Turk J Chem
25 (2001) , 55 – 61.

Evidence for a Hybrid Ping Pong-Semirandom Mechanism for Human Jejunal Glutathione Disulfide Reductase

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Received 29.01.1999

Initial rate versus substrate concentration, [S], profiles (at pH 7.4, 37°C) for human jejunal glutathione disulfide reductase indicated partial substrate inhibition by GSSG at fixed [NADPH] $\leq$ 40 μM. Saturation by NADPH assumed an increasingly sigmoidal character at fixed [GSSG] $\geq$ 20 μM. The results were interpreted in terms of a hybrid ping pong-semirandom mechanism proceeding through both E.NADPH (ping pong pathway) and E.GSSG (ordered pathway) complexes. When V(apparent) versus [GSSG] and [NADPH] were plotted, hyperbolic curves were obtained. Plotting 1/Vm(apparent) versus 1/[NADPH] and 1/[GSSG] yielded two lines with different slopes but intersecting at the same point on the y axis. A value of 26.3 U/ml was calculated for Vm, and Km values of 25 and 71 μM were determined for NADPH and GSSG, respectively.

Key Words: Glutathione disulfide reductase, ping pong-semirandom mechanism, human jejunum.

Introduction

As a part of the oxidized glutathione (GSSG)/reduced glutathione (GSH) redox system implicated in the maintenance of cellular integrity, glutathione disulfide reductase, GSSGGR (EC 1. 6. 4. 2.), has been the subject of detailed structural and kinetic studies[1−11]. The enzyme is a homodimer with Mr 100 000 - 150 000 and contains one FAD/subunit. Kinetic patterns observed with yeast[2,4,6] mouse liver[3] and human erythrocyte[8,11] glutathione disulfide reductases have been generally interpreted in terms of a branched mechanism in which the binary complex, E.NADPH, partitions between ping pong and sequential ordered pathways[4]. On the other hand, experiments with yeast GSSGGR at pH $\leq$ 7 reveal substrate inhibition by GSSG[6], which, together with the protection afforded by GSSG against heat inactivation of the enzyme, imply that the binary complex, E.GSSG, may also be kinetically relevant. Inhibition of the human erythrocyte enzyme by GSSG (and by NADPH) has been reported[8], but not corroborated[11].

The small intestine is not only the most important gate for the absorption of all kinds of foods but also a very important site for the detoxification of toxic chemicals and drugs[12,13]. GSSGGR, by reducing GSH at

the expense of NADPH, has a central role in GSH-dependent detoxication systems[14]. Therefore, it is very important to elucidate the kinetics of GSSGGR. In the present study, we therefore reexamined the kinetic behavior of human jejunal glutathione disulfide reductase over a wide range of substrate concentrations. The results obtained with enzyme isolated from human jejunal mucosa are compatible with a hybrid ping pong-semirandom mechanism employing both E.NADPH and E.GSSG.

Experimental

Materials: GSSG and NADPH were obtained from Boehringer-Mannheim (FRG) and Sigma (USA), respectively. All other chemicals were standard products from Sigma or Aldrich, USA.

Enzyme preparation: The tissue source of glutathione disulfide reductase was a surgical specimen obtained from the proximal jejunum of a 30-year-old female patient undergoing gastrojejunostomy indicated by chronic duodenal ulcer and pyloric stenosis. The part of the tissue used for GSSGGR isolation was healthy on pathological examination. The enzyme was purified to homogeneity by modification of a published procedure[1] detailed elsewhere[15,16]. In short, a homogenate was prepared from mucosal cells, was desalted on a Sephadex G25 column and applied to an S-hexylglutathione Sepharose 4B column for the removal of the human jejunal glutathione S-transferases[17]. The pass-through material, containing glutathione disulfide reductase, was applied to a 2',5'-ADP-Sepharose 4B affinity column and the enzyme was eluted by NADPH into a serially connected Polybuffer Exchanger 94 (PBE 94) column. NADPH was very efficiently removed from GSSGGR using PBE 94 column[15]. By this method, a 3814-fold purification with 76 per cent yield was achieved. The purified enzyme had a specific activity of 225 U/mg protein and gave a single peak on chromatofocusing and a single band on 12.5 percent SDS/PAGE. The isoelectric point and subunit molecular mass were 6.75 and 56 kDa, respectively[16].

Activity measurements: GSSGGR activity was determined at 37°C, in 100 mM potassium phosphate buffer (pH 7.4), containing 4 mM EDTA. The reaction was initiated by the addition of enzyme (3.3 nM GSSGGR) and followed by monitoring the change in absorbance at 340 nm, using an LKB Ultraspec Plus spectrophotometer equipped with a sipper system. No lag was observed in the progress curves in the substrate ranges covered. Any data spanning more than 20 percent substrate hydrolysis were excluded.

Results and Discussion

The data in Figures 1 and 2 show the variation of initial rate with [GSSG] and with [NADPH], respectively. Notable features of the kinetic patterns obtained are: i. Partial substrate inhibition by GSSG, the extent of which depends on the fixed concentration of NADPH (Fig. 1) and ii. a corresponding sigmoidal saturation with NADPH at high [GSSG] (Fig. 2). These observations do not fit the previously proposed ping pong-sequential ordered mechanisms[4] with E.NADPH at the branching point of the alternative pathways and predicting a hyperbolic dependence of initial rate on [NADPH], regardless of the concentration of GSSG. They are different also from the findings of Scott et al.[7] and Icen[2], where both substrates are inhibitory at high concentration. With the human jejunal GSSGGR, only GSSG was inhibitory and its inhibitory effect was observed only at low NADPH concentrations (Figs. 1, 2). The inhibition with both substrates was uncompetitive (Figs. 3, 4) and at [NADPH] below 40 μM, inhibition became mixed type (Fig. 3). With the erythrocyte enzyme, at low [GSSG], a strong inhibition by NADPH was observed[8]. This finding is also

contrary to the human jejunal GSSGGR, which is not inhibited by NADPH at low [GSSG] (Fig. 2).

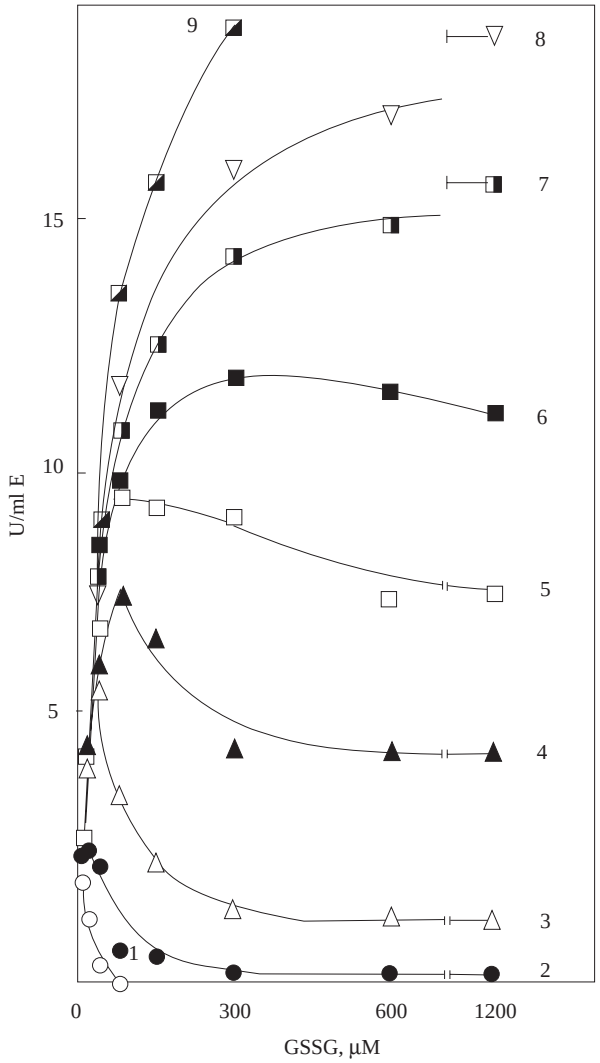


Figure 1. Dependence of initial rate on GSSG concentration. Curves 1 to 9 obtained at fixed [NADPH] = 5, 10, 15, 20, 25, 30, 40, 80, and 160 mM, respectively.

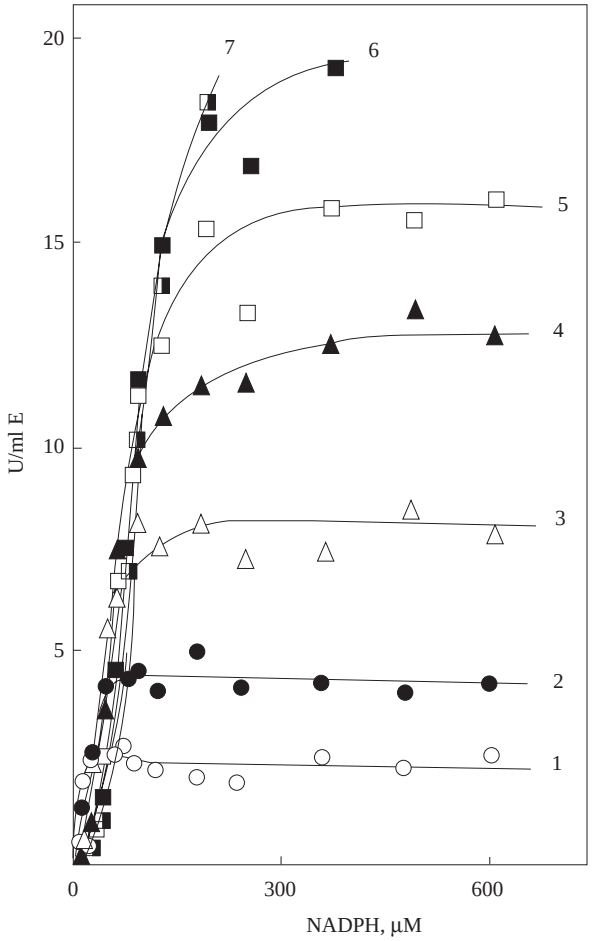


Figure 2. Dependence of initial rate on NADPH concentration. Curves 1 to 7 obtained at fixed [GSSG] =10, 20, 40, 80, 150, 600, and 2400 mM, respectively.

Vm(apparent) versus NADPH (and GSSG) plots shows hyperbolic saturation (Fig. 4a). 1/Vm(apparent) versus 1/[NADPH] and 1/[GSSG] plots give two lines with different slopes, which intercept at the same point on the 1/Vm axis, indicating the presence of different concentration-pairs to each intermediate Vm(apparent) value (Fig. 4b).

In the ordered Bi Bi mechanism, the Km(apparent) versus Vm(apparent) plot does not pass through the origin[8]. In human jejunal glutathione disulfide reductase, ordered the Bi Bi mechanism is ruled out because the Km(apparent) versus Vm(apparent) plot for both substrates passes through the origin (Fig. 5). Therefore, it appears that the human jejunal enzyme is committed to ping pong (NADPH first) or ordered (GSSG first) pathways, depending on the relative concentrations of E.NADPH and E.GSSG (Scheme). The rate equation for the proposed scheme would contain second-order terms in both substrates and, with proper

interplay of rate constants, could accommodate both the apparent sigmoidicity in NADPH binding and the maxima obtained in the v_i (initial rate) versus [GSSG] plots at low [NADPH][18].

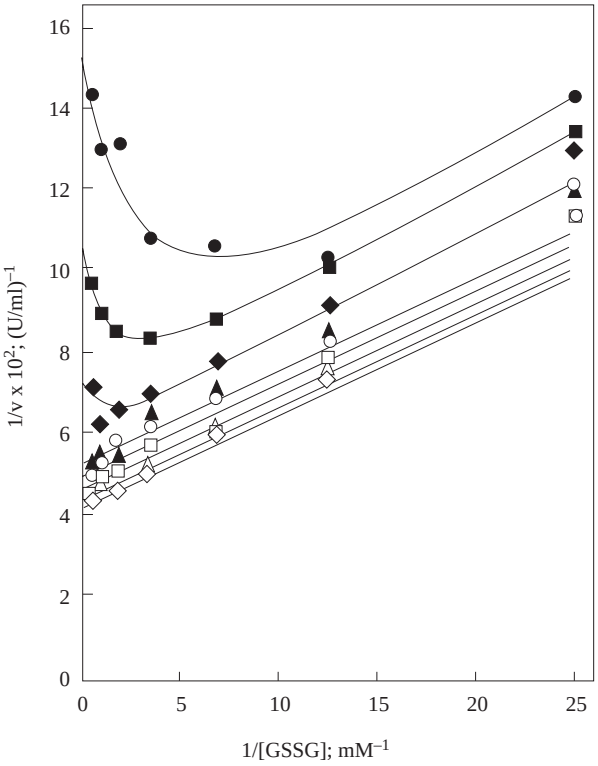


Figure 3. 1/v versus 1/[GSSG] and varying concentrations of [NADPH] plot: from top to bottom [NADPH] = 25, 30, 40, 60, 80, 120, 160 and 200 mM.

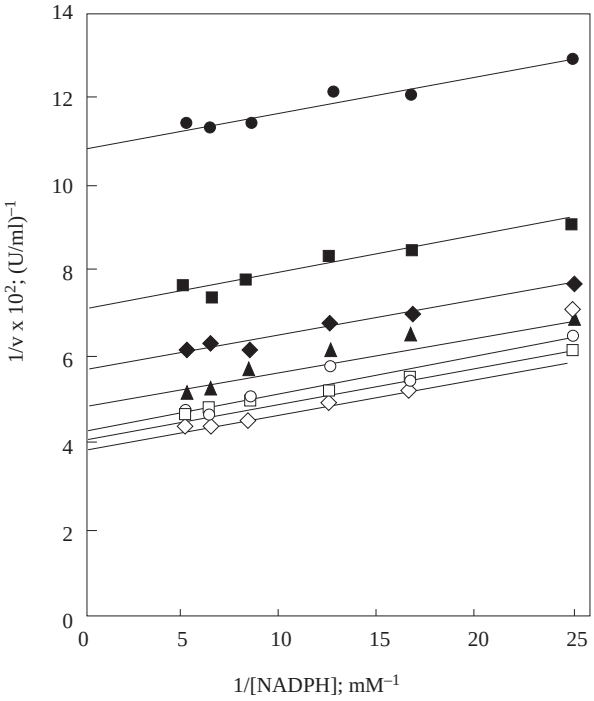
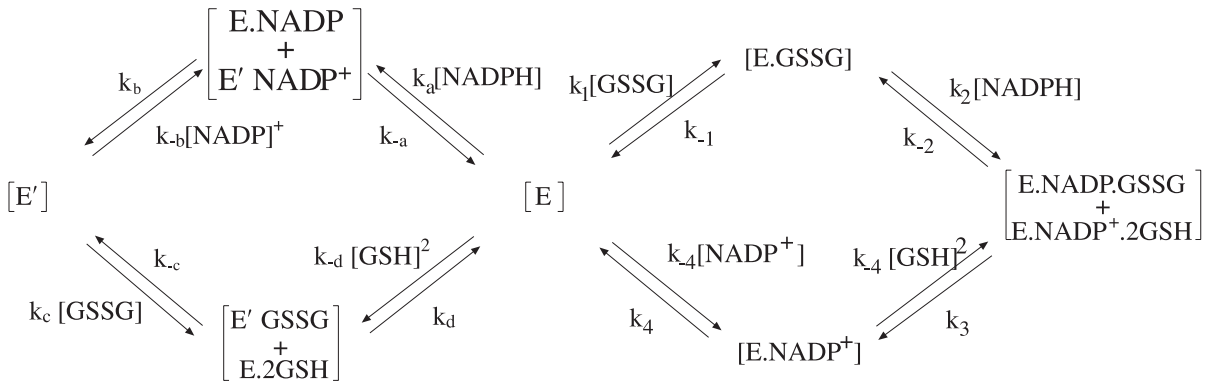


Figure 4. 1/v versus 1/[NADPH] and varying concentrations of [GSSG] plot: from top to bottom [GSSG] = 10, 20, 40, 80, 150, 600 and 2400 mM.



Scheme

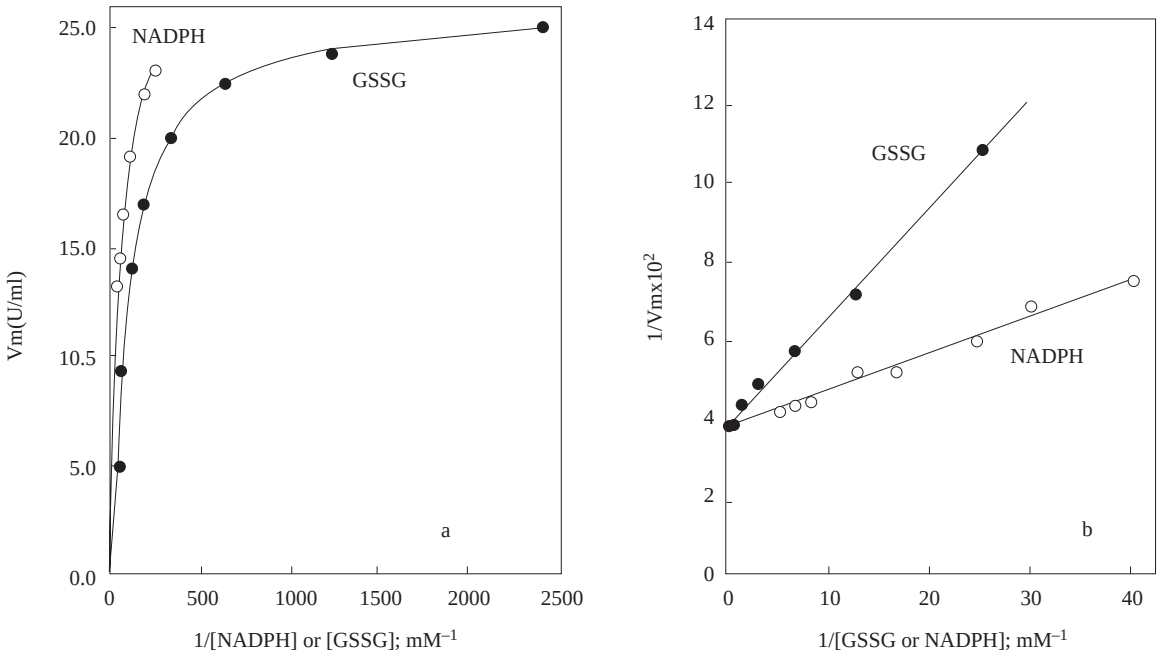


Figure 5. a. Vm(apparent) versus [GSSG] and [NADPH] plot; b. Lineweaver-Burk plot of "a"; (1/Vm(apparent)) x102 versus 1/[NADPH] and 1/[GSSG]).

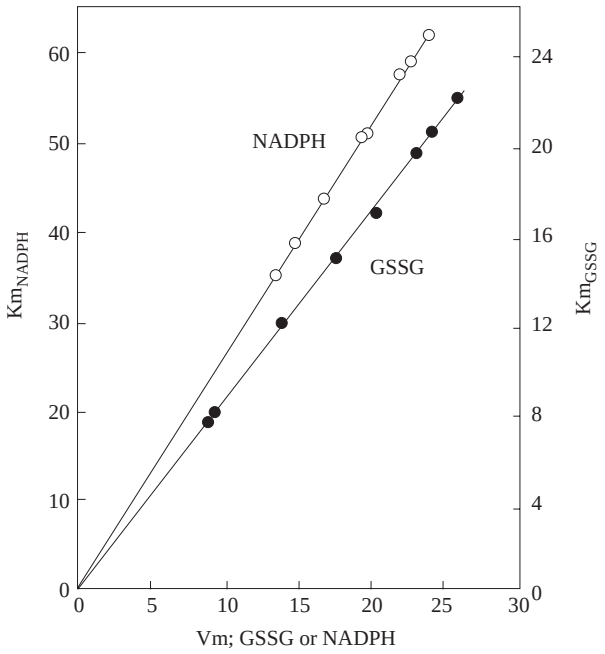


Figure 6. KmGSSG and NADPH (apparent) versus VmGSSG and NADPH(apparent) plot.

Available information on human glutathione disulfide reductase points to considerable variation in the kinetic properties of this enzyme (Table). The variability is likely to extend to inhibition patterns obtained with $NADP^+$, which is reported to be a competitive inhibitor of erythrocyte glutathione disulfide reductase with NADPH as the varied substrate and a noncompetitive inhibitor with respect to GSSG as the varied

substrate[11]. The ping pong-semirandom mechanism proposed for the jejunal enzyme requires that $NADP^+$ be a mixed (or competitive) inhibitor with respect to NADPH and a competitive inhibitor with respect to GSSG. This is what is observed with a similar enzyme from human erythrocytes[19]. Due to the paucity of material, this prediction has not been tested for jejunal GSSGGR.

Table. Information Relating to Substrate Inhibition of Human Glutathione Disulfide Reductase

Tissue Source	Experimental Conditions	Substrate Inhibition by		References
		NADPH	GSSG	
Erythrocyte	Phosphate Buffer, pH 7.0, 25°C $\mu \cong 0.06$ - 0.6 M [NADPH] = 25 - 200 μM [GSSG] = 20 - 1500 μM	+	+	16
Erythrocyte	Phosphate buffer, pH 7.0, 25°C $\mu \cong 0.1$ M, [NADPH] = 6.7 - 67 μM [GSSG] = 10 - 1000 μM	$(-)^a$	-	7
Human jejenum	Phosphate buffer, pH 7.4, 37°C $\mu \cong 0.2$ M, [NADPH] = 5 - 200 μM [GSSG] = 10 - 2400 μM	-	+	this study

[a] A narrower [NADPH] range (6.7 - 67 μM) was used in this study than in reference [16], where [NADPH] spanned 25 - 200 μM and inhibition became apparent at > 100 μM [NADPH]. Hence the absence of inhibition is not definite.

It appears that the catalytic sequence may be significantly affected by the tissue and batch origin of the enzyme[3,4,6,7,11] and possibly by the experimental conditions employed[6,8]. It was reported that changes in buffer concentration and pH will affect the kinetic parameters[6,8]. Moroff and Brandt, using yeast enzyme, showed that changing the pH from 4.5 to 8.0 resulted in changes in the Km values for GSSG and NADPH of 30 to 110 μM and 3 to 13 μM, respectively. They also showed that high salt concentration is a non-competitive inhibitor of glutathione disulfide reductase with respect to GSSG[6]. Substrate inhibition for both NADPH and GSSG at low buffer concentration and the removal of substrate inhibition for GSSG, by increasing buffer concentration (from 30 mM to 300 mM), were shown by Staal and Veeger[8]. A 10-fold increase in buffer concentration resulted in a 1.4-fold increase in the Km for NADPH, and a 6.6-fold increase in the Km for GSSG[8]. However, there seems to be a contradiction with the results presented in the literature[11], where there is no substrate inhibition for both NADPH and GSSG, in that they used 3.3 times more concentrated buffer and they did not use a NADPH concentration high enough ([NADPH] > 100 μM is inhibitory) to obtain substrate inhibition[11]. The discrepencies in the kinetic parameters obtained by different research groups are either due to the differences in the experimental conditions employed, or the use of an enzyme from a different source. In the present study on human jejunal GSSGGR, a 100 mM buffer concentration and a pH of 7.4 were used. Although the Km values for NADPH and GSSG obtained were in good agreement with the results of Moroff et al. and Staal et al.[6,8], existence of the substrate inhibition by GSSG at this buffer concentration could be explained by the origin of the enzyme.

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