THE EFFECT OF GLYCEROL IN THE STORAGE MEDIUM ON THE INHIBITORY KINETIC BEHAVIOR OF GLUTATHIONE REDUCTASE

GULNIHAL KULAKSIZ-ERKMEN[1], BETUL CAN[1], OZLEM DALMIZRAK[1], I. HAMDI OGUS[1] and NAZMI OZER[1,2]

[1] *Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University,* 06100, Sihhiye, Ankara, Turkey
[2] *Department of Basic Medical Sciences, Faculty of Medicine, Near East University,* Nicosia, 922022, Mersin 10, Turkey

Abstract - In this study we report on the effect of glycerol used in storage buffers, on the inhibitory kinetic behavior of $NADP^+$ on rat kidney glutathione reductase (rkGR) stored at -80°C in ± 10% (v/v) glycerol. At fixed [GSSG] and varied [NADPH], rkGR stored ± glycerol were inhibited competitively by $NADP^+$. Although the addition of glycerol decreased K_m (9.7 ± 0.9 μM) and $K_{i(NADP+)}$ (26.6 ± 2.2 μM) for NADPH, the catalytic efficiency ($k_{cat}/K_m = 2.75$ x 10^7 $M^{-1}.s^{-1}$) was increased 1.64 fold. At fixed [NADPH] and varied [GSSG], rkGR exhibited noncompetitive ($K_i = 397 ± 69$ μM) and linear mixed-type ($K_i = 59.6 ± 8.4$ μM; $K_s = 49.9 ± 3.2$ μM; α = 7.74) inhibitions with and without glycerol during storage. The k_{cat}/K_m of rkGR stored in glycerol was 5.16 x 10^6 $M^{-1}.s^{-1}$. Although the increase in k_{cat} was 1.25 fold, the k_{cat}/K_m was minimally affected (1.06).

Key words: Glutathione reductase, glycerol, rat kidney, $NADP^+$, inhibition kinetics

INTRODUCTION

Reduced glutathione (GSH) is the major form of intracellular glutathione that participates in the detoxification of harmful electrophilic compounds. It maintains proteins in their proper redox states and protects cells against oxidants through its antioxidant property (Rall and Lehninger, 1952; Gerard-Monnier and Chaudiere, 1996; Yang et al. 2006). In fulfilling these functions, GSH is converted to its oxidized disulfide form, GSSG, which is not able to accomplish these effects. Thus, it is necessary to recycle GSSG back to GSH. Glutathione reductase (GR, $NADP^+$ oxidoreductase; E.C. 1.8.1.7) catalyzes the reduction of GSSG to GSH at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced mainly by the pentose phosphate pathway (Rall and Lehninger, 1952; Meister, 1983; Dickinson and Forman, 2002). Thus, it is the pivotal enzyme in

regulating the redox thiol buffer against oxidative damage (Deneke, 2000).

The reaction catalyzed by GR can be summarized as:

$$NADPH + H^+ + GSSG \xrightarrow[\text{Reductase}]{\text{Glutathione}} NADP^+ + 2GSH$$
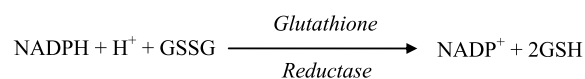
[12,19]

GR was purified from various sources and substrate and inhibitory kinetics have been studied in detail (Mavis and Stellwagen, 1968; Staal and Veeger, 1969; Carlberg and Mannervik; 1975; Worthington and Rosemeyer, 1976; Moroff and Kosow, 1978; Lopez-Barea and Lee, 1979; Carlberg and Mannervik, 1981; Scrutton et al., 1987; Acan and Tezcan, 1989; Takeda et al., 1993; Gutterer et al., 1999; Ulusu

and Tandoğan; 2007). The end-products of GR reaction, GSH and NADP⁺, have been shown to inhibit GR (Staal and Veeger, 1969; Carlberg and Mannervik; 1975; Worthington and Rosemeyer, 1976; Moroff G and Kosow, 1978; Lopez-Barea and Lee, 1979; Ulusu and Tandoğan; 2007) with some exceptions (Willmore and Storey, 2007). We could not find any study investigating the inhibitory behavior of NADP⁺ on rat kidney GR.

Polyols (glycerol, inositols, sorbitols) and derivatives (i.e. o-methyl inositol) are called osmolytes. They belong to the "compatible" solutes category, based on the concept that they do not perturb cellular macromolecules, even at high concentrations (Brown and Simpson, 1972; Yancey, 2005). They stabilize the native structures of proteins and reduce damage by ice crystals (Brown and Simpson, 1972; Timasheff, 1998; Shimizu and Boon, 2004). The *In vivo* effects of glycerol have inspired their use in laboratory work. Glycerol is commonly used in the storage of enzymes below zero degrees Celsius. Understanding the effect of glycerol on enzymes is crucial to provide the proper information on their kinetic parameters. To our knowledge, there are limited studies investigating the effect of glycerol on the enzyme kinetics (Levy et al., 1966, Olsen et al., 2007). It is reported that osmolytes, with some exceptions, are preferentially excluded from protein-water interfaces to different extents (Timasheff, 1998; Yancey, 2005), but their effects on the kinetics of different enzymes cannot be predicted.

In this study, the inhibitory behavior and kinetic parameters of NADP⁺ on GR purified from rat kidney (rkGR) stored at -80°C with and without glycerol was investigated. First, GR was purified from rat kidneys using conventional chromatographic methods (Can et al., 2010) and stored at -80°C in buffer only or buffer supplemented with 10% (v/v) glycerol. Second, the inhibitory behavior and inhibition kinetics of NADP⁺ on rkGR at fixed [GSSG] / varied [NADPH] and at fixed [NADPH] / varied [GSSG] were elucidated. The data obtained were processed using different kinetics models, and inhibition parameters were calculated using Statistica (version)

1999 for Windows. It was shown that both rkGR, stored in buffer only and buffer supplemented with 10% (v/v) glycerol, gave competitive inhibition at fixed [GSSG] and varied [NADPH]. However, with fixed [NADPH] and varied [GSSG] the kinetic behavior of rkGR was different; rkGR stored in glycerol gave a noncompetitive type of inhibition, whereas rkGR stored in buffer gave linear mixed-type inhibition. It seems that the storage in glycerol affects both the kinetic behavior and kinetic constants of rkGR; it decreases the kinetic constants for NADPH but increases for GSSG.

MATERIALS AND METHODS

Chemicals

Sephadex G25 was purchased from Pharmacia (Uppsala, Sweden), Polybuffer Exchanger 94 (PBE 94), 2',5'-ADP Agarose was purchased from Sigma (Steinheim, Germany). Other chemicals were analytical grade and obtained from Sigma (St. Louis, MO, USA) or Fluka (Germany).

Purification of rat kidney GR

This study was approved by Hacettepe University Local Ethic Committee. Wistar rats were sacrificed and their kidneys were removed. rkGR was purified according to Ogus and Ozer's method (Ogus and Ozer, 1991) with some modifications. Briefly, heat denaturation, Sephadex G25 gel filtration, 2',5'-ADP agarose affinity chromatography, ion-exchange chromatography (PBE 94) steps were used to purify the rkGR. The purified enzyme was stored as 0.2 ml aliquots in 50 mM potassium phosphate buffer (KPi), pH 7.4, at -80°C with and without 10% (v/v) glycerol and the specific activity of rat kidney GR was 250 units per milligram protein (Can et al., 2010).

Assay of rkGR activity

The stored enzyme was thawed in a 37°C waterbath and transferred onto ice, and the reaction was initiated by transferring 4 μl of enzyme directly from stock into 500 μl of incubation mixture preincubated

at 37°C for 3 min. Enzyme from the stock thawed once was not reused. In all experiments, the final concentration of glycerol in the reaction mixture was ≤ 0.08% (v/v). Rat kidney GR stored without glycerol exerted the same kinetic behavior in the presence and absence of 0.08 % (v/v) glycerol in the reaction mixture.

The activity of rkGR was determined spectro-photometrically at 37°C in an incubation mixture of 500 μl, containing 100 mM KPi pH 7.4, 4 mM ethyl-enediamine tetraacetic acid (EDTA), 1 mM GSSG, 0.1 mM NADPH, by using Shimadzu UV-1700 spec-trophotometer. The reaction was initiated by the ad-dition of rkGR directly from the stock kept on ice and the decrease of absorbance due to the oxidation of NADPH was recorded at 340 nm for 10 seconds. One unit of the enzyme activity was defined as the amount of the enzyme that catalyzes the consump-tion of 1 μmol of NADPH per min under the de-scribed assay conditions (Ogus and Ozer, 1991; Ogus and Ozer, 1998; Can et al., 2010).

Kinetic studies

In substrate and inhibition kinetic experiments cau-tion was taken to ensure the extent of NADPH oxida-tion was ≤ 10%. When determining K_m for NADPH, [GSSG] was kept at 1 mM and [NADPH] varied from 10 to 200 μM. When determining K_m for GSSG, [NADPH] was fixed at 0.1 μM and [GSSG] was var-ied from 20 to 1200 μM. For the determination of the inhibition type and the inhibitory kinetic parameters of NADP$^+$ for rat kidney GR, one substrate was used at constant concentration and the concentrations of the second substrate and the concentrations of NADP$^+$ were varied. The obtained initial rates were converted to units/milligram protein and the data were evaluated by Dixon or Cornish-Bowden plots (Cornish-Bowden, 1974; Segel, 1975).

Protein concentration determination

After thawing the samples stored with or without glycerol, protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Ten percent SDS-PAGE was carried out as described by Laemmli (1970) and protein bands were stained with silver nitrate.

Statistical analysis

The data were evaluated by using Statistica (version) 1999 for Windows.

RESULTS AND DISCUSSION

Rat kidney GR was purified to homogenity by using heat denaturation, gel filtration on Sephadex G25, af-finity chromatography on 2',5'-ADP agarose and ion-exchange chromatography using PBE 94 (Can et al., 2010). The purified rkGR gave a single band on 10% SDS-PAGE with an MW of 53 kDa confirming that purification was successful (Fig. 1).

The Michaelis-Menten constants were evaluated in 0.1 M KPi, pH 7.4 buffer containing 4 mM EDTA at 37°C for both enzymes; stored with and without glycerol. At fixed [GSSG] and varied [NADPH], the V_m values obtained for enzymes stored in buffer and buffer supplemented with 10% (v/v) glycerol were similar; 258 ± 5 and 267 ± 5 U/mg protein, respec-tively (Table 1) (Can et al., 2010). On the other hand, the Michaelis-Menten constant K_m and the k_{cat}/K_m values were affected; the K_m values of 9.7 ± 0.9 and 15.3 ± 1.4 μM and k_{cat}/K_m values of 2.75 x 10^7 and 1.68 x 10^7 M^{-1}.s^{-1} for the rat kidney GR stored with and without glycerol (Can et al., 2010) were obtained, re-spectively (Table 1).

The inhibition constant, K_i was also affected by storage in glycerol. The values for K_i at fixed [GSSG] (1 mM), varied [NADPH] (10-200 μM) and varied [NADP$^+$] (5-80 μM), were different; the K_i values for NADP$^+$ for the enzyme stored with and without glyc-erol were 26.6 ± 2.2 and 118 ± 11 μM, respectively (Table 1). These data indicate that glycerol caused a
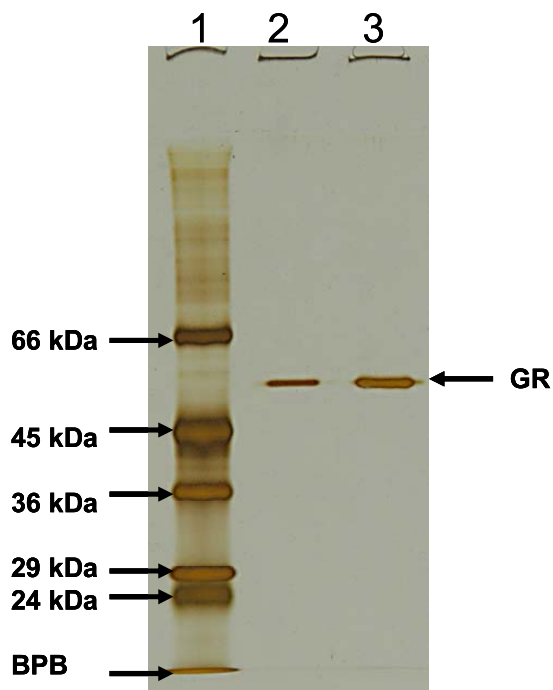
Fig. 1. SDS/PAGE (10%) of the rat kidney GR. Lane 1, Protein standards (From top to down): 66 kDa, Albumin; 45 kDa, Ovalbumin; 36 kDa, Glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, Carbonic anhydrase; 24 kDa, Trypsinogen; BPB, Bromophenol blue. Lane 2 and Lane 3, 250 and 750 ng of protein, respectively.
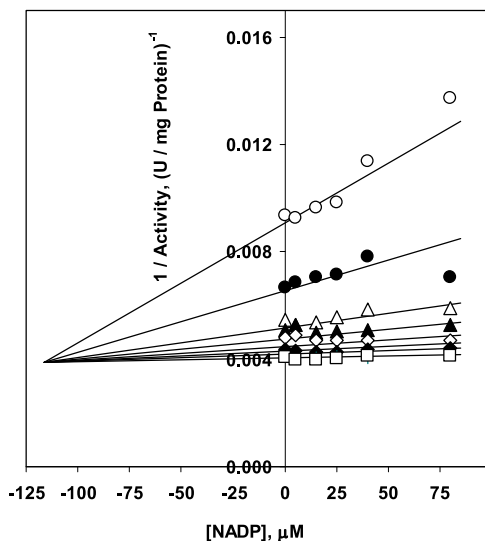

Fig. 2. The inhibition of rat kidney GR (-Glycerol) by NADP$^+$. [GSSG] = 1 mM; [NADPH] = (o), 10; ($\bullet$), 20; ($\triangle$), 40; ($\blacktriangle$), 60; ($\lozenge$), 80; ($\blacklozenge$), 120; ($\square$), 160 and ($\blacksquare$), 200 μM. All the points are average of at least two separate measurements.

Fig. 3. The inhibition of rat kidney GR (+Glycerol) by NADP$^+$. [GSSG] = 1.0 mM; [NADPH] = (o), 10; ($\bullet$), 20; ($\triangle$), 40; ($\blacktriangle$), 60; ($\lozenge$), 80; ($\blacklozenge$), 120; ($\square$), 160 and, ($\blacksquare$), 200 μM. All the points are average of at least two separate measurements.

Fig. 4. Slopes of lines in Figures 2 and 3 vs 1 / [NADPH] plot; (o), (-Glycerol) and ($\bullet$) (+Glycerol).

conformational change in rkGR that results in an enzyme with higher affinity for NADPH and NADP$^+$. The k_{cat}/K_m value obtained for rkGR stored in glycerol is in good correlation with the published data (Arias et al., 2010).

Fig. 5. The inhibition of rat kidney GR (-Glycerol) by NADP⁺. [NADPH] = 0.1 mM; [GSSG] = (o), 20; (●), 40; (Δ), 80; (t), 160; (◊), 320; (♦), 640 and (□), 1200 μM. All the points are average of at least two separate measurements.

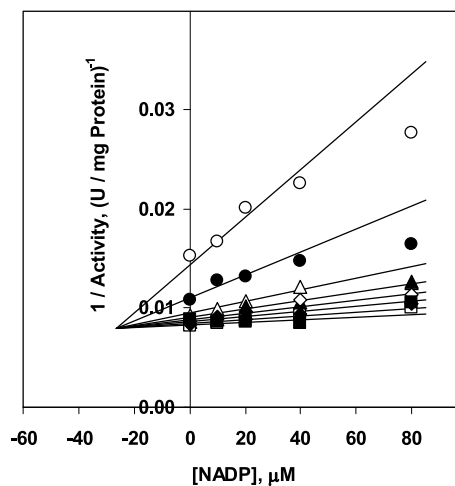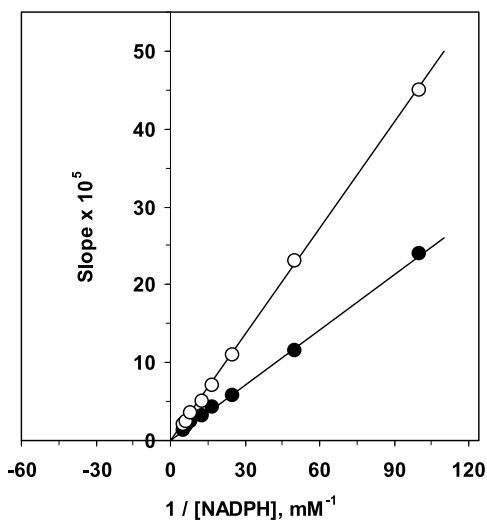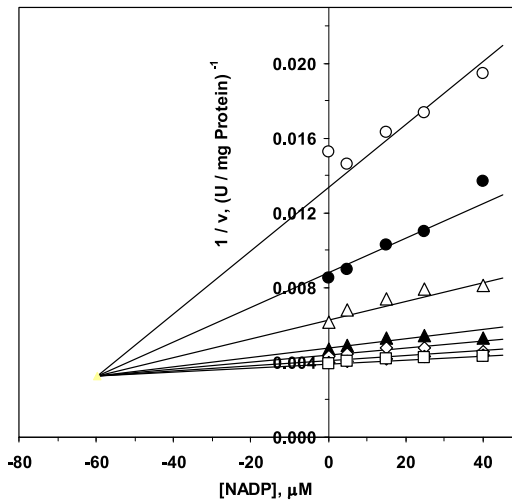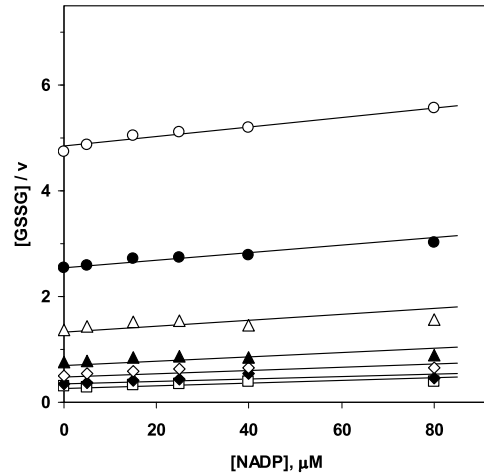**Fig. 7.** The inhibition of rat kidney GR (-Glycerol) by NADP⁺. [NADPH] = 0.1 mM; [GSSG] = (o), 20; (●), 40; (Δ), 80; (▲), 160; (◊), 320; (♦), 640 and (□), 1200 μM (Cornish – Bowden Plot). All the points are average of at least two separate measurements.
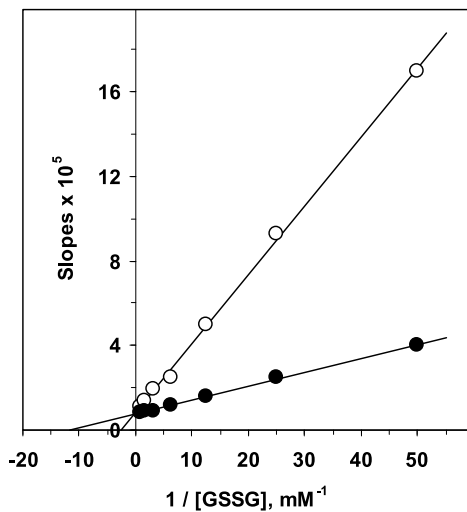

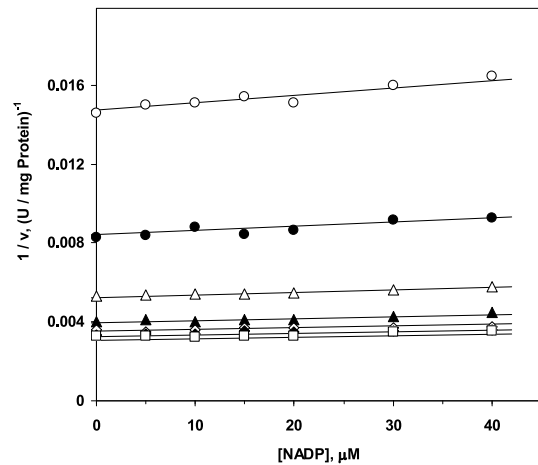**Fig. 6.** Slopes of lines in Figures 5 and 8 vs 1 / [GSSG] plot; (o), (-Glycerol) and (●) (+Glycerol).

Fig. 8. The inhibition of rat kidney GR (+Glycerol) by NADP⁺. [NADPH] = 0.1 mM; From top to bottom; [GSSG] = (o), 20; (●), 40; (Δ), 80; (▲), 160; (◊), 320; (♦), 640 and (□), 1200 μM. All the points are average of at least two separate measurements.

However, the inhibitory behavior was not affected by storage in glycerol. Both enzymes were inhibited competitively (Fig. 2: -glycerol and Fig. 3: +glycerol). The competitive inhibition characteristics of rkGR (stored with and without glycerol) at fixed [GSSG], varied [NADPH] and varied [NADP⁺] were strengthened by the secondary plots (slopes of lines obtained from Figs. 2 and 3 vs 1 / [NADPH]). In the secondary plots, lines passing through the origin indicate competitive inhibition (Fig. 4).

There are several contradictory reports about the inhibitory behavior of GR isolated from different organisms, such as competitive inhibition (Staal and Veeger, 1969; Worthington and Rosemeyer, 1976; Lopez-Barea and Lee, 1979; Ogus and Ozer N, 1999; Ulusu and Tandoğan, 2007; Tandoğan and Ulusu, 2010), mixed type inhibition (Acan and Tezcan, 1991), noncompetitive inhibition (Carlberg and Mannervik, 1975) and no inhibition at all (Willmore and Storey, 2007). The K_i values reported were from 43 to 115 μM (Lopez-Barea and Lee, 1979; Worthington and Rosemeyer, 1976; Ulusu and Tandoğan, 2007; Tandoğan and Ulusu; 2010). In this study, the K_i values obtained with NADP⁺ for rkGR, 26.6 ± 2.2 and 118 ± 11 μM for the enzyme stored in glycerol and buffer (Table 1) are in good agreement with the values reported in the literature. Several GRs isolated from different organisms (Staal and Veeger, 1969; Worthington and Rosemeyer, 1976; Lopez-Barea and Lee, 1979; Ogus and Ozer N, 1999, Ulusu and Tandoğan, 2007; Tandoğan and Ulusu; 2010) were inhibited competitively and rkGR was also inhibited competitively, regardless of storage conditions (Figs. 2, 3 and 4).

On the other hand, at fixed [NADPH] and the varied [GSSG] and varied [NADP⁺], the V_m value obtained from rkGR stored in glycerol (323 ± 4 U/mg protein) was higher than the rkGR stored in buffer (258 ± 5 U/mg protein) (Can et al., 2010) (Table 1). The kinetic behavior and kinetic parameters were also different for the rkGR stored with and without glycerol. At fixed [NADPH] and the varied [GSSG], the Michaelis-Menten constant K_m and K_s values for GSSG were 67.5 ± 2.1 and 49.9 ± 3.2 μM and the k_{cat}/K_m and k_{cat}/K_s values were 5.16 x 10⁶ and 4.85 x 10⁶ M⁻¹.s⁻¹ for the rkGR stored with and without glycerol (Can et al., 2010), respectively (Table 1). The kinetic behavior of the rkGR stored only in buffer was linear mixed-type with K_s of 49.9 ± 3.2 μM and α value of 7.74 (Table 1). In the Dixon plot, 1/v vs [I] at different concentrations of substrate (GSSG), the characteristics of the linear mixed-type inhibition are almost identical to that obtained for a pure competitive inhibitor, but here the intersection point is lower

than 1/V_m and at the left site of 1/v axis (Fig. 5). The secondary plot (slopes vs 1/[GSSG]) does not go through the origin, indicating linear mixed-type inhibition (Fig. 6) (Segel, 1975). The parallel lines obtained from the Cornish-Bowden plot ([GSSG]/v vs [NADP⁺] plot) further supports that the inhibition type is linear mixed-type inhibition (Fig. 7) (Cornish-Bowden, 1974; Segel, 1975). At fixed [NADPH] and varied [GSSG], the rkGR stored in 10% (v/v) glycerol inhibited noncompetitively by NADP⁺ with a K_i value of 397 ± 69 μM (Table 1, Fig. 8). The secondary plot using the slopes of lines of Fig. 8 vs 1/[GSSG] did not pass from the origin, indicating a noncompetitive type of inhibition (Fig. 6) (Segel, 1975).

At fixed [NADPH], varied [GSSG] and varied [NADP⁺], there are again contradictory reports in literature for the inhibition of GRs obtained from different organisms or tissues; noncompetitive (Staal and Veeger, 1969; Worthington and Rosemeyer, 1976; Lopez-Barea and Lee, 1979), uncompetitive (Ogus and Ozer N, 1999; Ulusu and Tandoğan, 2007; Tandoğan and Ulusu; 2010), and at low [GSSG] concentrations, mixed type (Ogus and Ozer N, 1999). Rat kidney GR showed linear mixed-type and noncompetitive type of inhibition in the absence and presence of 10% (v/v) glycerol in storage buffer (Figs. 5-8). This finding is also in good agreement with the data reported in the literature. The K_i values reported ranged from 219 to ≥ 6000 μM (Worthington and Rosemeyer, 1976; Lopez-Barea and Lee, 1979; Ulusu and Tandoğan, 2007; Tandoğan and Ulusu, 2010). The GR purified from rat kidneys stored in the absence of glycerol had a higher affinity for both its substrate NADPH and its inhibitor (product) NADP⁺. The K_s and K_i values for GSSG and NADP⁺ were 49.9 ± 3.2 and 59.6 ± 8.4 μM, respectively. The storage in glycerol decreased the affinity of the rkGR for both its substrate, GSSG (K_m = 67.5 ± 2.1 μM) and for its inhibitor, NADP⁺ (K_i = 397 ± 69 μM) (Table 1).

Glycerol is an organic compound that belongs to polyols, found in some organisms in which it serves to maintain adaptation to environmental

Table 1. Some kinetic and inhibitory parameters of rat kidney GR.

	Kinetic Parameters	- Glycerol	+ Glycerol
[GSSG] fixed; [NADPH] varied	V_m, U / mg protein	258 ± 5*	267 ± 5
	K_m, μM	15.3 ± 1.4*	9.7 ± 0.9
	k_{cat} / K_m; M^{-1}.s^{-1}	1.68 x 10^7*	2.75 x 10^7
	**CER ((+Gly) / (-Gly))	1.64	
	K_i, μM	118 ± 11	26.6 ± 2.2
	Inhibition type	Competitive	Competitive
[NADPH] fixed; [GSSG] varied	V_m, U / mg protein	258 ± 5*	323 ± 4
	K_m, μM	53.1 ± 3.4*	67.5 ± 2.1
	k_{cat} / K_m; M^{-1}.s^{-1}	4.85 x 10^6*	5.16 x 10^6
	**CER ((+Gly) / (-Gly))	1.06	
	K_s, μM	49.9 ± 3.2	-
	α	7.74	-
	K_i, μM	59.6 ± 8.4	397 ± 69
	Inhibition type	Linear mixed-type	Noncompetitive

* These data were taken from Can et al. (2010); the source of GR is the same.
**CER: Catalytic Efficiency Ratio

conditions. It is considered as a compatible solute since it does not alter macromolecular structures, even at high concentrations (Timasheff, 1998; Yancey, 2005). However, limited studies show that it may lead an alteration in the steady-state kinetics of different enzymes (Levy et al., 1966; Olsen et al., 2007). In a previous study investigating the osmolyte effects of glycerol on hexokinase kinetics, it was shown that glycerol alters the kinetic parameters; a decrease in k_{cat}, K_m and V_m values was observed and this decrease was not affected by BSA (Olsen et al., 2007).

In this study, it was found that when [GSSG] is fixed and [NADPH] is the varied substrate, glycerol decreases K_m for NADPH and causes an increase in catalytic efficiency (k_{cat}/K_m) by a factor of 1.64. On the other hand, when [NADPH] is fixed and

[GSSG] is the varied substrate, although glycerol increased the turnover number by a factor of 1.25, the catalytic efficiency (k_{cat}/K_m) was minimally affected and a small increase (1.06) was observed (Table 1). In contrast to our findings, Levy et al. (1966) reported increases of all kinetic parameters for glucose-6-phosphate dehydrogenase. However, this difference in response to osmolytes could be explained by the difference in enzyme systems (Levy et al., 1966).

In summary, our results show that glycerol causes a decrease in both K_m and K_i values with respect to NADPH, an increase with respect to GSSG for rkGR, and that the catalytic efficiency is more affected when the varied substrate is NADPH than when it is GSSG (Table 1). This finding could be explained by the fact that glycerol causes a conformational change on the rat kidney enzyme which results in a tighter binding of NADPH and NADP+ when the varied substrate is NADPH and a more loose binding of GSSG and NADP+ when the varied substrate is GSSG. As written in the methods section, our study was performed using rkGR stored in ± glycerol and the final concentration of glycerol in the reaction mixture was ≤ 0.08% (v/v): glycerol at this concentration was ineffective in creating a conformational change of the enzyme stored without glycerol. Compared to the present study, Levy et al. (1966) performed their assay in the presence of 40% glycerol. They hypothesized that in the absence of glycerol, more than one molecule of NADPH per mole of enzyme is involved. This hypothesis does not have a strong basis and obviously more studies are required. The competitive inhibition by the product NADP+ against the corresponding substrate NADPH could be interpreted by various sequential mechanisms (Theorell-Chance, ordered or rapid equilibrium random) (Segel, 1975; Carlberg and Mannervik, 1986; Ogus and Ozer, 1998) but not via a ping-pong mechanism. Our data imply that although kinetic parameters are different and the conformational change created by glycerol remains after dilution, the rkGR stored in ± glycerol followed the same kinetic mechanism.

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