Volume 243, number 1, 33–36 FEB 06689 January 1989

A 'branched' mechanism of the reverse reaction of yeast glutathione reductase

An estimation of the enzyme standard potential values from the steady-state kinetics data

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Received 10 November 1988

The reduced glutathione-linked $NADP^+$ reduction, catalyzed by yeast glutathione reductase, follows a 'sequential' or 'ping-pong' mechanism at high or low $NADP^+$ concentrations, respectively. The pattern of the NADPH and $NADP^+$ cross-inhibition reflects not only the competition for the binding site, but the shift of the reaction equilibrium as well. A 'branched' scheme of the glutathione reductase reaction is presented. The enzyme standard potential (−255 mV, pH 7.0) was estimated from the ratio of the NADPH and $NADP^+$ rate constants corresponding to the ping-pong mechanism.

Glutathione reductase; Steady-state kinetics; Redox potential

1. INTRODUCTION

Glutathione reductase (EC 1.6.4.2) catalyzes the reversible oxidation of NADPH by glutathione and contains FAD and redox active disulfide in the active centre [1]. The structure and mechanism of this enzyme are well known [2–6], but the data on its redox potential except glutathione reductase from cyanobacteria [7] are lacking. It seems likely that the potentiometry of glutathione reductase is complicated by side-reactions and inactivation of the enzyme under reducing conditions in the corresponding time scale [7–9].

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Abbreviations: GSSG, oxidized glutathione; GSH, reduced glutathione; E°, standard potential; TN, turnover number; TN/K_m, bimolecular rate constant; E_o, oxidized enzyme; E_r, reduced enzyme; DTT, dithiothreitol

The redox potential of glutathione reductase may be estimated from the NADPH and $NADP^+$ TN/K_m ratio in the steady-state kinetics, assuming that both reactions proceed via the same intermediates. The TN/K_m value of NADPH in the direct reaction proceeding via a 'hybrid ping-pong' mechanism [5,10] due to two structurally distinct NADPH and GSSG-binding sites [2] is equal to the rate constant of the active centre reduction [5,6]. However, according to [11] the reverse reaction follows a 'sequential' mechanism, so it seems probable that TN/K_m of $NADP^+$ corresponds to the alternative reaction pathway, as it follows from the hypothesis of a 'branched' mechanism of glutathione reductase [12].

In this paper we show that at low $NADP^+$ concentrations a transition from a sequential to ping-pong mechanism for the reverse reaction of glutathione reductase is observed. These data enabled us to estimate the redox potentials of glutathione reductase.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. MATERIALS AND METHODS

Yeast glutathione reductase (Sigma, grade III) was additionally purified as described in [3] up to $A_{280}/A_{460} = 7.7–7.9$. The enzyme concentration was determined using $\epsilon_{460} = 11.3\ mM^{-1} \cdot cm^{-1}$. NADPH, $NADP^+$ (Calbiochem), GSH and GSSG (Chemapol, Czechoslovakia), 2′,5′-ADP and DTT (Sigma) were used as received. The reaction rate was determined using a Hitachi-557 spectrophotometer according to the NADPH absorption change ($\Delta\epsilon_{340} = 6.2\ mM^{-1} \cdot cm^{-1}$) at 25 ± 0.1°C. The enzymatic GSH-dependent reduction of $NADP^+$ was done in the presence of 10 mM DTT [11]. 0.1 M K-phosphate-citrate (pH 6.5–8.0) and tris-citrate (pH 8.0–9.0) buffer solutions containing 1 mM EDTA were used. TN of the enzyme corresponds to a number of NADP(H) consumed by FAD per 1 s.

3. RESULTS

By analogy to the data of [1,3,5], the GSSG-linked NADPH oxidation is described by a ping-pong mechanism. TN_{max} of the enzyme (at saturating concentrations of both substrates) and TN/K_m of NADPH reach their maxima at pH 7.5–8.0. They are equal to 260 s^{-1} and $2.0 \times 10^7\ M^{-1} \cdot s^{-1}$, respectively. Beyond these limits TN/K_m of NADPH decreases up to $1.4–1.5 \times 10^7\ M^{-1} \cdot s^{-1}$ (pH 6.5 and 9.0). At pH ⩽7.5, $NADP^+$ acts as a competitive inhibitor for NADPH ($K_i = 60\ \mu M$). However, one must note that at pH 8.0 the $NADP^+$ inhibition exhibits a mixed pattern (fig.1). Under these conditions 2′,5′-ADP remains a competitive inhibitor for NADPH. The $NADP^+$ inhibition at pH 8.0 is linear with respect to the slopes in reciprocal coordinates and the intercepts on the ordinate axis as well.

A change from a sequential to ping-pong mechanism is observed in the reverse reaction of glutathione reductase at $NADP^+$ concentrations lower than that used in [11]. This is indicated by a break in a series of the converging lines in reciprocal coordinates and its transition to the series of parallel lines (fig.2A). The pH-optimum for this reaction is 8.0, and TN_{max} reaches 25 s^{-1}. The inverse TN values, obtained by extrapolation of converging or parallel lines to infinite concentrations of $NADP^+$, nonlinearly depend on the inverse concentrations of GSH (fig.2B). This phenomenon shown in [11] may be explained by the reaction of higher than the second order. The $NADP^+$ TN/K_m value, calculated according to the

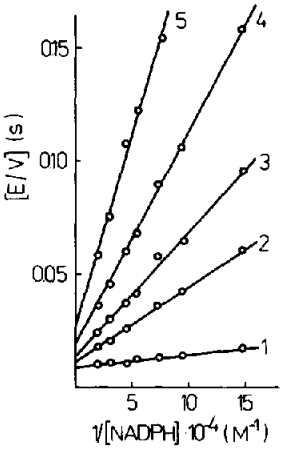

Fig.1. The dependence of the initial rate of GSSG-dependent oxidation of NADPH (pH 8.0) on the $NADP^+$ concentration. $NADP^+$ concentration: 0 (1), 0.25 (2), 0.5 (3), 1.0 (4) and 1.5 mM (5). GSSG concentration, 100 μM.

slope of parallel lines, is $3.0–3.5 \times 10^5\ M^{-1} \cdot s^{-1}$ (pH 8.0–9.0). It decreases with the pH decrease and makes up $3.2 \times 10^4\ M^{-1} \cdot s^{-1}$ at pH 6.5. The constant values do not depend on the buffer composition. As it is noted in [11], at $NADP^+$ concentrations corresponding to a sequential mechanism NADPH acts as a competitive inhibitor for

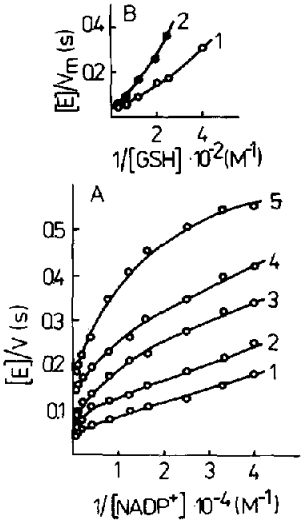

Fig.2. The initial rates of GSH-dependent reduction of $NADP^+$ at pH 8.0. (A) GSH concentration: 30 (1), 15 (2), 8 (3), 5 (4) and 4 mM (5). (B) Dependence of the maximal rate at saturating $NADP^+$ concentrations on the GSH concentration, obtained by extrapolation of the converging (1) and parallel (2) lines in A.

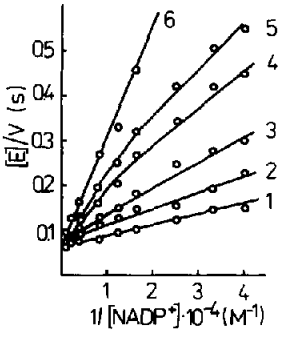

Fig.3. The dependence of the initial rate of GSH-dependent reduction of $NADP^+$ at pH 8.0 on the NADPH concentration. NADPH concentration: 0 (1), 5.5 (2), 11.1 (3), 25.5 (4), 35.5 (5) and 57.5 μM (6). GSH concentration, 20 mM.

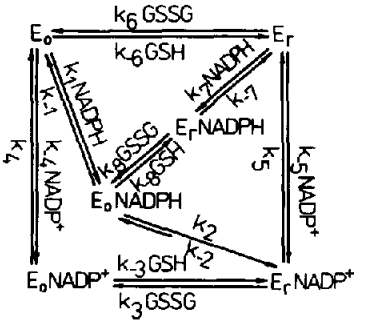

Fig.4. A proposed mechanism of a branched reaction of glutathione reductase.

$NADP^+$ (fig.3). However, with a decrease in the $NADP^+$ concentration, the NADPH inhibition assumes a mixed character.

4. DISCUSSION

The data of the present work show that a branched mechanism is characteristic of the GSH-linked reduction of $NADP^+$, catalyzed by glutathione reductase (fig.2). An analogous mechanism is known for the reduction of NAD^+ by dihydrolipoamide, catalyzed by lipoamide dehydrogenase [13,14]. It reflects the difference in the reduction rates of free and bound oxidized pyridine nucleotide active centres. A proposed scheme of the glutathione reductase reaction involving both sequential and ping-pong mechanisms is presented in fig.4. It includes the complexes of reduced enzyme with NADPH and $NADP^+$ which according to [5] are oxidized by GSSG at the same rate as a free enzyme. The figure shows that the NADPH oxidation by GSSG must follow a formal ping-pong mechanism when $k_3 \approx k_6 \approx k_8$, and $NADP^+$ must act as a competitive inhibitor for NADPH when $k_{-2} \approx 0$. However, when k_{-2} increases (the enzyme is efficiently oxidized by $NADP^+$) a mixed inhibition must occur. This is observed experimentally at pH 8.0 (fig.1). For the reverse reaction a change from sequential to a ping-pong mechanism occurs when $k_{-4}[NADP^+]k_{-3} \ll k_4k_{-6}$ [14] (fig.4). The pattern of the NADPH inhibition at low $NADP^+$ concentrations (fig.3) reflects not only the competition for the binding site, but the shift of the reaction equilibrium as well (fig.4), which is also in accord with a ping-pong mechanism [15]. So, one can conclude, that TN/K_m of $NADP^+$, determined by the slope of parallel lines in reciprocal coordinates, equals the rate constant of the reduced glutathione reductase oxidation (fig.4) in the following way:

$$E_o \underset{k_{-1}}{\overset{k_1[NADPH]}{\rightleftharpoons}} E_oNADPH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_rNADP^+ \underset{k_{-5}[NADP^+]}{\overset{k_5}{\rightleftharpoons}} E_r \qquad (1)$$

The equilibrium constant (K), corresponding to the ratio TN/K_m of NADPH and $NADP^+$, enables the determination of the standard redox potential of glutathione reductase, which equals $E^\circ_{(NADP^+/NADPH)} + 29.5\log K$ (mV). The pH-dependent TN/K_m of NADPH and $NADP^+$ and the calculated redox potential are shown in fig.5. E°_7 of -255 mV is 30 mV higher than that of pig

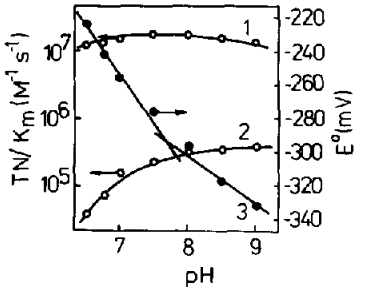

Fig.5. The pH profile of TN/K_m of NADPH (1) and $NADP^+$ (2) in the reactions, following a ping-pong mechanism, and the calculated standard potential of glutathione reductase (3).

heart lipoamide dehydrogenase [16] and is markedly lower than the potential of glutathione reductase from cyanobacteria ($E^{\circ}_{7.5} = -217$ mV [7]), determined by potentiometric titration. A change of the slope $\Delta E^{\circ}/\Delta$pH from -60 mV/pH to -30 mV/pH is observed at pH 7.6 (fig.5), which reflects, evidently, the pK_a value of a reduced active centre.

The validity of the approach used was tested on pig heart lipoamide dehydrogenase. Using the TN/K_m value of NADH (4.9×10^6 $M^{-1} \cdot s^{-1}$) and NAD^+ (1.5×10^6 $M^{-1} \cdot s^{-1}$), corresponding to the reverse reaction following a ping-pong mechanism [14], we obtained a difference in the standard potentials of lipoamide dehydrogenase and NAD^+/NADH at pH 7.5 of 15 mV, thus giving a $E^{\circ}_{7.5}$ value of -320 mV. This value is close to that obtained by potentiometric measurements of -315 mV [16].

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