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Purification and kinetic properties of glutathione reductase from bovine liver

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Abstract Glutathione reductase (GR, NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as reducing cofactor. The aim of the present work was to purify and characterize GR from bovine liver. GR was purified using 2′, 5′ ADP-Sepharose 4B and DEAE-Sepharose Fast Flow columns. The enzyme has been purified 5456-fold and with a yield of 38.4%. The molecular and catalytic properties of bovine liver GR have been studied. Optimum temperature and pH was found to be 50°C and 7, respectively. The activation energy of the reaction catalyzed by the enzyme was 9.065 kcal/mole. The molecular weight of the enzyme was found to be 55 kDa by SDS-PAGE. Kinetic characterization of bovine liver GR was also investigated, Km_{NADPH} 0.063 ± 0.008 mM and Km_{GSSG} 0.154 ± 0.015 mM were determined. It is accepted that parallel lines observed in these double reciprocal plots obeys Ping Pong mechanism and we have showed this in our steady state study. According to our results of statistical analysis, the Ping Pong mechanism is a suitable model since the loss function is less than the other mechanisms. However, competitive inhibition by a product could be accepted in sequential mechanisms but not in a Ping Pong mechanism. In this study, kinetic data are consistent with a branching reaction mechanism previously proposed for GR from other sources by other studies.

Keywords Glutathione reductase · Purification · Kinetics · Liver · Branched and Ping Pong mechanism

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Introduction

Glutathione reductase is a crucial enzyme, catalyzing the NADPH-dependent reduction of GSSG to GSH [1]. GR is mainly localized in cytosol, mitochondria, and chloroplast [2]. GRs had been purified from a variety of sources, from bacteria to mammalian cells, show great similarity both in physical and kinetic parameters, with different purification folds and yields; including the *E. coli* [3] human erythrocytes [4], rat liver [5], bovine brain [6], bovine ciliary body [7], calf liver [8], gerbil liver [9], anoxia-tolerant turtle, *Trachemys scripta elegans* [10], *Rhodospirillum rubrum* [11], *Moniezia expansa* [12], *Cyanobacterium anabaena* [13], malarial parasite *Plasmodium falciparum* [14], and pea leaves [15]. Purification and kinetic properties of bovine liver GR have not been reported previously. We have purified the GR from bovine liver by using a two step chromatographic procedure consist of 2′,5′-ADP Sepharose affinity and DEAE Sepharose anion exchange Fast Flow. In the previous years, ammonium sulphate fractionation, DEAE-Sephadex, Sephadex G-100, hydroxyapatite [8], Sephadex G-75, CM- cellulose, and sephacryl S-200 [5] columns have been used frequently for purification of GR. The enzyme has been purified very rapidly in high yield (47%) by employing N6-(6-aminohexyl)-adenosine-1′, 5′-bisphosphate Sepharose (N6-2′5′-ADP-Sepharose) [16] or 2′,5′-ADP-Sepharose 4B [8, 17] as affinity columns. Reactive Red-120-Agarose, Sephacryl S–300 [16], fast protein liquid chromatography FPLC-anion exchange, and FPLC-hydrophobic interaction chromatography [17] are also used for purification of the GR.

Kinetic mechanism of GR obeys to the Ping Pong mechanism [18] but some studies have proposed an ordered sequential mechanism [19]. Mannervik suggested that GR acting according to both Ping Pong and sequential

mechanism; this type of mechanism named as branched mechanism [20]. The kinetic data of GR from the filamentous C*yanobacterium anabaena* and the mouse liver are consistent with a branched mechanism [13, 21], the kinetic mechanism of human erythrocyte GR follows the Ping Pong/sequential ordered hybrid model [22]. In the kinetic studies of the GR from mycelium of *Phycomyces blakesleeanus*, at low concentrations of GSSG the Ping Pong mechanism prevails, whereas at high concentrations the ordered mechanism appears to dominate [23].

Materials and methods

Materials

Bovine liver, obtained from a local slaughterhouse was kept on ice and processed within 2–3 h after death.

Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), oxidized glutathione (GSSG), Tris [Tris (hydroxymethyl) aminomethane], DEAE Sepharose Fast Flow were obtained from Sigma Chemical Co., MO, USA. 2′, 5′-ADP-Sepharose 4B, were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) was from British Drug Houses Ltd. All other reagents were analytical grade and obtained from Sigma Chemical Co., MO, USA.

Assay of glutathione reductase

Glutathione reductase activity was determined according to modified Stall method [24]. The incubation mixture contained 100 mM sodium phosphate buffer, pH 7.4; 1 mM GSSG; 200 μM NADPH. Decrease in the absorbance of NADPH at 340 nm was monitored spectrophotometrically, at 37°C.

A unit of activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 μmole of NADPH in 1 min under these conditions. Specific activity is defined as units per mg of protein.

Protein assay

Protein concentrations of column fractions were determined measuring the absorbance at 280 nm and, while pooled protein concentrations of the purification steps were determined by the method of Bradford [25] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

SDS-PAGE was done using the method of Laemmli [26]. 10% slab gels were used for molecular weight determination. The gels were stained by the silver staining method of Merril [27].

Statistical analysis of kinetic data

The data were analyzed and the kinetic constants were calculated by means of a nonlinear curve-fitting program Statistica.

Purification of glutathione reductase

Step 1. Homogenization and ultracentrifugation

Bovine liver was purified by two subsequent chromatography consists of two steps after ultracentrifugation: 2′, 5′-ADP Sepharose 4B affinity and DEAE Sepharose Fast Flow anion exchange chromatography. All the procedures were carried out at +4°C. Bovine liver was minced with scissors after washing with physiologic serum and homogenized using an IKA ultra-turrax homogenizer with S18N-10G probe at 22, 000 l/min approximately 3 min with 3 volumes of 10 mM Tris/HCI buffer, pH 7.6, containing 1 mM 2-mercaptoethanol and 1 mM EDTA (buffer A). The homogenate was then centrifuged at 105,000 × *g* for 60 min.

Step 2. Affinity chromatography

The supernatant obtained was loaded onto a 2′, 5′-ADP-Sepharose 4B column (1.5 × 6.7 cm) equilibrated with buffer A. The column was washed with the same buffer (the flow rate was 10.8 ml/h) until the absorbance at 280 nm decreased to 0.045 O.D. to remove all non-specifically bound compounds. 6PGD was not bound and eluted while washing the column with buffer A. GR and G6PD were bound to the column. Then GR and G6PD were co eluted with buffer A containing 0.1 mM $NADP^+$. But we have pooled the fractions, which were showed just only GR activity (the flow rate was 10.8 ml/h). Elution profile of GR from 2′, 5′-ADP-Sepharose 4B column is given in Fig. 1.

Step 3. DEAE chromatography

The enzyme solutions obtained in the previous step loaded onto DEAE Sepharose Fast Flow column (1.5 × 7.5 cm) equilibrated with 5 mM potassium phosphate buffer, pH 6.9 (buffer B). The flow rate was maintained at 16.8 ml/h, the column was washed with buffer B until the absorbance at 280 nm decreased to 0.03 O.D. and GR was eluted with buffer B containing 200 mM KCl (Fig. 2).

The whole purification procedure took two working days. The enzyme was stable for one week at 4°C. No stabilizers were added to pure GR.

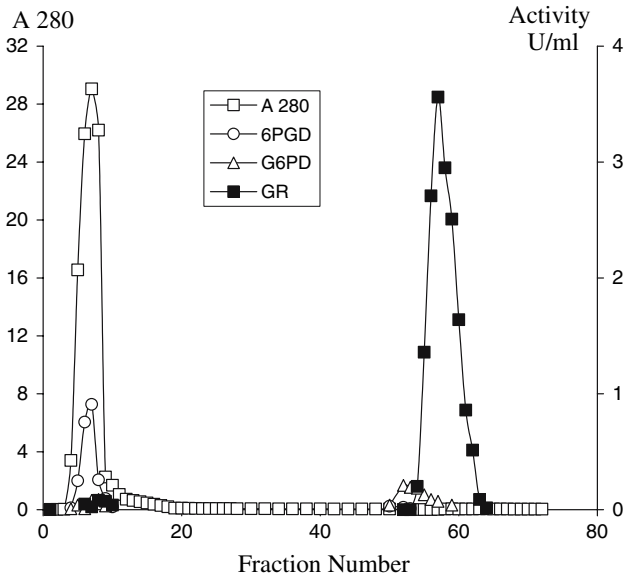

Fig. 1 Affinity chromatography of GR on 2′, 5′-ADP-Sepharose 4B. Column size, 1.5 × 6.7 ml; column equilibration and washing buffer, 10 mM Tris/HCl pH 7.6 containing 1 mM 2-ME and EDTA. Enzyme elution buffer: same as the washing buffer containing 0.1 mM $NADP^+$; flow rate, 10.8 ml/h. Fractions of 1.52 ml were collected

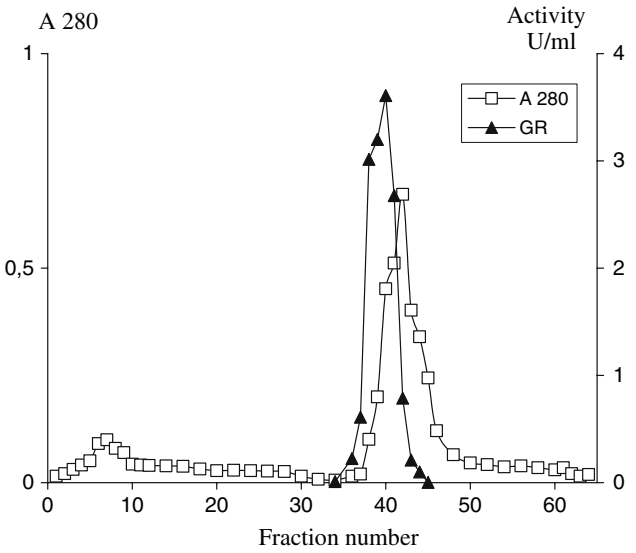

Fig. 2 Elution profile of GR from DEAE Sepharose Fast Flow. Column size, 1.5 × 7.5 cm. GR was eluted with 200 mM KCl prepared in 5 mM potassium phosphate buffer, pH 6.9 at a flow rate of 16.8 ml/h. Fractions of 1.35 ml were collected

Results and discussion

Some properties of glutathione reductase

GR has been purified from bovine liver 5,456 fold and with a yield of 38.4% and specific activity of the enzyme 185. The purification procedures included high-speed ultracentrifugation, 2′, 5′-ADP Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow anion exchange chromatography. Previously GR has been purified from bovine brain by procedures included, 35–70% ammonium sulphate fractionation and DEAE cellulose, 2′, 5′-ADP Sepharose, Superdex 200 prep grade column chromatography respectively [28]. The purification procedure which we presented includes mainly two steps and allows the purification of the enzyme in a very short time with a high yield. Also in our purification protocol we have centrifuged at 105,000 × *g* for 1 h to get cytosolic fraction. In addition, GR was separated well from G6PD at the end of the purification as seen in Fig. 2. A summary of the purification is presented in Table 1. Similar purification fold and specific activity results were observed for the enzymes from bovine brain 5,000 fold, specific activity 145 [28]. In this study we have preferred to elute enzyme by $NADP^+$ because it was shown that preincubation with NADPH resulted in 90% loss of activity which could be partially reversed by 2 mM GSSG but not GSH. GSH also caused inactivation which potentially amounted to >80%. This inactivation could not be reversed by GSSG [29]. Mouse liver GR has been purified by using 2′, 5′-ADP-Sepharose from which was specifically eluted by using $NADP^+$ gradients [30]. But in another study bovine erythrocyte GR was eluted with a gradient of 0–0.5 mM GSH and 0–1 mM NADPH [31].

For the optimal pH determination, the enzyme activity was measured in 10 mM potassium phosphate buffer within the pH range of 5–10. The enzyme was found to be stable between pH 6.5 and 10 and two optima occurred at pH 7 and 8 (data was not shown). This type of curve may be seen for diprotic systems and indicate that the active site of the enzyme contains several ionizable groups [32]. Optimum pH of the enzyme purified from various sources was found to be between pH 6.5 and 7.0 [4, 33]. However, in *Phycomyces blakesleeanus* [23] and *Mytulis edulis* [34] optimum pH of the GR was determined to be pH 7.5; in *Eugleuna gracilis* [35] and *Cyanobacterium Anabaena* sp. Strain 7119 optimum pH of GR was found to be 8.2, 9.0, respectively [13].

To obtain the optimum temperature, the activities of the enzyme were measured at saturating substrate concentrations in 100 mM sodium phosphate buffer, pH 7.4; between 20 and 80°C (data was not shown). The optimum temperature was obtained from the graph as 50°C. But in our experiments we preferred to study at physiological temperature (37°C). GR from wheat grain was relatively resistant to high temperatures and was very resistant to very low temperatures [36]. To obtain Arrhenius plot, the activities of the enzyme were measured between 20 and 55°C. Activation energy (Ea) was determined from the slope of the plot as 9.065 kcal/mole (data was not shown).

Table 1 Summary of the typical purification procedure of glutathione reductase from bovine liver

Purification step	Volume(ml)	GR activity (U/ml)	Total GR activity	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Homogenate	20 ml	2.6	52.5	75	1500	0.03	100	1
105,000 × *g* supernatant	9.35 ml	3.5	32.5	14.5	135.4	0.24	62.3	7.1
2′,5′-ADP-Sepharose 4B	10.7 ml	2.0	21.5	0.6	6.3	3.39	41.2	99.8
DEAE Sepharose Fast Flow	5.4 ml	3.7	20	0.02	0.1	185.5	38.4	5456

The subunit molecular weights of GR, from different sources are between 70 and 140 kDa [7, 23, 34]. The molecular weight of the GR from bovine liver was found to be 55 kDa by SDS-PAGE (Fig. 3). The purified enzyme gave a single band on and SDS-PAGE (Fig. 4).

Kinetics of bovine liver glutathione reductase

In general, multi-substrate reactions were defined as Ping Pong or sequential mechanisms [32]. We have also reported that kinetic mechanism of the sheep kidney cortex G6PD found to operate according to a Ping Pong Bi Bi mechanism; the reciprocal plots have parallel lines and the loss function of the Ping Pong mechanism was less than the sequential mechanism [37].

In this study we have determined the kinetic mechanism of bovine liver GR. Initial-rate studies with the enzyme were performed; GSSG was used as variable substrate and the double reciprocal plots were drawn at different fixed concentrations of the other substrate, NADPH (Fig. 5).

The parallel lines observed in these double reciprocal plots are consistent with the widely accepted idea that GR operates according to a Ping Pong mechanism (Fig. 5). According to the results of statistical analysis, the Ping Pong mechanism is a convenient model since the loss function is less than the others. This kinetic behavior is typical of an enzyme reaction where the enzyme reacts with one substrate to be converted to a modified form before reacting with the second substrate which reconverts it to the original form. The K_m values for NADPH and GSSG and V_m were determined to be 0.063 ± 0.008 mM, 0.154 ± 0.015 mM and 239.463 ± 15.469 respectively (Table 2).

Product inhibition of bovine liver glutathione reductase by NADP+

To identify the real type of the mechanism, product inhibition studies were also performed. Figure 6 illustrates the

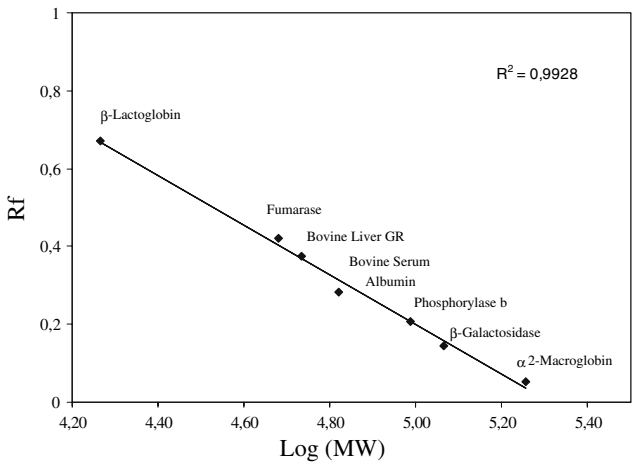

Fig. 3 Estimation of the subunit molecular weight of GR by SDS-PAGE (10% acrylamide gel was used). Line 1. Homogenate, Line 2. 105,000 × *g* supernatant, Line 3. 2′,5′-ADP-Sepharose 4B eluant Line 4. DEAE Sepharose Fast Flow eluant Line 5,The protein standards: α_2-Macroglobulin, 180,000 Da; *β*-Galactosidase (*E. coli*), 116,000 Da; Phosphorylase b (Rabbit Muscle), 97,400 Da; Serum Albumin (Bovine), 66,000 Da; Fumarase (Porcine Heart) 48,500 Da; *β*-Lactoglobulin (Bovine Milk) 18,400 Da; The protein band is shown by an arrow. The molecular weight of the enzyme was found to be 55 kDa

Fig. 4 Standard Rf-LogMW graph of bovine liver GR by SDS-PAGE

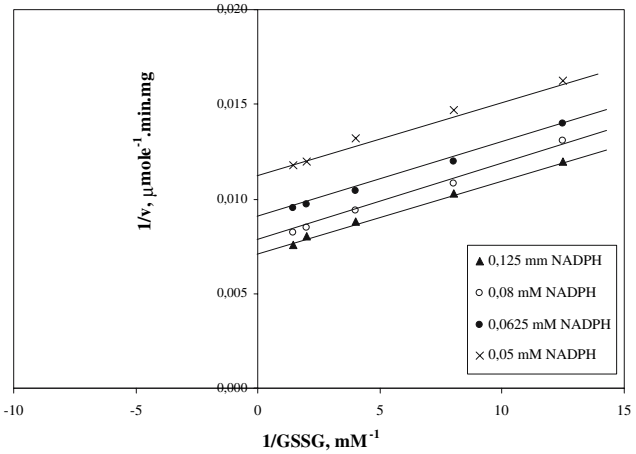

Fig. 5 Double reciprocal plots of initial velocity of the bovine liver GR against as GSSG varied substrate at different fixed NADPH concentrations. The assays were carried out at 37°C in100 mM sodium phosphate buffer, pH 7.4, as described under Materials and methods

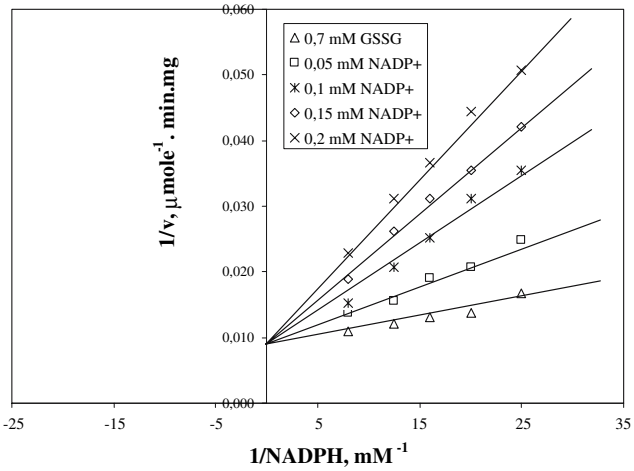

Fig. 6 Product inhibiton by $NADP^+$ of the reaction catalyzed by bovine liver GR with NADPH as the variable substrate at fixed GSSG concentrations. The assays were carried out at 37°C in100 mM sodium phosphate buffer, pH 7.4, as described under Materials and methods

Table 2 Kinetic parameters of GR from bovine liver

Parameter	Value
Km NADPH	0.063 ± 0.008 mM
Km GSSG	0.154 ± 0.015 mM
Ki $NADP^+$ (varied NADPH)	0.043 ± 0.003 mM
	Competitive
Ki GSH (varied NADPH)	8.506 ± 0.563 mM
	non-competitive
Ki $NADP^+$ (varied GSSG)	0.219 ± 0.01 mM
	Uncompetitive
Ki GSH (varied GSSG)	7.985 ± 0.466 mM
	non-competitive

inhibition pattern obtained with $NADP^+$ as the product inhibitor when NADPH was the variable substrate at fixed GSSG concentrations (0.7 mM). From these steady state kinetics data we have calculated the inhibition constant (Ki) of $NADP^+$ as 0.043 ± 0.003 mM. The inhibition by $NADP^+$ was competitive with respect to NADPH (Fig. 6).

The study was repeated by varying GSSG at constant NADPH concentrations. Initial velocities were determined at the same concentrations of $NADP^+$. From the statistical analysis of data, the inhibition type was found to be uncompetitive with respect to GSSG and Ki was calculated as 0.219 ± 0.01 mM (Fig. 7).

Product inhibition of bovine liver glutathione reductase by GSH

GSH is also used in product inhibition studies. We have found that GSH inhibited the reaction, but only at high concentrations. GSH was found to be a non-competitive inhibitor with respect to both substrates. First, NADPH was varied substrate (Fig. 8) and GSSG was fixed substrate (0.7 mM), initial velocities were measured at 1, 2, and 4 mM concentrations of GSH. From the statistical analysis of data Ki was calculated as 8.506 ± 0.563 mM.

With regard to GSSG as variable substrate at fixed NADPH concentration (0.1 mM), GSH appears to be a non-competitive inhibitor (Fig. 9). From the double reciprocal plots and statistical analysis, Ki was calculated as 7.985 ± 0.466 mM.

In previous studies both Ping Pong mechanism [38] and branched mechanism have been proposed for GR [38–40].

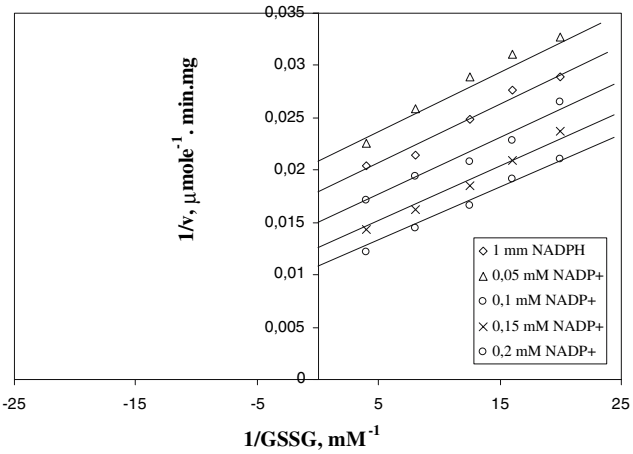

Fig. 7 Product inhibition by $NADP^+$ of the reaction catalyzed by bovine liver GR with GSSG as the variable substrate at fixed NADPH concentrations. The assays were carried out at 37°C in100 mM sodium phosphate buffer, pH 7.4, as described under Materials and methods

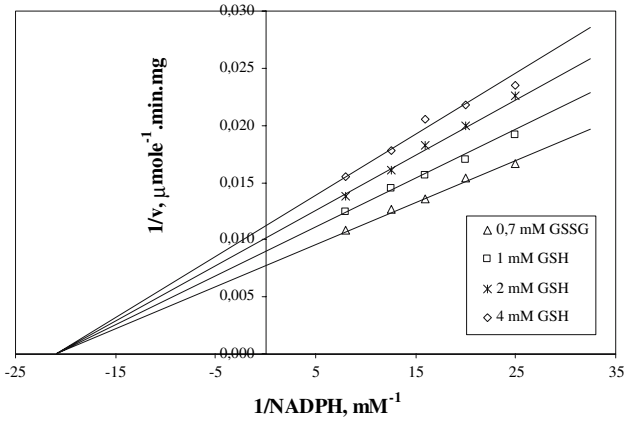

Fig. 8 Product inhibition by GSH of the reaction catalyzed by bovine liver GR with NADPH as the variable substrate at fixed GSSG concentrations. The assays were carried out at 37°C in100 mM sodium phosphate buffer, pH 7.4, as described under Materials and methods

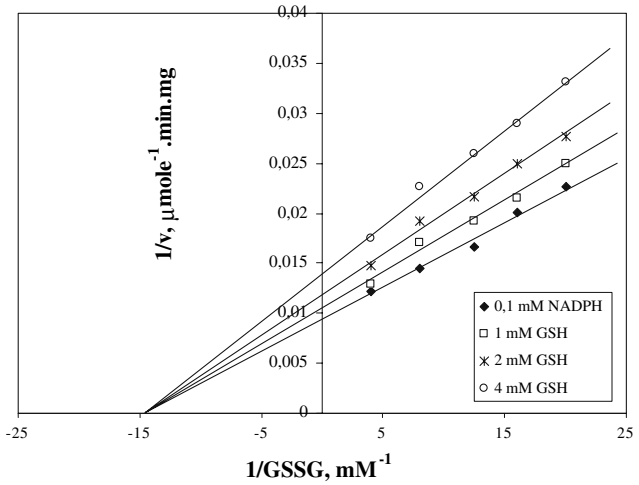

Fig. 9 Product inhibition by GSH of the reaction catalyzed by bovine liver GR with GSSG as the variable substrate at fixed NADPH concentrations. The assays were carried out at 37°C in100 mM sodium phosphate buffer, pH 7.4, as described under Materials and methods

However, at low GSSG concentrations the rate equation can be approximated by that of a simple Ping Pong mechanism [5]. Yeast GR, follows a sequential or Ping Pong mechanism at high or low $NADP^+$ concentrations, respectively [40].

In this study we have studied the steady state kinetics of GR at pH 7.4 and data are consistent with a branching reaction mechanism previously proposed for GR from yeast [20]. The steady-state kinetic studies of yeast GR, performed when [GSSG] = 10[NADPH] in the assay mixture, show that at concentrations of GSSG under 450 microM the enzymatic mechanism pathway is Ping Pong. Furthermore, in the case of higher values, the enzymatic kinetics follows a sequential pathway. However, when the GR reaction passes to the Ping Pong mechanism, the inhibition effect by excess of NADPH is stronger than when the reaction takes place over the sequential mechanism [41].

Conclusion

In this study, GR has been purified from bovine liver by using 2′, 5′ ADP-Sepharose 4B and DEAE-Sepharose Fast Flow columns. Some properties and kinetic characterization of the purified bovine liver GR have been investigated. This enzyme is essential for the antioxidative system that maintains adequate levels of reduced cellular GSH. Since GR is cytoprotective against ROS-induced damage, a better understanding the molecular and catalytic properties of this enzyme is important.

In the studies about the kinetic mechanisms of GR from different sources such as, *Cyanobacterium Anabaena* sp. Strain 7119 [13], *E. coli* [3], *Chromatium vinosum* [42], rat liver [5] seems to be a binary complex or two-site Ping Pong mechanism. Several previously investigated GRs give a steady-state kinetic pattern which is typical of a Ping Pong reaction mechanism [3, 5, 13]. The yeast enzyme, like the erythrocyte enzyme, is inhibited by $NADP^+$ and inhibition is competitive with NADPH [20]. We have also found that inhibition of $NADP^+$ is competitive with NADPH. 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 (Theorell-Chance, ordered, or rapid equilibrium random), but not in a Ping Pong mechanism [43]. In this study, steady state kinetics shows that bovine liver GR follows the Ping Pong mechanism. However, the kinetic data obtained from product inhibition studies was indicated sequential ordered mechanism. This could be explained by supposing that the GR normally functions by branching mechanism. As is the case with the same enzyme from other sources, the kinetic data is consistent with a branched mechanism.

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