KINETICS OF THE REVERSE REACTION CATALYZED BY GLUTATHIONE REDUCTASE OF YEAST

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1. Introduction

Glutathione reductase (EC 1.6.4.2.) catalyzes the following reaction:

$$GSSG + NADPH + H^+ \rightleftharpoons 2\,GSH + NADP^+.$$

To a lesser extent the enzyme is also active with NADH. The steady-state kinetic behavior of the forward reaction has been widely investigated using enzymes prepared from yeast [1] and several other sources [2–6]. Initial velocity studies have in all instances given parallel double reciprocal plots, thus being consistent with a ping-pong [7] reaction mechanism involving successive binary complexes of the enzyme and one of the substrates at a time. Some more recent findings concerning the mode of inhibition of glutathione reductase of human [6, 8] and porcine [9] erythrocytes, and yeast [10] by the reaction products are, however, difficult to explain in terms of a simple ping-pong reaction model.

Kinetic data on the reverse reaction would be useful in solving the above apparent discrepancy, but such data are lacking presumably due to difficulties caused by the unfavorable reaction equilibrium [2, 3, 5].

It will be shown here that the reduction of $NADP^+$ by GSH, catalyzed by glutathione reductase of yeast, is greatly accelerated at pH values above neutrality and by the presence of a low molecular weight thiol, e.g. dithiothreitol. In this way it is possible to follow the kinetics of this reaction. The results are inconsistent with a ping-pong reaction mechanism for yeast glutathione reductase in the reverse direction, but indicate a 'sequential' [7] mechanism involving ternary complexes of the enzyme and both of the substrates.

2. Materials and methods

Yeast glutathione reductase was obtained commercially from Boehringer (Mannheim). At the time of the experiments it had a specific activity of 95–100 μmoles $\times$ min^{-1} $\times$ mg of $protein^{-1}$ according to Racker [11]. Before use the enzyme was incubated for 10 min in the presence of 1 mM dithiothreitol and 1 mM EDTA followed by dilution with a solution of bovine serum albumin (1 mg/ml). The concentration of dithiothreitol introduced into the final assay mixture in this way, was always less than 2 μM. $NADP^+$ and GSH were obtained from Sigma (St. Louis) and dithiothreitol from Calbiochem (Lucerne).

The assay mixtures contained in a final volume of 1.0 ml: 0.12 mmole each of tris and phosphate (pH was adjusted to 8.3 with NaOH), 1 μmole of EDTA, 100 μg of bovine serum albumin, and dithiothreitol and substrates as indicated in the figure legends. GSH and dithiothreitol were preincubated together at pH 8.3 for at least 10 min before their addition to the incubation to start the reaction. The formation of NADPH was followed at 340 nm using a Beckman model DU monochromator with a Gilford model 200 attachment and a recorder. Full scale sensitivities from 0.2 to 1.0 absorbance units were used. All experiments were carried out at 25°. A slow enzymic reduction of $NADP^+$ by dithiothreitol in the absence of GSH was observed and corrections were made using appropriate blanks.

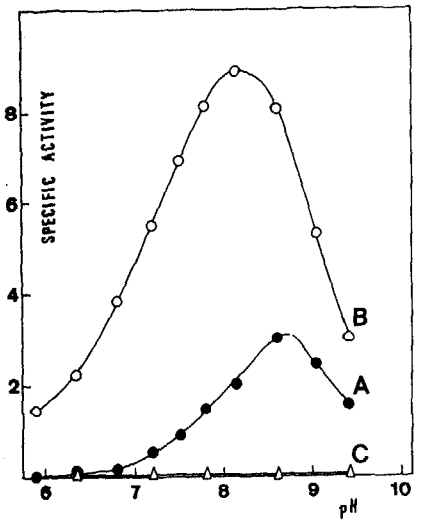

Fig. 1. Dependence of the reaction rate on hydrogen ion concentration without added dithiothreitol (curve A) and in the presence of 10 mM dithiothreitol (curve B). Enzyme, 1.7 μg of protein per assay; $NADP^+$, 3 mM; GSH, 10 mM; buffer, tris-phosphate, 0.12 M in respect to both components, adjustment of pH was made with NaOH. The indicated pH values were measured directly from each assay mixture after completion of the activity measurement. Curve C: reduction of $NADP^+$ by dithiothreitol in the absence of GSH; these values have been subtracted from curve B.

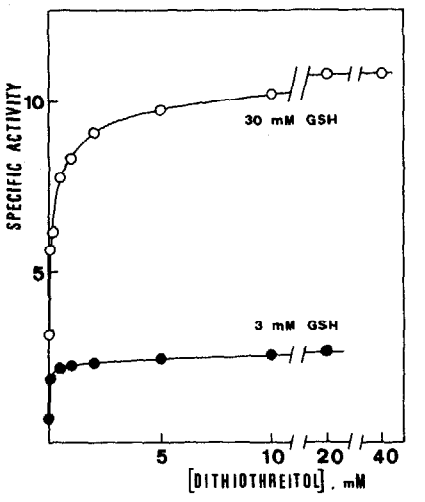

Fig. 2. Dependence of the reaction rate on dithiothreitol concentration at two different concentrations of GSH. Enzyme, 1.7 μg of protein per assay; $NADP^+$, 3 mM. For other conditions see Methods.

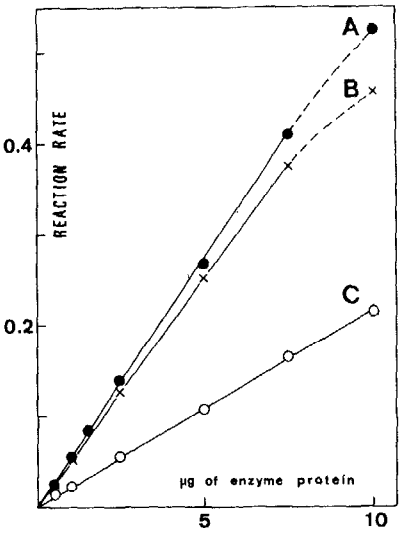

Fig. 3. Dependence of the reaction rate on the amount of enzyme added. $NADP^+$, 3 mM; GSH, 10 mM; dithiothreitol, 10 mM (curve A), 1 mM (curve B) or 0.1 mM (curve C). For other conditions see Methods. The reaction rate is expressed in change of absorbance at 340 nm per min.

All experiments described have been repeated at least twice, each time with identical results.

3. Results

3.1. *Effect of pH and dithiothreitol*

Curve A in fig. 1 shows that the pH optimum for the reduction of $NADP^+$ by GSH was 8.6–8.7. A rise of pH from neutrality to these values resulted in an approximate tenfold increase in reaction rate. The effect of added dithiothreitol at different pH values is represented by curve B. In this case the pH optimum was slightly lower (8.2–8.3), stimulation being most marked on the acid side. The rate of the slow and apparently pH-independent reduction of $NADP^+$ by dithiothreitol in the absence of GSH is represented by curve C.

The effect of the dithiothreitol concentration on the reaction rate at two different concentrations of GSH is shown in fig. 2. Qualitatively similar results were obtained with 2-mercaptoethanol although higher concentrations of this monothiol were necessary.

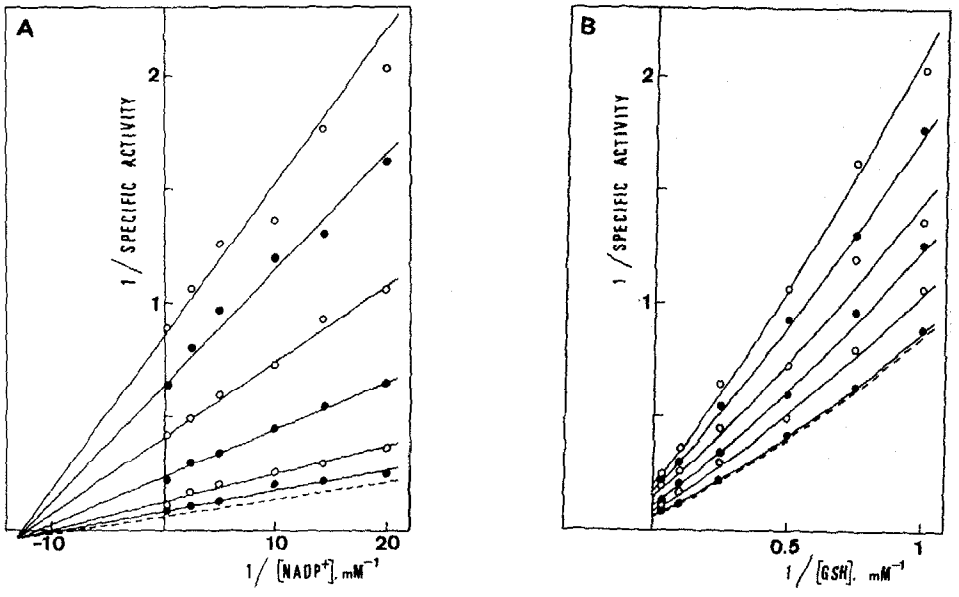

Fig. 4. Dependence of the reaction rate on the concentrations of $NADP^+$ and GSH. Enzyme, 3.3 μg of protein per assay; dithiothreitol, 10 mM. For other conditions see Methods.
(A) GSH concentrations (top to bottom): 1.0, 1.3, 2.0, 4.0, 10 and 30 mM. The broken curve represents an extrapolation to an infinite concentration of GSH drawn from the intercepts with ordinate in fig. 4B.
(B) $NADP^+$ concentrations (top to bottom): 0.05, 0.07, 0.10, 0.20, 0.40 and 3.0 mM. The broken curve represents an extrapolation to an infinite concentration of $NADP^+$ drawn from the intercepts with ordinate in fig. 4A.

Fig. 3. shows that a linear correlation existed between the reaction rate and a broad range of enzyme concentrations when dithiothreitol concentrations from 0.1 to 10 mM were used. At the 0.1 mM dithiol concentrations the individual progress curves were strongly nonlinear making calculation of the reaction rates difficult. This was not the case with the two higher concentrations.

3.2. *Initial velocity studies*

When the concentration of $NADP^+$ was varied at several fixed concentrations of GSH, the double reciprocal plots were linear and gave a series of converging lines (fig. 4A). When GSH was varied and the concentration of $NADP^+$ was kept constant, the individual plots were curved even at an infinite concentration of $NADP^+$, giving again a converging pattern (fig. 4B). A K_m value of 0.14 mM was calculated for $NADP^+$ at an infinite concentration of GSH. The nonlinearity of the plots did not permit an exact determination of the K_m value for GSH; it was estimated to be of the order of 10 mM.

3.3. *Product inhibition by NADPH*

In order to obtain additional information on the reaction mechanism, the effect of NADPH as an inhibitor was also studied. Fig. 5 shows that the inhibition by NADPH was competitive against $NADP^+$. As can be seen in the inset, the effect of NADPH on the slope of the individual plots was a linear function of inhibitor concentration.

4. Discussion

The pH optimum of the reverse reaction (8.6–8.7) was higher than the optimum (7.1) reported for the forward reaction [1]. In this respect the reaction catalyzed by yeast glutathione reductase resembles most of the oxidoreductions involving nicotinamide nucleotides, where higher pH values favor the reduction of the nucleotide and vice versa. This results from participation of the hydrogen ion in the reaction equilibrium.

The most attractive explanation for the stimula-

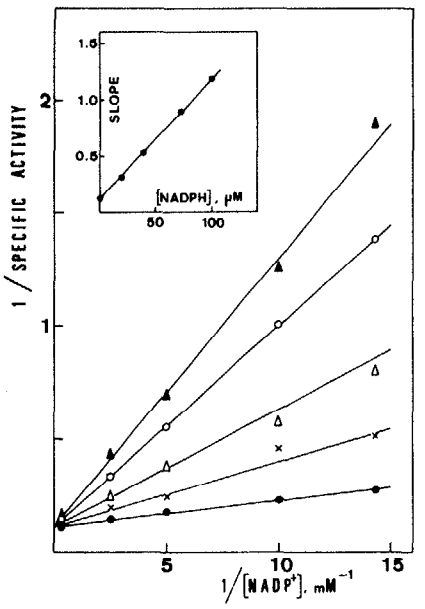

Fig. 5. Inhibition of the reaction by NADPH with $NADP^+$ as the variable substrate. Enzyme, 4.2 μg of protein per assay; dithiothreitol, 10 mM; GSH, 10 mM, NADPH concentrations (bottom to top): none, 19, 39, 72 and 99 μM. For other conditions see Methods. Inset: a secondary plot of the slopes of the lines versus the concentration of NADPH.

ting effect of the low molecular weight thiols is that they act by reducing GSSG. In addition to a continuous removal of this reaction product from the equilibrium, indicated by increase of linearity of the individual progress curves when the dithiol concentration was increased, one also has to take into account the presence of GSSG even in freshly made solutions of GSH. This phenomenon together with the fact that previous attempts to measure the reverse reaction were performed near neutrality, probably explains why satisfactory reaction rates for kinetic studies were not obtained.

The intersecting initial velocity pattern observed in this study differs from the parallel pattern reported for the forward reaction by several authors [1–6, 9]. This parallel pattern is mainly responsible for the widely accepted idea that glutathione reductase operates according to a ping-pong mechanism. The intersecting pattern observed in this work rather suggests a sequential mechanism for the reverse direction.

The finding that NADPH was a competitive inhibitor towards $NADP^+$ is also in disagreement with a ping-pong mechanism. Competitive inhibition by a product against the corresponding substrate could be expected in various sequential mechanisms (ordered, Theorell-Chance or rapid equilibrium random), but not in a ping-pong mechanism [7].

Studies on the reverse reaction catalyzed by glutathione reductase purified from human erythrocytes [4] are also in progress in this laboratory. The results so far obtained indicate behavior similar to the yeast enzyme.

Acknowledgements

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