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Steady-State Kinetic Studies of Glutathione Reductase

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The steady-state kinetic studies of yeast glutathione reductase, performed when [GSSG] = 10[NADPH] in the assay mixture, show that at concentrations of GSSG under 450 μM the enzymatic mechanism pathway is ping-pong. Furthermore, in the case of higher values, the enzymatic kinetics follows a sequential pathway. However when the glutathione reductase 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.

Key words: Mechanism pathway, Glutathione reductase, Glutathione.

Glutathione reductase (EC 1.6.4.2) is a flavoprotein found in the soluble fraction of both eukaryotic and procaryotic cells (8). The primary role of the enzyme is apparently the maintenance of the intracellular reduced glutathione by means of a reaction between oxidized glutathione (GSSG) and NADPH (7). MANNERVIK, has proposed a branching mechanism for the glutathione reductase reaction (6), that implies that at high concentration of acceptor substrate, GSSG, the sequential mechanism dominates, whereas at lower concentrations of GSSG the importance of the ping-pong mechanism increases. The aim of the present paper is to describe the steady-state kinetic studies of yeast glutathione reductase performed with various concentrations of NADPH and GSSG in the assay mixture, emphasizing the GSSG concentration that determines the mechanism followed by the enzyme. Furthermore the effect of the GSSG concentration on the inhibitory power of high NADPH concentration is also included.

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Materials and Methods

Glutathione reductase from *S. cerevisiae*, GSSG and NADPH were purchased from Boehringer Mannheim. The glutathione reductase reaction system contained a suitable amount of enzyme (diluted in 1 % albumin and buffer), varying concentrations of NADPH as well as GSSG, 100 mM of KH_2PO_4-NaOH buf-

fer pH 7.2, 5 μM of FAD, 1 ml of assay mixture. The initial rates were determined spectrophotometrically by recording at 339 nm the oxidation of NADPH with a Beckman UV 5260 double beam spectrophotometer. The individual constants for glutathione reductase (maximum velocity and the true Michaelis constants of the substrates) were obtained either by the simple graphical procedure described by FLORINI and VESTLING (3) or by the EISENTHAL and CORNISH-BOWDEN (2) plot procedure. The GSSG concentration that determines the kinetic pathway —two-step transfer or compulsory-order for the glutathione reductase reaction—, was worked out by the method described by DIXON and WEBB (1).

Results

In figure 1 the results of steady-state kinetic studies, of yeast glutathione reductase performed with various concentrations of NADPH and GSSG are presented. The values of the reciprocals of the maximum velocities that were obtained at the fixed concentration of GSSG plotted against 1/[GSSG], give a straight line (R^2: 0.999) and the true Michaelis constant for GSSG was 93.2 μM. The reciprocals of the maximum velocities that were obtained at the fixed concentrations of NADPH plotted against 1/[NADPH], also give a straight line (R^2: 0.993) and the true Michaelis constant for NADPH was 17.8 μM. The values of the true Michaelis constants for NADPH and for GSSG determined by the method of EISENTHAL and CORNISH-BOWDEN plot were 72.5 μM and 15.5 μM respectively. Figure 2 shows the plot of 1/v against 1/[NADPH]. When the concentrations of both NADPH and GSSG were held at a fixed ratio: [GSSG] = 10[NADPH]. Under these conditions the reciprocal plot of initial velocity against [NADPH] is linear at concentrations of oxidized glutathione lower

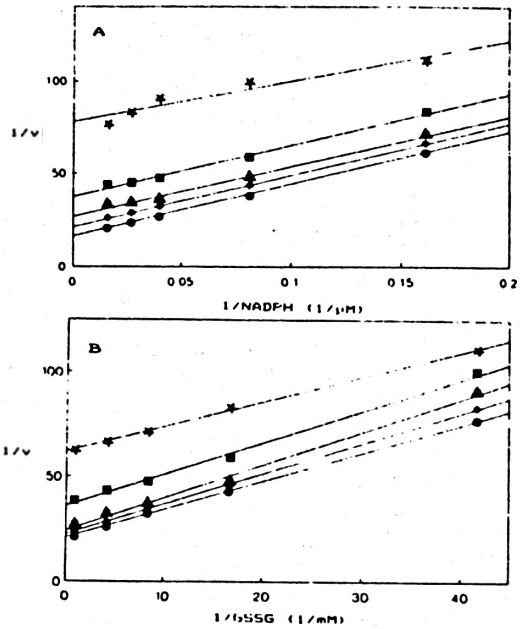

Fig. 1. *Effect of different NADPH and GSSG concentrations on the steady-state kinetics of yeast glutathione reductase.*
Enzyme 0.41 μg/assay. v, is expressed as the rate of absorbance increase per minute. A) Different fixed concentration of GSSG: (●), 1.21 mM (R^2 = 0.999); (✲), 240 μM (R^2 = 0.999); (▲), 127 μM (R^2 = 0.995); (■), 60 μM (R^2 = 0.997); (★), 24 μM (R^2 = 0.957). B) Different fixed concentration of NADPH: (●), 62 μM (R^2 = 0.999); (✲), 38 μM (R^2 = 0.999); (▲), 25 μM (R^2 = 0.997); (■), 12 μM (R^2 = 0.998); (★), 6 μM (R^2 = 0.999).

than 450 μM, becoming a curve for higher concentrations. For two-step transfer system, the reciprocal equation becomes: $1/v = (k_{M\ NADPH} + k_{M\ GSSG}/10)\ 1/[NADPH]\ 1/V + 1/V$ and thus the reciprocal plot will be linear (R^2 = 0.997 of the straight line defined by points 6-11); $1/v = 5.26 + 148.3\ 1/[NADPH]$. Although for systems which obey an orderely sequential pathway, the reciprocal equation of the form is: $1/v = (k_{M\ NADPH} + k_{M\ GSSG}/10 + k_a\ k_{M\ GSSG}/10\ [NADPH])$

1/[NADPH] 1/V + 1/V (K_a is the dissociation constant of the binary complex enzyme-first substrate) at which it yields a reciprocal plot that would curve upwards ($R^2 = 0.656$ of the curve defined by points 1-6). In figure 3 the inhibition effect of high NADPH concentrations is presented, illustrating that a strong inhibition by excess of NADPH was found, especially at low GSSG concentrations.

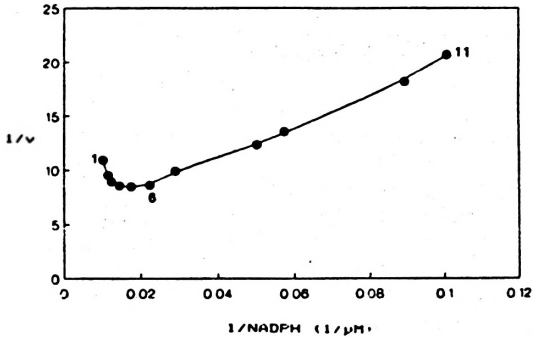

Fig. 2. *Reciprocal plot of initial velocity against NADPH concentration, when the concentrations of both NADPH and GSSG were held at a fixed ratio: [GSSG] = 10[NADPH].*
Enzyme 0.41 μg/assay. v, is expressed as the rate of absorbance increase per minute.

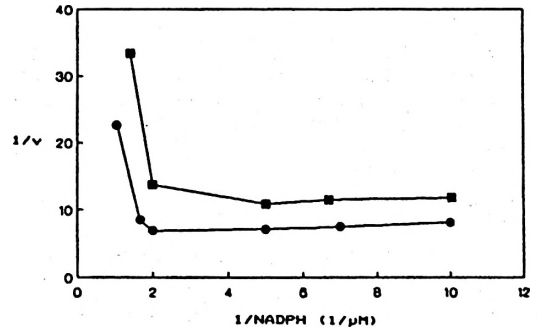

Fig. 3. *The inhibition effect of a high NADPH concentration on the yeast glutathione reductase system at non-saturant and saturant levels of GSSG, respectively.*
Enzyme 0.41 μg/assay. v, is expressed as the rate of absorbance increase per minute. (●), 1,000 μM of GSSG and (■), 100 μM of GSSG.

Discussion

The kinetic characteristics of glutathione reductase are so particular that its mechanism of reaction has been designated as «anomalous ping-pong» (5). It is well established that in the glutathione reductase system NADPH is the first substrate that reacts with the free enzyme and the GSSG is the acceptor substrate of the reduction power of NADPH (6). The kinetic study shown in figure 1 suggests either a two-step transfer pathway or that glutathione reductase obeys a sequential mechanism. It is sometimes difficult to distinguish experimentally whether the true two-step transfer takes place orf the reaction of the first substrate —NADPH— implies an irreversible step before acceptor substrate —GSSG— is bound. Hence, in the latter the value of the apparent dissociation constant for the binary complex will be zero. The data in figure 2 establishes that at lower than 450 μM concentrations of oxidized glutathione the mechanism pathway is ping-pong for glutathione reductase and in the case of GSSG higher value concentrations the enzymatic kinetics follows a sequential pathway. It is important to emphasize that this value of oxidized glutathione concentration is the threshold that implies a change of kinetics behaviour. The outcome of this paper is consistent with the branched mechanism previously described for the glutathione reductase, conditioned by GSSG levels (4, 5).

Furthermore, the concentration of GSSG should also determine other features of kinetic behaviour of glutathione reductase in addition to the reaction pathway (9). As the data in figure 3 show, the inhibition effect of high —NADPH— substrate concentration is also a function

of GSSG concentration. For our purpose, only two concentrations of GSSG have been chosen, corresponding to a different kinetic pattern in each range, to show the inhibitory effect of NADPH excess. The conclusion is drawn that when the glutathione reductase reaction passes to the ping-pong mechanism, the inhibition effect of an excess of NADPH is stronger than when the enzymatic system takes place over the sequential mechanism.

Resumen

Estudios cinéticos realizados sobre la glutatión reductasa de levadura, bajo las condiciones experimentales de [GSSG] = 10[NADPH] en la mezcla de reacción muestran que, a concentraciones de GSSG inferiores a 450 μM, el mecanismo de reacción es *ping-pong* y para valores superiores, el mecanismo se adapta a uno secuencial. Cuando el mecanismo de la glutatión reductasa es *ping-pong*, la inhibición por exceso de NADPH es mayor que cuando la reacción transcurre a través de un mecanismo secuencial.

Palabras clave: Mecanismo de reacción, Glutatión reductasa, Glutatión.

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