the dehydration of the α-ketoaldehyde, the reaction was performed in the presence of carbonic anhydrase (0.5 mg ml^{-1}), which is known to catalyze the reversible hydration of aldehydes [8]. No effect of this enzyme was observed either on the shape of the progress curve, or on the rate of adduct formation with methylglyoxal as substrate. However,

Fig.1. Rate of nonenzymatic hemimercaptal adduct formation as a function of phenylglyoxal concentration. After mixing in a stopped-flow spectrophotometer, the assay system (30°C) contained 50 mM Tris–HCl (pH 7.0), 5 mM glutathione and phenylglyoxal (0.026–0.26 mM).

the rate of adduct formation from 5 mM glutathione and 0.13 mM phenylglyoxal was 0.64 ± 0.04 mM min^{-1} ($n = 3$), which is 1.8 times higher than in the absence of carbonic anhydrase. The finding of an effect on the reaction of phenylglyoxal but not on that of methylglyoxal may be explained by the assumption that phenylglyoxal is a better substrate for carbonic anhydrase (cf. [9]). Another explanation is that phenylglyoxal is more extensively hydrated than is methylglyoxal.

3.2. The enzymatic reaction

The glyoxalase I catalyzed formation of S-D-lactoylglutathione and S-D-mandeloylglutathione, respectively, was studied under three different conditions with respect to premixing of the reactants: glutathione, α-ketoaldehyde, and enzyme. The combination of enzyme and glutathione or enzyme and α-ketoaldehyde in one syringe was used to study the reaction in the absence of preformed hemimercaptal adduct. The formation of product under these conditions in which one of the two reactants was premixed with enzyme was estimated to be 0.45 ± 0.04 mM min^{-1} ($n = 3$) for methylglyoxal and 0.34 ± 0.03 mM min^{-1} ($n = 3$) for phenylglyoxal. These figures are not significantly different from those of the rate of adduct formation under the same conditions. The preincubation of glutathione and α-ketoaldehyde before mixing with the enzyme, results in a significantly higher reaction rate than preincubation of enzyme and a single reactant.

Also the enzymatic reactions were performed in the presence of carbonic anhydrase (0.5 mg ml^{-1}). These experiments gave the same results as those of the adduct formation discussed above. In the case of methylglyoxal, no increase in the rate of product formation was found. With 0.13 mM phenylglyoxal a rate of 0.61 ± 0.06 mM min^{-1} ($n = 3$) was recorded in the presence of carbonic anhydrase (0.5 mg ml^{-1}). Thus, also for the glyoxalase I catalyzed reaction did carbonic anhydrase increase the rate 1.8-fold. Attempts were made to increase the concentration of carbonic anhydrase in the assay system in order to detect any additional effects, but the high absorbance of the enzyme solution prevented use of concentrations above 0.5 mg ml^{-1}.

3.3. Steady state kinetics

An explanation of the finding that the rate of product formation catalyzed by glyoxalase I was
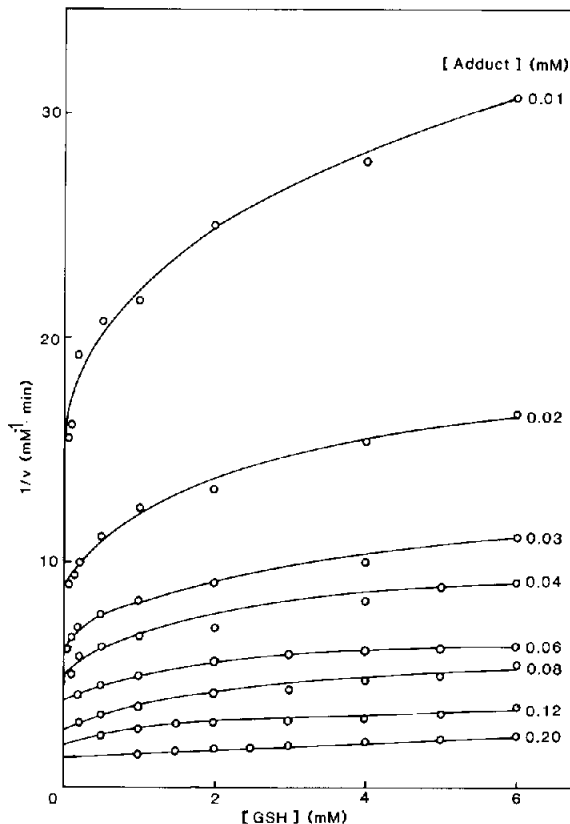

Fig.3. Dixon plot [14] of the influence of glutathione (GSH) concentration on the steady-state kinetics of glyoxalase I at different fixed concentrations of the adduct of phenylglyoxal and glutathione. Initial velocities were measured after equilibration of the reactants in the absence of enzyme. The concentrations were calculated on the basis of a dissociation constant of 0.6 mM for the equilibrium between phenylglyoxal, glutathione, and their hemimercaptal adduct [2].

higher after equilibration of glutathione and the α-ketoaldehyde might be that the concentration of free glutathione had decreased. Glutathione is known to be an inhibitor of the enzyme [3,4,6,10]. This possibility was investigated by a kinetic analysis under steady-state conditions. Fig.3 shows an experiment in which the glutathione concentration was varied at several fixed concentrations of the adduct, using phenylglyoxal as the α-ketoaldehyde. It is evident that the velocity of the enzymatic reaction is essentially unchanged if the glutathione concentration is lowered somewhat from 5 mM (the total concentration of glutathione) by the adduct formation. The adduct concentrations in fig.3 relevant to the stopped-flow experiments are approx. 0.1 mM. Consequently, this explanation is not valid.

4. Discussion

The present study was undertaken with the aim of revealing if, under any experimental conditions, the rate of product formation in the reaction catalyzed by glyoxalase I could exceed the rate of nonenzymatic formation of the hemimercaptal adduct. If the enzymatic reaction were more rapid than the spontaneous reaction, the hemimercaptal adduct of glutathione and the α-ketoaldehyde could not be an obligatory intermediate of the enzymatic reaction and a simple one-substrate reaction involving the adduct as substrate would be exluded. The rate of a two-substrate reaction involving free glutathione and free α-ketoaldehyde would not be restricted by the rate of adduct formation. The results of the experiments show that under the different conditions tested, the rate of the enzymatic reaction was, within the limits of experimental error, identical with the rate of formation of the hemimercaptal adduct. The experiments involved the use of methylglyoxal as well as phenylglyoxal in the presence and absence of carbonic anhydrase. No conditions could be found under which the rate of the enzymatic reaction exceeded the nonenzymatic rate of the adduct formation. These findings do not rigorously exclude the two-substrate pathway, because it cannot, a priori, be excluded that the rate of the enzymatic reaction could be less or equal to the rate of adduct formation. However, it seems unlikely that under the different conditions investigated the two rates would be equal (as found) unless the adduct were a true intermediate of the enzymatic reaction. We therefore conclude that the results of the present investigation exclude a significant contribution of the two-substrate pathway proposed for glyoxalase I [3,4]. The same conclusion was drawn by Vander Jagt et al. [2] on the basis of different experiments.

It remains to explain the non-Michaelian steady-state kinetics of glyoxalase I, which show that the reaction scheme has to involve alternative pathways [3,4]. The yeast enzyme is a monomer [11] with one binding site for glutathione derivatives [12]; facts which seem to exclude cooperativity between subunits or binding sites. A model involving isomerization of free enzyme according to the concept of enzyme memory [13] is at present considered to give the best explanation of the available information.

References

[1] Ekwall, K. and Mannervik, B. (1973) Biochim. Biophys. Acta 297, 297–299.
[2] Vander Jagt, D. L., Daub, E., Krohn, J. A. and Han, L.-P. B. (1975) Biochemistry 14, 3669–3675.
[3] Mannervik, B., Górna-Hall, B. and Bartfai, T. (1973) Eur. J. Biochem. 37, 270–281.
[4] Mannervik, B., Bartfai, T. and Górna-Hall, B. (1974) J. Biol. Chem. 249, 901–903.
[5] Marmstål, E., Aronsson, A.-C. and Mannervik, B. (1979) Biochem. J. 183, 23–30.
[6] Cliffe, E. E. and Waley, S. G. (1961) Biochem. J. 79, 475–482.
[7] Marmstål, E. (1979) Ph.D. thesis, University of Stockholm.
[8] Pocker, Y. and Meany, J. E. (1965) Biochemistry 4, 2535–2541.
[9] Pocker, Y., Meany, J. E. and Davis, B. C. (1974) Biochemistry 13, 1411–1416.
[10] Bartfai, T., Ekwall, K. and Mannervik, B. (1973) Biochemistry 12, 387–391.
[11] Marmstål, E. and Mannervik, B. (1978) FEBS Lett. 85, 275–278.
[12] Marmstål, E. and Mannervik, B. (1979) FEBS Lett. 102, 162–164.
[13] Ricard, J., Meunier, J.-C. and Buc, J. (1974) Eur. J. Biochem. 49, 195–208.
[14] Dixon, M. (1953) Biochem. J. 55, 170–171.