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THE REACTION MECHANISM OF GLUTATHIONE REDUCTASE FROM HUMAN ERYTHROCYTES

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SUMMARY

1. The reaction mechanism of glutathione reductase (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) was studied. By varying the GSSG concentration, a series of parallel lines was obtained in the $1/v$ *vs.* $1/[NADPH]$ plots. $NADP^+$ is a competitive inhibitor towards NADPH and a noncompetitive one towards GSSG. Both GSSG and NADPH were inhibitory at high concentrations. In the $1/v$ *vs.* $1/[GSSG]$ plots only parallel lines were obtained at low NADPH concentrations. The maximum velocity was dependent on the Na^+ concentration.

2. With NADH as electron donor, a series of converging lines was obtained in the $1/v$ *vs.* $1/[NADH]$ plots; at high [GSSG] activation was observed. The maximum velocity was not affected by the Na^+ concentration. NAD^+ did not inhibit these reactions.

3. The results were interpreted in terms of a combination of simplified ordered bi-bi and ping-pong bi-bi mechanism and individual rate constants were calculated.

Opposite effects of the Na^+ concentration on the reaction rate of GSSG with the NADPH- and the NADH-reduced enzyme were found.

4. The influence of the temperature on the velocity of the NADPH dependent reaction in relation to different concentrations of buffer and GSSG led to either linear or nonlinear Arrhenius plots.

INTRODUCTION

Glutathione reductase (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) catalyzes the reaction GSSG + NADPH + $H^+ \rightleftharpoons$ 2 GSH + $NADP^+$. The enzyme is also active with NADH though less effectively. The difference in activity between NADPH and NADH as substrate is probably caused by the more effective binding of NADPH by the enzyme[1]. The reaction is reversible but high concentrations $NADP^+$ and GSH are necessary for significant NADPH formation, and the rate of the reaction in this direction is very low[1].

MASSEY AND WILLIAMS[2] proposed a reaction mechanism which is similar to that of lipoamide dehydrogenase. From their experiments with yeast glutathione reductase it was concluded that the enzyme has a reactive disulfide as part of the active center in addition to the flavin.

SCOTT *et al.*[1] investigated the effect of substrate concentration on the reaction velocity. Both NADPH and GSSG were inhibitory at high concentrations. K_m and V for both substrates were determined, and the Lineweaver–Burk plots showed parallel lines. Similar results were found by ICÉN[3]. SCOTT *et al.*[1] proposed a reaction mechanism which was based on the formation and decomposition of binary complexes of the two substrates with the enzyme in a mechanism essentially identical with that of MASSEY AND WILLIAMS[2].

SCOTT *et al.*[1] and ICÉN[3] found that many ions influence the reaction velocity considerably. We have investigated the influence of sodium phosphate at different temperatures on the reactions with NADH and NADPH.

MATERIALS AND METHODS

Chemicals

NADPH, $NADP^+$, NADH and NAD^+ were obtained from the Sigma Chemical Co. or from Boehringer and Soehne. GSSG from Boehringer and Soehne.

Glutathione reductase was prepared by the method described in the previous paper[4]. Glutathione reductase assays were carried out spectrophotometrically at 25° by following the change in absorbance at 340 nm due to oxidation of NADPH or NADH with GSSG as acceptor as described previously[4]. Further conditions are given in the various experiments described.

RESULTS

Several investigators[1–3] have proposed a reaction mechanism which was based on the formation and decomposition of binary complexes of the two substrates with the enzyme:

$$H^+ + E + NADPH \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E' \cdot NADP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E' + NADP^+$$

$$E' + GSSG \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E' - GSSG \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E + 2\,GSH$$

in which E is the oxidized and E' the reduced enzyme. In the presence of a competitive inhibitor (I), the rate equation is

$$v = \frac{V}{1 + \frac{K_{NADPH}}{[NADPH]}\left(1 + \frac{[I]}{K_i}\right) + \frac{K_{GSSG}}{[GSSG]}} \qquad (1)$$

in which K_i is the dissociation constant of the enzyme inhibitor complex. It is clear from Eqn 1 that either in the absence or in the presence of a competitive inhibitor, the lines of the $1/v$ *vs.* $1/[NADPH]$ plots at different GSSG concentrations are parallel.

Figs. 1 and 2 show that parallel lines are obtained at different GSSG concen-

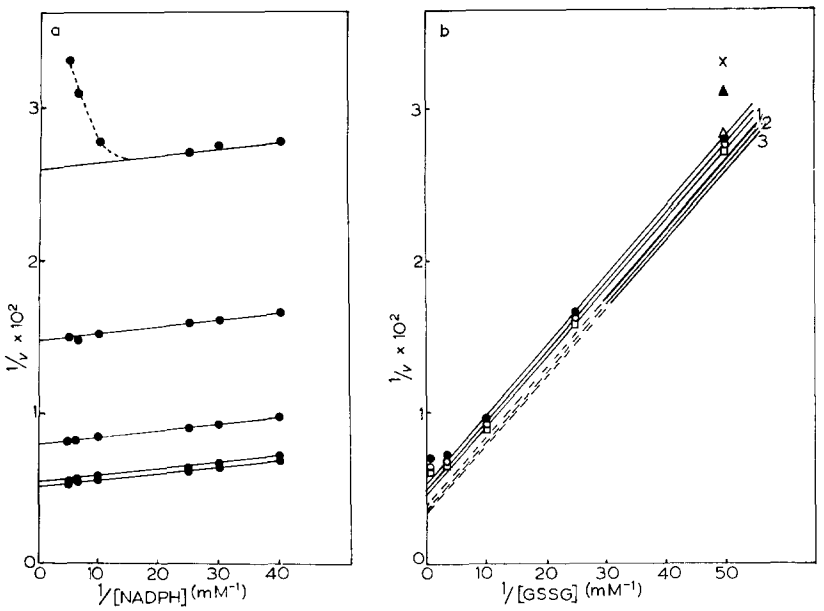


Fig. 1. (a) $1/v$ *vs.* 1/[NADPH] plots at different GSSG concentrations. Top to bottom: 20, 40, 100, 300 and 1500 μM GSSG. Buffer: 0.3 M sodium phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADPH concentrations as calculated from (a) Top to bottom: 25 (●), 33 (○), 40 (□), 100 (1, △) 150 (2, ▲) and 200 (3, ×) μM NADPH. Buffer: 0.3 M sodium phosphate (pH 7.0). v in μmoles NADPH oxidized per min per mg.

trations in the $1/v$ *vs.* 1/[NADPH] plots; the results are dependent on the buffer concentration used.

Figs. 1a and 1b show the effect on the enzyme activity by varying the concentrations of both GSSG and NADPH in 0.3 M sodium phosphate buffer (pH 7.0). At low GSSG concentrations a strong substrate inhibition by NADPH is found; this inhibition is overcome at higher GSSG concentrations. In the $1/v$ *vs.* 1/[GSSG] plots only at the lower concentrations of NADPH are parallel lines obtained. Due to the substrate inhibition observed at high and low GSSG concentrations, the slope of the lines at high NADPH concentration can be calculated only over a limited GSSG concentration range. The results show that they are parallel.

The same investigations were carried out in 0.03 M sodium phosphate buffer (pH 7.0); Fig. 2a shows the effect of varying the concentrations of both GSSG and NADPH. As is the case with 0.3 M sodium phosphate at low GSSG concentrations a substrate inhibition by NADPH, however, to a much less extent, is found. On the other hand inhibition at high GSSG concentrations is found in 0.03 M sodium phosphate; at 1.5 mM GSSG strong inhibition is found at all NADPH concentrations, a result different from that in 0.3 M sodium phosphate. By plotting $1/v$ *versus* 1/[GSSG] only parallel lines are obtained at low NADPH concentrations. At NADPH concentrations higher than 100 μM, the lines converge due to inhibition by this substrate (Figs. 2b and 2c). From these results an apparent K_m for GSSG of 19 μM (Fig. 3a) and a K_m for NADPH of 9.5 μM were calculated. These values are considerably lower than obtained in 0.3 M sodium phosphate: K_m (GSSG) 125 μM and K_m (NADPH)

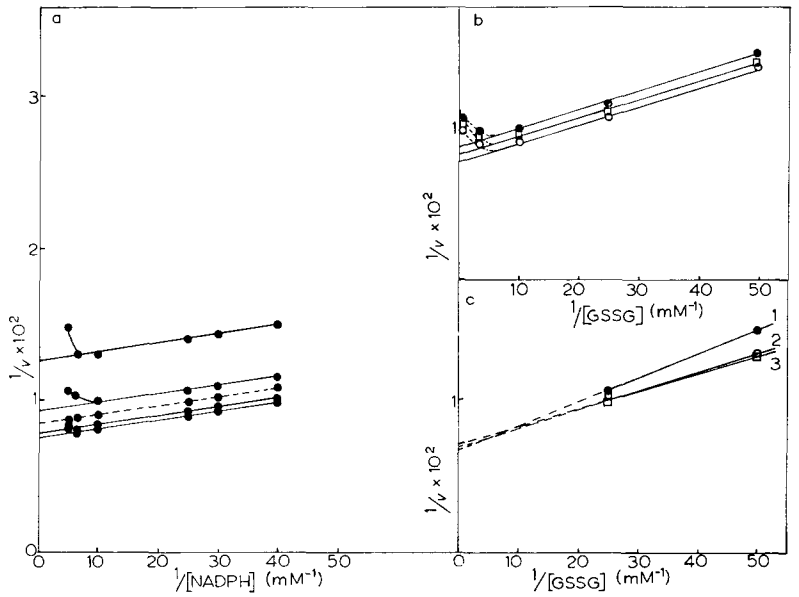


Fig. 2. (a) $1/v$ *vs.* 1/[NADPH] plots at different GSSG concentrations. Top to bottom: 20, 40, 1500, 100 and 300 μM GSSG. Buffer: 0.03 M sodium phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADPH concentrations. ●—●, 25; □—□, 33; and ○—○, 40 μM NADPH. Buffer: 0.03 M sodium phosphate (pH 7.0). (c) $1/v$ *vs.* 1/[GSSG] plots at different NADPH concentrations. Top to bottom: 200 (1), 150 (2) and 100 (3) μM NADPH. Buffer: 0.03 M sodium phosphate (pH 7.0). v in μmoles NADPH oxidized per min per mg.

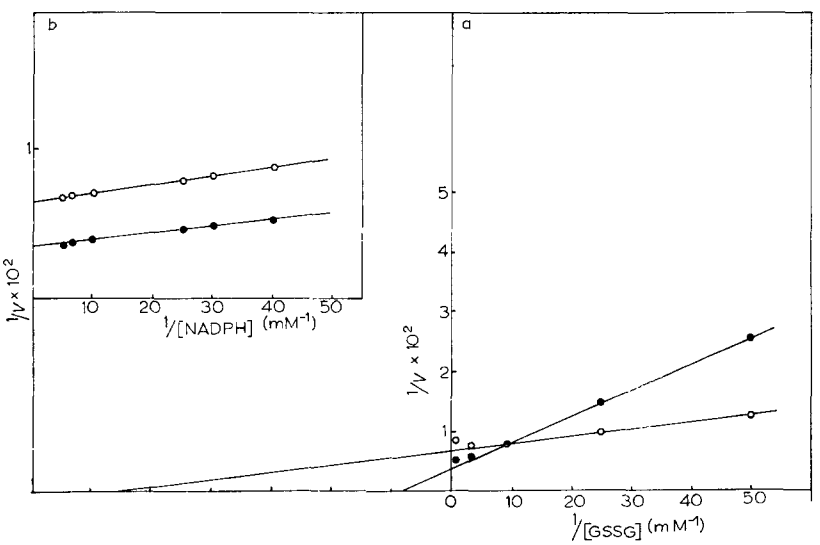


Fig. 3. (a) The plot $1/V$ (NADPH) *vs.* 1/[GSSG]. These values are calculated from Fig. 1a (●—●) and Fig. 2a (○—○). (b) The plot $1/V$ (GSSG) *vs.* 1/[NADPH]. These values are calculated from Fig. 1b (●—●) and from Figs. 2b and 2c (○—○). V in μmoles NADPH oxidized per min per mg.

TABLE I

RATE CONSTANTS FOR THE NADPH- AND NADH-DEPENDENT REACTIONS AT DIFFERENT SODIUM PHOSPHATE CONCENTRATIONS

Conditions as described in the text.

Rate constants	*Substrate:* *NADPH*	*NADH*	*NADH*	*NADPH*
	0.3 M sodium phosphate	*0.1 M sodium phosphate*	*0.03 M sodium phosphate*	*0.03 M sodium phosphate*
k_{-1} (sec^{-1})		7	15	
k_{+1} ($mole^{-1} \cdot l \cdot sec^{-1}$)				
Fig. 3	$2 \cdot 10^7$			$2 \cdot 10^7$
Fig. 9		$2 \cdot 10^5$	$2.5 \cdot 10^5$	
Fig. 10b		$1.5 \cdot 10^5$	$2.0 \cdot 10^5$	
k_{+2} ($mole^{-1} \cdot l \cdot sec^{-1}$)	$2 \cdot 10^6$	$1 \cdot 10^6$	$0.4 \cdot 10^6$	$8 \cdot 10^6$
k_{+3} (sec^{-1})	260	50	50	140
k_{+4} (sec^{-1})		43	36	
k_{+5} ($mole^{-1} \cdot l \cdot sec^{-1}$)		$3 \cdot 10^6$	$3 \cdot 10^6$	
K_D (M)				
from Fig. 10		$3 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	
this table		$4 \cdot 10^{-5}$	$6 \cdot 10^{-5}$	

13.3 μM, respectively. On the other hand the maximum velocity in 0.3 M sodium phosphate is two times higher (Table I).

SCOTT *et al.*[1] and ICÉN[3] have obtained parallel lines; these authors reported that $NADP^+$ is a competitive inhibitor towards NADPH but did not discuss the consequences for the reaction mechanism as given above. The studies of MASSEY AND WILLIAMS[2] with yeast GSSG reductase show that it is rapidly reduced by NADPH which indicates that NADPH is the first substrate combining with the enzyme. In a binary ping-pong bi-bi mechanism[5], as given above, the product $NADP^+$ can only be a noncompetitive inhibitor towards NADPH, since it dissociates as the first product. Competitive inhibition can only be expected in case of either a rapid equilibrium random bi-bi or an ordered bi-bi or Theorell–Chance mechanism (*cf.* ref. 5).

One could consider that $NADP^+$ forms an abortive complex by binding to the oxidized enzyme which acts in a binary complex bi-bi mechanism. In that case and [GSH] = 0 the rate equation will modify into

$$v = \frac{V}{1 + \frac{K_{NADPH}}{[NADPH]}\left(1 + \frac{[NADP]}{K_{NADP}}\right) + \frac{K_{GSSG}}{[GSSG]} + \frac{k_{-2}[NADP]\,(k_{-3} + k_4)}{k_3[GSSG]\,(k_2 + k_4)}\left\{1 + \frac{k_{-1}}{k_1[NADPH]}\left(1 + \frac{[NADP]}{K_{NADP}}\right)\right\}} \quad (1A)$$

in which K_{NADP} is the dissociation constant of the abortive complex. It is clear that $NADP^+$ is a competitive inhibitor toward NADPH only in the case that k_{-2} [NADP] $\ll$ k_3 [GSSG]. Fig. 4a shows that a 15-fold variation of the GSSG concentration does not affect the competitive behavior.

Two things are obvious from this equation: (1) only at [NADPH] = ∞, $NADP^+$ acts competitively towards GSSG. (2) There exists a nonlinear relationship between the amount of inhibition and [NADP] (*cf.* ref. 5).

In case of the random bi-bi mechanism the product will also act competitively

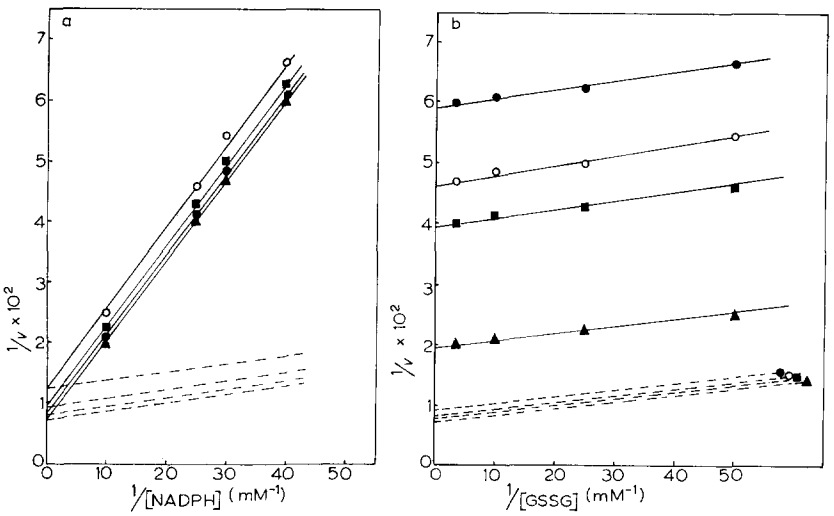


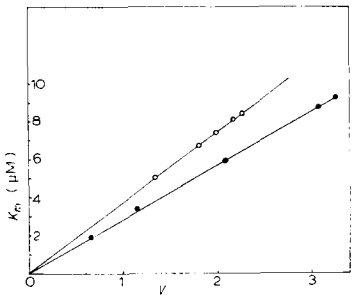
Fig. 4. (a) $1/v$ *vs.* $1/[NADPH]$ plots in the presence of 0.5 mM $NADP^+$ at different GSSG concentrations. ○—○, 20; ■—■, 40; ●—●, 100; and ▲—▲, 300 μM GSSG. Buffer: 0.03 M sodium phosphate (pH 7.0). The dotted lines represent the non-inhibited reactions (Fig. 2a). (b) $1/v$ *vs.* $1/[GSSG]$ plots in the presence of 0.5 mM $NADP^+$ at different NADPH concentrations. ●—●, 25; ○—○, 33 ;■—■, 40; and ▲—▲, 100 μM NADPH. The dotted lines represent the corresponding non-inhibited reaction (Fig. 2b). v in μmoles NADPH oxidized per min per mg.

towards the second substrate. Fig. 4a shows the same experiment as is given in Fig. 2, carried out in the presence of the product and competitive inhibitor $NADP^+$ (0.5 mM). (The plot with 1.5 mM GSSG is omitted.) It is clear that under this condition the lines hardly converge but are not parallel.

It can be concluded from the $1/v$ *vs.* $1/[GSSG]$ plots (Fig. 4b) that inhibition of this substrate is not competitive which rules out the random bi-bi mechanism. The plots of Fig. 4b, however, do not permit the conclusions that the lines are parallel. Actually such a behavior cannot be expected under the conditions of the experiments for any mechanism (*cf.* ref. 4). It has been demonstrated[6–8] by the additions of competitive inhibitors (*cf.* refs. 9 and 10) that the postulated ping-pong bi-bi mechanisms of D-amino-acid oxidase and of succinate dehydrogenase rather are ordered bi-bi mechanisms. The presence of the inhibitor increases the influence of the term $K_{SA}/[S]$ in the total rate equation

$$v = \frac{V}{1 + \frac{K_s}{[S]}\left(1 + \frac{[I]}{K_i}\right) + \frac{K_A}{[A]} + \frac{K_{SA}}{[S][A]}\left(1 + \frac{[I]}{K_i}\right)} \qquad (2)$$

in which S is the donating first substrate and A is the accepting second substrate. This converts the apparent parallel lines obtained into series of nonparallel Lineweaver–Burk plots (*cf.* ref. 6). It also can be shown (*cf.* refs. 6–10) that in case of an ordered bi-bi mechanism the K_m *vs.* V plot does not pass through the origin, although the intercept might be close to zero. Fig. 5 shows that at both buffer concentrations the K_m *vs.* V plot yields a straight line which passes through the origin. In the K_m/V *vs.*



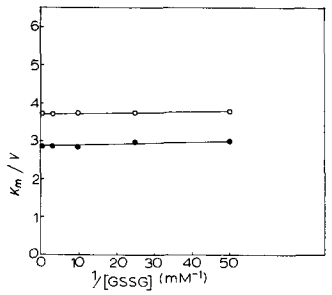


Fig. 5. The plot K_m *vs.* V. The values of K_m and V are calculated from Fig. 1a (●—●) and Fig. 2a (○—○). V in μmoles NADPH oxidized per sec per mg.

Fig. 6. The K_m/V *vs.* 1/[GSSG] plot. The values of K_m/V are calculated from Fig. 1a (●—●) and Fig. 2a (○—○).

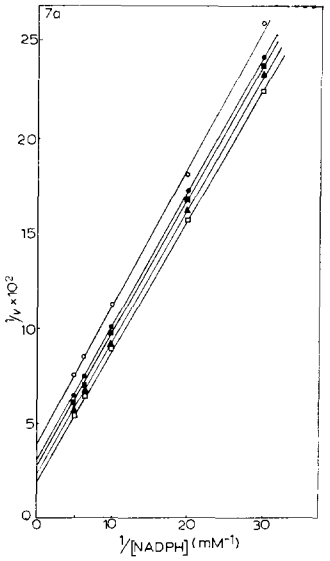
1/[GSSG] plot the lines at both buffer concentrations are almost parallel with the abcissa (Fig. 6). Such a result only can be expected in case the presence of a considerable amount of the competitive inhibitor does not lead to a large increase the two substrate term in the rate equation. These results show, therefore, that rather than the slope of the lines of the Lineweaver–Burk plots, the observation of competitive product inhibition leads to a conclusion about the actual kinetic mechanism which in a minimum hypothesis is:

$$E + \text{NADPH} + \text{H}^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E' - \text{NADP}^+$$

$$E' - \text{NADP}^+ + \text{GSSG} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E - \text{NADP}^+ + 2\,\text{GSH}$$

$$E - \text{NADP}^+ \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + \text{NADP}^+$$

NADH as an electron donor in this reaction gives much lower activities. It was of interest to carry out the same investigations with this compound. Figs. 7 and 8 show the relationships between rate, GSSG concentration and NADH concentration at two different buffer concentrations. It can be seen from Figs. 7a and 8a that the plots are converging to a point in the third quadrant, an observation in agreement with the ordered bi-bi mechanism for this enzyme as concluded from the experiments with NADPH. The presence of $NADP^+$, competitive inhibitor with respect to NADH, accentuates the nonparallel relationship between the plots. Figs. 7b and 8b show that at low GSSG concentrations in the $1/v$ *vs.* 1/[GSSG] plots the lines are parallel, but at high GSSG concentrations activation is observed. This activation is dependent on the NADH and buffer concentrations. Therefore 2 types of extrapolations towards infinite GSSG were made from 2 parts of the plots (Fig. 9). The points show a straight line relationship in the $1/V$ *vs.* 1/[NADH] plot giving different V values, the lines intersecting at the same point on the abscissa. These relationships which have been observed in two independent experiments are in contrast with those reported by IcÉN[3], who



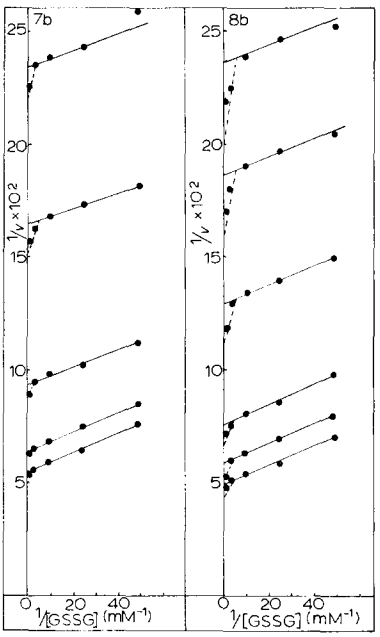


Fig. 7. (a) $1/v$ *vs.* 1/[NADH] plots at different GSSG concentrations. ○—○, 20; ●—●, 40; ■—■, 100; ▲—▲, 300; and □—□, 1500 μM GSSG, Buffer: 0.1 M sodium phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADH concentrations. Top to bottom: 33, 50, 100, 150 and 200 μM NADH. Buffer: 0.1 M sodium phosphate (pH 7.0). v in μmoles NADH oxidized per min per mg.

Fig. 8. (a) $1/v$ *vs.* 1/[NADH] plots at different GSSG concentrations. ○—○, 20; ●—●, 40; ■—■, 100; ▲—▲, 300; and □—□, 1500 μM GSSG. Buffer: 0.03 M sodium phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADH concentrations. Top to bottom: 25, 33, 50, 100, 150 and 200 μM NADH. Buffer: 0.03 M sodium phosphate (pH 7.0). v in μmoles NADH oxidized per min per mg.

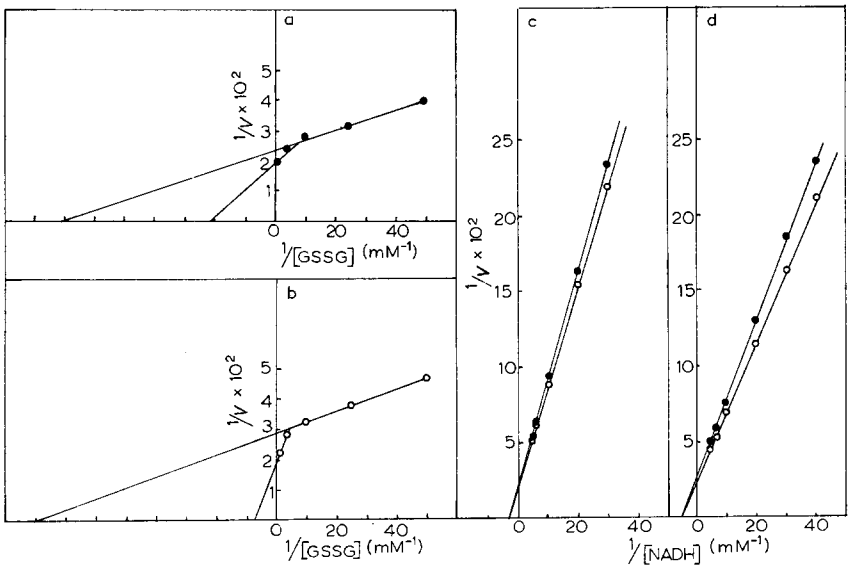


Fig. 9. (a) The plot $1/V$ (NADH) *vs.* $1/[GSSG]$. These values are calculated from Fig. 7a. (b) The plot $1/V$ (NADH) *vs.* $1/[GSSG]$. These values are calculated from Fig. 8a. (c) The plot $1/V$ (GSSG) *vs.* $1/[NADH]$. These values are calculated from Fig. 7b. ●—●, not activated; ○—○, activated. (d) The plot $1/V$ (GSSG) *vs.* $1/[NADH]$. These values are calculated from Fig. 8b. ●—●, not activated; ○—○, activated. V in μmoles NADH oxidized per min per mg.

reported that $NADP^+$ is competitive inhibitor of the NADH-dependent reaction. We have confirmed this result and also found that NAD^+ does not inhibit the NADH-dependent reaction. Similar results were obtained with the yeast enzyme.

GAWRON *et al.*[11,12] observed deviation from straight line relationship in the Lineweaver–Burk plots obtained with succinate dehydrogenase. They proposed the existence of a side reaction in addition to the ordered bi-bi mechanism in which the product dissociates from the reduced enzyme prior to oxidation by the acceptor; this is followed by oxidation of the reduced enzyme with the acceptor. This will lead to a quadratic relationship between velocity and acceptor concentration, however, a linear relationship is obtained with respect to the donor. Although it was recently shown[8] that under the conditions of GAWRON *et al.* this mechanism is not valid for succinate dehydrogenase, we propose as a minimum hypothesis for the NADH-dependent reaction of glutathione reductase a similar mechanism:

$$E + \mathrm{NADH} + \mathrm{H^+} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E' \cdot \mathrm{NAD^+} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E' + \mathrm{NAD^+}$$

$$E' \cdot \mathrm{NAD^+} + \mathrm{GSSG} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E \cdot \mathrm{NAD^+} + 2\,\mathrm{GSH}$$

$$E' + \mathrm{GSSG} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} E + 2\,\mathrm{GSH} \qquad\qquad E \cdot \mathrm{NAD^+} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + \mathrm{NAD^+}$$

with the following rate equation:

$$\frac{1}{v} = \frac{1}{E_0}\left\{\frac{k_4}{k_5[A]\,(k_4+k_2[A])} + \frac{1}{k_4+k_2[A]} + \frac{1}{k_1[S]} + \frac{k_{-1}}{k_1[S]\,(k_4+k_2[A])} + \frac{k_2[A]}{k_3(k_4+k_2[A])}\right\} \quad (3)$$

in which A = GSSG and S = NADH. From this equation it can be derived that in case $k_2[\mathrm{GSSG}] \gg k_4$, the rate equation for the ordered bi-bi mechanism is obtained; while in case $k_4 \gg k_2[\mathrm{GSSG}]$ the ping-pong bi-bi mechanism is obtained which is responsible for the parallel line relationship at low GSSG concentrations. The intercepts on the abcissa of the $1/V$ *vs.* $1/[\mathrm{NADH}]$ plots, taken for the two different cases, are k_1/k_3 and $k_1/(k_1 + k_4)$ for the ordered bi-bi and ping-pong bi-bi mechanism, respectively. The intercepts on the ordinate are k_3 and k_4, respectively. In the same way it can be shown that the intercepts on the abscissa of the $1/V$ *vs.* $1/[\mathrm{GSSG}]$ plots are k_2/k_3 for the ordered bi-bi mechanism and k_5/k_4 for the ping-pong bi-bi mechanism.

The values of the different constants are summarized in Table I. In the first place it must be noticed that the buffer concentration has no influence on the maximum velocity of the NADH- dependent reaction which is in contrast with the reaction with NADPH as electron donor. Sodium phosphate has its main influence on the reaction

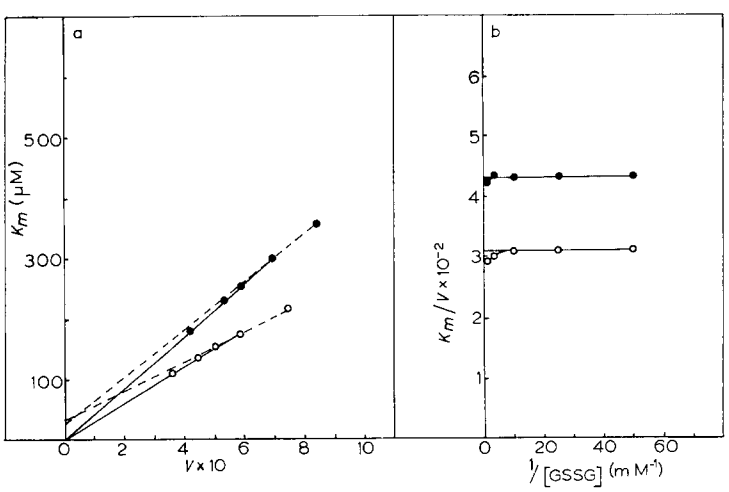


Fig. 10. (a) The plot K_m *vs.* V. The values of K_m and V are calculated from Fig. 7a (●—●) and Fig. 8a (○—○). (b) The K_m/V *vs.* $1/[\mathrm{GSSG}]$ plot. The values of K_m/V are calculated from Fig. 7a (●—●) and Fig. 8a (○—○). V in μmoles NADH oxidized per sec per mg.

rate of GSSG with the reduced enzyme–NAD^+ complex, while the dissociation of this complex is affected to some extent. SCOTT *et al.*[1] and ICÉN[3] reported that many ions influence the reaction velocity of glutathione reductase, *e.g.* K^+, Na^+ and NH_4^+. When sodium phosphate was replaced by NaCl the same activities were obtained. The same was found when potassium phosphate was replaced by KCl. This indicates that K^+ and Na^+ influence the reaction velocity and not phosphate. K^+ and Na^+ gave similar

results. SCOTT *et al.*[1] reported that the maximum velocity of the NADH-dependent reaction was not influenced by Na^+ and K^+, which is in agreement with our results.

Straight line relationships are not obtained in the secondary plots (Fig. 10). In the K_m *vs.* V plot the line through the points at the low GSSG concentrations passes through the origin, but the line through the points at the high GSSG concentrations intersects the ordinate. In the K_m/V *vs.* $1/[GSSG]$ plot the line at low GSSG concentrations is parallel with the abscissa, as expected for a ping-pong bi-bi mechanism; at high GSSG concentrations deviation from linearity occurs and the plot is not parallel with the abscissa as expected for an ordered bi-bi mechanism. For the mechanism proposed the intercept with the ordinate is equal with $1/k_{+1} \cdot e$ (*cf.* refs. 6 and 9): the value calculated is close to that from Fig. 9.

These results strongly support the ordered bi-bi mechanism for the enzyme. The fact that NAD^+ shows no competitive inhibition towards NADH indicates that NAD^+ has very little affinity for the oxidized enzyme. The observation that 1 mM NAD^+ has no influence on the reaction with NADH allows the conclusion that the affinity for the reduced enzyme is also low.

Accepting this kinetic mechanism for both pyridine nucleotides, it is possible to calculate from Fig. 3 some rate constants for the NADPH-catalyzed reaction (Table I). It is clear that in this case the Na^+ effect is completely different; rather than lowering the rate constant as in the NADH reaction, the lowest Na^+ concentration enlarges this constant for the reaction of GSSG with the reduced enzyme $NADP^+$

TABLE II

THE ACTIVATION ENERGIES, ΔE, CALCULATED FROM THE ARRHENIUS PLOTS OF THE CATALYTIC REACTION OF GLUTATHIONE REDUCTASE WITH TWO DIFFERENT GSSG CONCENTRATIONS (50 μM AND 1.5 mM) AT DIFFERENT BUFFER CONCENTRATIONS

GSSG concn. (μM)	*Sodium phosphate buffer (M)*	*ΔE ($cal \cdot mole^{-1}$)*	*Temp. range*
1500	0.3	14 400	9–28.5°
50	0.3	8 000	9–28.5°
1500	0.03	16 900	9–17°
1500	0.03	9 200	17–28.5°
50	0.03	11 000	9–17°
50	0.03	7 800	17–28.5°

enzyme. Also the effect of Na^+ on the catalytic center activity (k_{+3}) is different with the two pyridine nucleotides. Table I also gives the values of the dissociation constant for the first reaction as calculated from Figs. 7 and 8 and the value calculated from Fig. 10a (*cf.* refs. 6 and 9).

The influence of the temperature on the velocity of the NADPH-dependent reaction in relation to different concentrations of Na^+ and GSSG is shown in Fig. 11. In all cases V values were obtained by extrapolating to infinite NADPH concentration. At low temperatures (9–17°), the V values with 50 μM GSSG are higher with respect to the values obtained with 1.5 mM GSSG. However at higher temperatures just the

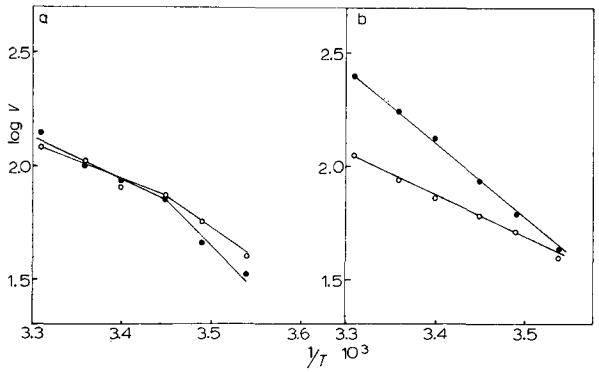


Fig. 11. (a) The Arrhenius plot of human erythrocyte glutathione reductase in 0.03 M phosphate (pH 7.0) with NADPH as electron donor. ●—●, 1.5 mM GSSG; ○—○, 50 μM GSSG. (b) The Arrhenius plot of glutathione reductase in 0.3 M sodium phosphate buffer (pH 7.0) with NADPH as electron donor. ●—●, 1.5 mM GSSG; ○—○, 50 μM GSSG.

reverse is found. At both GSSG concentrations a break occurs around 17° in 0.03 M buffer. At high Na^+ concentrations linear Arrhenius plots are obtained at low as well as high GSSG concentrations. However the activation energies at the two GSSG concentrations are different (Table II).

DISCUSSION

The data presented here show that the kinetic behavior of human erythrocyte glutathione reductase is strongly dependent on the buffer concentration. Although as a minimum hypothesis a simplified mechanism was used to make a comparison of rate constants possible under a variety of conditions with respect to buffer, donor and acceptor concentrations and temperatures, it cannot be excluded that a more complicated mechanism is operating. In that case, calculation of the individual rate constants is impossible; the results only lead to relations between the different rate constants (*cf.* refs. 6–8) which, however, does not lead to different conclusions. The more important conclusion which is derived from these results is that the ping-pong bi-bi mechanism is not operating in the human erythrocyte glutathione reductase which is in disagreement with previous results[1–3] with the yeast and erythrocyte enzyme. Although at high GSSG and NADPH concentrations complications due to substrate inhibition occur, the results of the NADPH-dependent reaction with respect to the inhibition by $NADP^+$ towards NADPH (competitive) and towards GSSG (non-competitive) completely support the ordered bi-bi mechanism derived from the NADH-dependent reaction. The fact that we found competitive inhibition by $NADP^+$ in case of the NADPH-catalyzed reaction of the yeast enzyme, while similar activation at high GSSG concentrations was observed with NADH as electron donor indicates that the yeast enzyme operates with a similar mechanism. In contrast with the inhibition occurring in 0.03 M sodium phosphate in the NADPH-dependent reaction at 1.5 mM GSSG with respect to much lower concentrations of the acceptor is the

activation observed in the NADH-dependent reaction at this GSSG concentration. It is likely to be due to the lower affinity of NADH. The kinetic relation derived from the NADH-dependent reaction accounts partly for the data observed in the NADPH-dependent reaction, *e.g.* the ordered bi-bi mechanism; it does not account for the strong substrate inhibition by both NADPH and GSSG.

From the constants at 25° at high sodium phosphate it can be easily derived that for a [GSSG] $\gg$ 126 μM the rate constant k_{+3} is the rate limiting factor in the rate equation. Below this concentration k_{+2}[GSSG] is the rate limiting factor. It is then likely that in the Arrhenius plot at [GSSG] = 50 μM the dependency of k_{+2}[GSSG] with the temperature at 1.5 mM k_{+3} is measured. If this is the case, it can be concluded that k_{+3} is more temperature dependent than k_{+2}. At low sodium phosphate concentrations the rate limiting concentration of GSSG is about 20 μM at 25°. The used GSSG concentrations are higher, so it can be concluded that in this case k_{+3} is measured.

MASSEY *et al.*[15] have listed a number of enzymes which show a discontinuous Arrhenius plot. Several of them are flavoproteins[16]. It is quite possible that the break occurring in a low sodium phosphate is due to a conformational change in the protein. Evidence for the existence of two conformations with different activities in temperature dependent equilibrium has been given for D-amino-acid oxidase and L-amino-acid oxidase[16]. The fact that below the break temperature a lower velocity is obtained with 1.5 mM GSSG than with 50 μM may be an indication that GSSG has a higher affinity for the low-temperature form, provided that the latter has a lower activity than does the high temperature form. Above the temperature of the break, only the high temperature form is present, while below this temperature the line represents a transition range between the two conformations. On the other hand one would expect a temperature dependence of the break on the GSSG concentration. Studies with succinate dehydrogenase show that similar temperature-dependent phenomena occur (*cf.* ref. 8 and 17).

Although a straight Arrhenius plot does not allow the conclusion that one is dealing with one conformation of the enzyme (*cf.* ref. 16), the straight lines observed in Fig. 11b could be explained in terms of a shift of such an equilibrium towards the more active high temperature conformation by Na^+. Only a detailed kinetic analysis at many temperatures may provide more definite conclusions.

Among the many similarities between glutathione reductase and lipoamide dehydrogenase (*cf.* ref. 4) is also the large influence of Na^+, K^+ and NH_4^+ on these enzymes (*cf.* ref. 18). In contrast to the activating effect of the ions, in the case of glutathione reductase, the effect is with the latter enzyme much more complex. It was shown[19] that a low protein concentrations the enzyme dissociates in sodium phosphate which occurs to a much less extent in potassium phosphate or in the presence of NH_4^+. The results from Table II also indicate that in glutathione reductase the role of these ions is rather complex, *e.g.* enhancement of the reaction rate of GSSG but a decline in V at the lower ion concentration in the case of NADPH as donor. The fact that the binding rate of GSSG is affected in an opposite way in the case of NADH as donor, may be an indication for the formation of a ternary complex.

The recent observations[6–8] of ordered bi-bi mechanisms occurring in flavoproteins in combination with the results of this paper lead to the question whether all enzymes of this type are acting with an ordered bi-bi mechanism. In the light of the recently isolated N-5 adducts[20,21] of flavin such a hypothesis is very attractive. The

consequences for the mechanism of action then will be very large for lipoamide dehydrogenase for which also a ping-pong bi-bi mechanism is postulated. It is interesting to recall in this connection that an intermediate of the NADH-reduced lipoamide dehydrogenase complexed with NAD^+, which is formed at a very high rate, has been reported[22].

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