Biochimica et Biophysica Acta, 527 (1978) 327—336

BBA 68612

CHARACTERIZATION OF HUMAN PLATELET GLUTATHIONE REDUCTASE

GARY MOROFF and DAVID P. KOSOW

American Red Cross Blood Research Laboratory, Bethesda, Md. 20014 (U.S.A.)

(Received March 17th, 1978)
(Revised manuscript July 11th, 1978)

Summary

Glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2) has been purified 1000-fold from the cytoplasmic fraction of human platelets. Salts, including the heretofore unreported effect of sodium citrate, activate the NADPH-dependent reduction of oxidized glutathione. Sodium citrate and monovalent salt activation appears to involve multiple sites having different binding affinities. At sub-saturating sodium phosphate, non-linear double reciprocal plots indicative of substrate activation by oxidized glutathione were observed. Initial velocity double reciprocal plots at sub-saturating and saturating concentrations of phosphate generate a family of converging lines. $NADP^+$ is a partial inhibitor, indicating that the reduction of oxidized glutathione can proceed by more than one pathway. FMN, FAD, and riboflavin inhibit platelet glutathione reductase by influencing only the V while nitrofurantoin inhibition is associated with an increase $K_{\text{oxidized glutathione}}$ and a decreased V.

Introduction

Glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2) catalyzes the pyridine nucleotide-dependent reduction of oxidized glutathione to reduced glutathione and accordingly maintains a high reduced glutathione/oxidized glutatione ratio in cells. Although the glutathione reductase isolated from various sources has been studied in depth [1—15], little information is available about the properties of the enzyme present in human blood platelets.

Abbreviation: Nbs_2, 5,5'-dithio-bis-(2-nitrobenzoic acid).

Materials and Methods

Oxidized glutathione, NADPH, bovine serum albumin (fatty-acid-free), FAD, FMN, riboflavin and nitrofurantoin were obtained from Sigma Chemical Co. Ammonium sulfate (special enzyme grade) was purchased from Schwarz/Mann. DEAE-cellulose (DE-52) was obtained from Whatman. Hydroxyapatite was purchased from Bio-Rad.

Assay of glutathione reductase activity

NADPH utilization was measured spectrophotometrically at 340 nm using a Cary 118 spectrophotometer temperature-controlled at 30°C. Concentrations of NADPH solutions were determined using an extinction coefficient of 6220 $M^{-1} \cdot cm^{-1}$ at 340 nm [16]. Protein concentrations were determined by the method of Warburg and Christian [17].

The standard assay system consisted of 60 mM sodium phosphate (pH 7.4), 100 mM NaCl, 1 mM EDTA, 2 mM oxidized glutathione and 0.08 mM NADPH in a final volume of 1 ml. Oxidized glutathione and EDTA solutions were adjusted to pH 7.4 with NaOH. NADPH solutions were prepared fresh daily in 0.001 M sodium phosphate (pH 8.0).

The assay system used in the steady-state kinetic experiments consisted of 10 or 100 mM sodium phosphate (pH 7.4), 1 mM EDTA and variable concentrations of oxidized glutathione and NADPH. Reactions were initiated with 0.05 ml of an enzyme solution. Stock enzyme solutions were diluted with 10 mM sodium phosphate, 0.5 mM EDTA (pH 7.4) and 1 mg/ml fatty-acid-free bovine serum albumin (previously dialyzed against 10 mM sodium phosphate, 0.5 mM EDTA, pH 7.4). Bovine serum albumin prevented a loss of glutathione reductase activity.

Purification of glutathione reductase

Preparation of platelet lysates. Human platelet concentrates (3—7 days old) in citrate/phosphate/dextrose anticoagulant were obtained from the Washington Regional Blood Program-American Red Cross. Contaminating leukocytes and erythrocytes were removed from pooled concentrates by centrifugation (four times) at 1800 $\times g$ in a Sorval GLC-1 centrifuge at room temperature. There was some loss of platelets by this procedure. Hemocytometer counting showed that a typical platelet suspension contained less than 0.2% of either leukocytes or erythrocytes. Based on the data showing the relative amount of glutathione reductase in human blood cells [18] this level of contamination is not significant. The platelet suspension, prepared from 50 to 75 units platelet concentrates was then lysed by the glycerol-lysis technique [19] and the cytoplasmic fraction was used for the isolation of the enzyme.

Isolation of glutathione reductase. The protein precipitating between 45 and 60% saturation of $(NH_4)_2SO_4$ contained the glutathione reductase activity. Following dialysis against 5 mM sodium phosphate/0.5 mM EDTA, the sample was put on a DEAE-cellulose column and eluted with the dialysis buffer. A protein peak containing glutathione reductase was detected after elution of the main protein peak. After dialysis and concentration by ultrafiltration, the enzyme fraction was applied to a hydroxyapatite column and one peak of

glutathione reductase was eluted with a linear gradient of 10—200 mM sodium phosphate (pH 7.4). After concentration and dialysis against 10 mM Tris-HCl/0.5 mM EDTA (pH 8.7), the enzyme preparation was separated further by DEAE-cellulose chromatography. One peak of glutathione reductase was eluted using a linear gradient of 0—200 mM NaCl in the Tris-HCl buffer. The enzyme was stored in 10 mM sodium phosphate/0.5 mM EDTA (pH 7.4). Approx. 1000-fold purification was obtained with a 20—30% recovery. Due to the limited quantity of human platelets available, the protein yield was less than 1 mg.

Polyacrylamide gel electrophoresis

Discontinuous gel electrophoresis on 7.5% polyacrylamide gels was carried out at pH 8.9 [20]. The gels were stained for activity with *p*-iodonitrotetrazolium essentially by the procedure used to detect rabbit erythrocyte glutathione reductase [21]. Persulfate was removed prior to the addition of the enzyme.

Purification of flavin compounds

FAD, FMN and riboflavin were purified on DEAE-cellulose at 4°C by linear gradient elution using the step-wise gradient elution procedure [22]. The procedures were carried out with columns wrapped with aluminum foil to decrease light exposure. The fractions in the middle of the flavin peaks were pooled and used within 72 h of preparation. The concentration of flavins were determined by their absorbance at 450 nm [22]. The 260 nm/450 nm and 375 nm/450 nm ratios of the flavin were very similar to those of Beinert [23].

Results

Characteristics of platelet glutathione reductase

The specific activity of the partially purified platelet glutathione reductase obtained from the cytoplasmic fraction ranged between 20 and 30 units/mg protein. Staining of polyacrylamide gels for enzyme activity showed one broad band and a trailing band which by densitometer measurements amounts to 7.5% of the major band. Since the staining procedure is based on reduced glutathione acting as the dye reductant, the minor band cannot be due to an NADPH oxidase enzyme. Furthermore, no NADPH oxidase activity could be detected in our preparations by spectrophotometric assay. Highly purified human erythrocyte glutathione reductase shows comparable electrophoretic heterogeneity under similar experimental conditions [5].

When 80 μM NADH is substituted for NADPH, utilizing the standard assay system, the rate is about 2% of that observed with NADPH. It is probable that the NADH and NADPH activities are due to one enzyme as has been shown for the human erythrocyte enzyme [24].

Platelet glutathione reductase is considered to be a flavoprotein, on the basis of its visible spectrum. No identification of the flavin component has been made, but it should be noted that only FAD has been identified as the coenzyme of other glutathione reductases [1,2,4,5,7,11,15].

An NADPH-Nbs_2 reductase activity cochromatographs with NADPH-depen-

dent platelet glutathione reductase activity on both hydroxyapatite and DEAE-cellulose. The ratio of the Nbs_2 to oxidized glutathione activity was 0.015 after each of the column treatments indicating that the reduction of Nbs_2 is catalyzed by glutathione reductase and not by an impurity. The yeast and rat liver glutathione reductase also have activity with Nbs_2 [1,25].

Platelet glutathione reductase has a broad pH optimum between pH 6.8 and 7.6.

Effect of salts on glutathione reductase activity

Inorganic salts increase platelet glutathione reductase activity. Sodium phosphate activation follows saturation kinetics with a K_A of 13 mM (Fig. 1A). Monovalent salts at non-saturating sodium phosphate concentrations also activate. In contrast to sodium phosphate the activation by NaCl, NaBr and sodium acetate does not give linear double reciprocal plots. The activation by NaBr and NaCl in the presence of 5 mM sodium phosphate is shown in Fig. 1B.

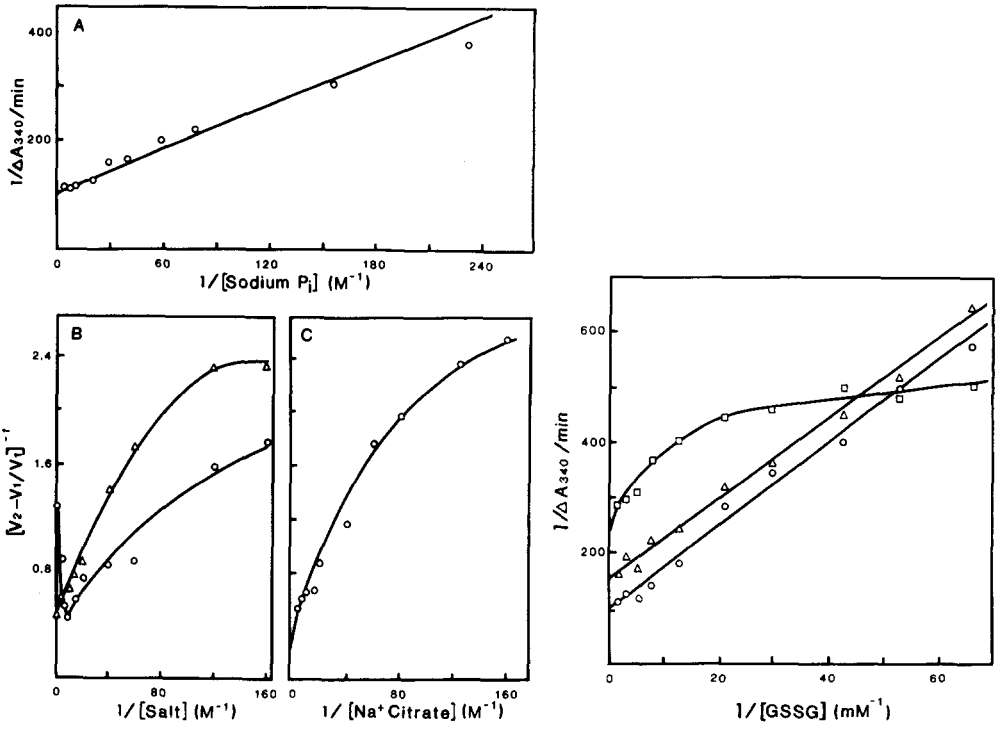

Fig. 1. The influence of salts on glutathione reductase activity. The reaction mixture contained: 80 μM NADPH, 2 mM oxidized glutathione, 1 mM EDTA, and $1.6 \cdot 10^{-3}$ units of enzyme. (A) Effect of sodium phosphate, pH 7.4. (B) effect of NaBr (○) and NaCl (△) in the presence of 5 mM sodium phosphate (pH 7.4). Data expressed as the increase in activity above that observed with sodium phosphate alone. V_1 is velocity with sodium phosphate alone, V_2 is velocity on addition of monovalent salt. (C) Effect of sodium citrate in the presence of 5 mM sodium phosphate (pH 7.4).

Fig. 2. The influence of sodium phosphate on the initial velocity. Oxidized glutathione (GSSG) was the varied substrate. The reaction mixture contained: 80 μM NADPH, 1 mM EDTA and $1.6 \cdot 10^{-3}$ units of enzyme. Sodium phosphate concentration was: (○) 100 mM; (△) 30 mM; (□) 10 mM.

Sodium citrate also activates in a non-linear manner (Fig. 1C), indicating the involvement of at least two sites having different binding constants. Potassium phosphate and sodium phosphate gave the same activation profile while identical reaction rates are seen with NaCl, KCl and LiCl. Inhibition of glutathione reductase occurs at concentrations of NaBr greater than 0.2 M (Fig. 1B).

The activation by sodium phosphate as a function of oxidized glutathione concentration is shown in Fig. 2. As the sodium phosphate concentration is increased from 30 to 100 mM the $K_{\text{oxidized glutathione}}$ (apparent) increases from 40 to 77 μM and the V increases about 2-fold. Similar results have been obtained with the yeast and erythrocyte enzymes [3,9]. The K_{NADPH} is not affected by changes in the sodium phosphate concentration. Non-linear kinetics indicative of substrate activation by oxidized glutathione are observed only at non-optimal sodium phosphate concentration (10 mM).

Initial velocity studies

Initial velocity studies were carried out at both 10 and 100 mM sodium phosphate. A plot of reciprocal velocity against the reciprocal concentration of NADPH at various fixed concentrations of oxidized glutathione in the presence of 100 mM sodium phosphate gave a family of lines which intersect at a common point (Fig. 3). A similar situation occurred when oxidized glutathione was the variable substrate. Replots of the slopes and the intercepts of the primary data were linear (Insert, Fig. 3). A K_{NADPH} of 4 μM and a $K_{\text{oxidized glutathione}}$ of 66 μM were calculated.

In the presence of 10 mM sodium phosphate similar primary plots were obtained. In the case of replots of the slopes and intercepts as a function of oxidized glutathione concentration non-linear secondary plots were obtained in agreement with the oxidized glutathione activation shown in Fig. 2. $K_{\text{oxidized glutathione}}$ (apparent) of 11 μM is obtained by extrapolating the linear segment in the low oxidized glutathione concentration range. The K_{NADPH} is 3 μM.

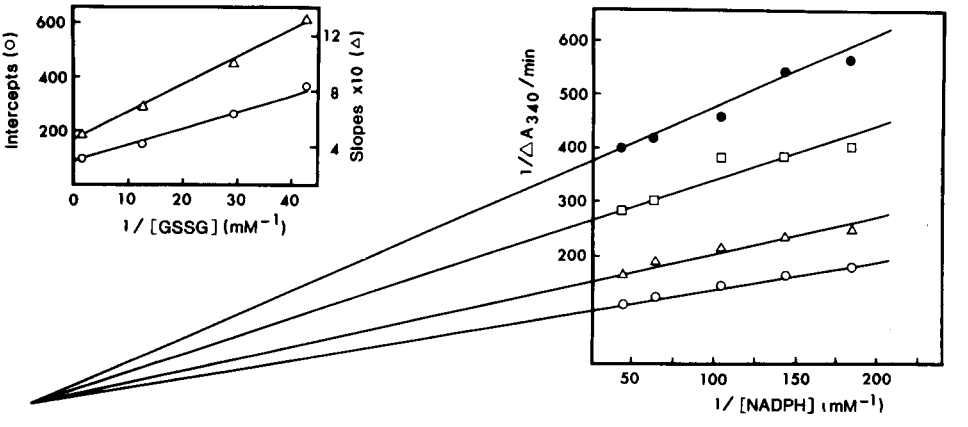

Fig. 3. Double reciprocal plot of the initial velocities with NADPH as the varied substrate at pH 7.4. The reaction mixture contained: 100 mM sodium phosphate, (pH 7.4) 1 mM EDTA and $1.6 \cdot 10^{-3}$ units of enzyme. Oxidized glutathione (GSSG) concentration was: (○) 800 μM; (△) 80 μM; (□) 34 μM; (●) 23.5 μM. The inset represents a replot of the slopes (△) and intercepts (○) vs. the corresponding reciprocal of the oxidized glutathione concentration.

$NADP^+$ product inhibition

$NADP^+$ is a competitive inhibitor with respect to NADPH and a non-competitive inhibitor with respect to oxidized glutathione at both 10 and 100 mM sodium phosphate. The data obtained with 100 mM sodium phosphate are shown in Fig. 4. A replot of the slopes of Fig. 4A vs. $NADP^+$ is linear while replots of the slopes and intercepts of Fig. 4B vs. $NADP^+$ are hyperbolic with non-horizontal asymptotes. This type of replot is a 2/1 function as defined by Cleland [26] and is indicative of alternate reaction pathways in the mechanism. Plots of the reciprocal of the velocity vs. $NADP^+$ concentration at various fixed levels of oxidized glutathione (Dixon plots) are also 2/1 functions. The $NADP^+$ inhibition patterns obtained in the presence of 10 mM sodium phosphate are shown in Fig. 5. The non-linearity of the $1/v$ vs. 1/[oxidized glutathione] plot observed at low phosphate persists in the presence of $NADP^+$. The replot of the slopes of Fig. 5A is linear and a replot of the primary data of Fig. 5B, in terms of the reciprocal of the velocity vs. $NADP^+$ concentration at various fixed levels of oxidized glutathione, is linear at 20 μM oxidized glutathione and above and hyperbolic below 20 μM oxidized glutathione. The $K_{i,NADP^+}$ determined from a replot of the slopes of the reciprocal plot versus NADPH was 60 μM with 100 mM sodium phosphate and 28 μM with 10 mM sodium phosphate (Table I).

Inhibition by flavin compounds

FMN is a non-competitive inhibitor with respect to both NADPH and oxidized glutathione (Fig. 6) at saturating salt concentration. Neither the

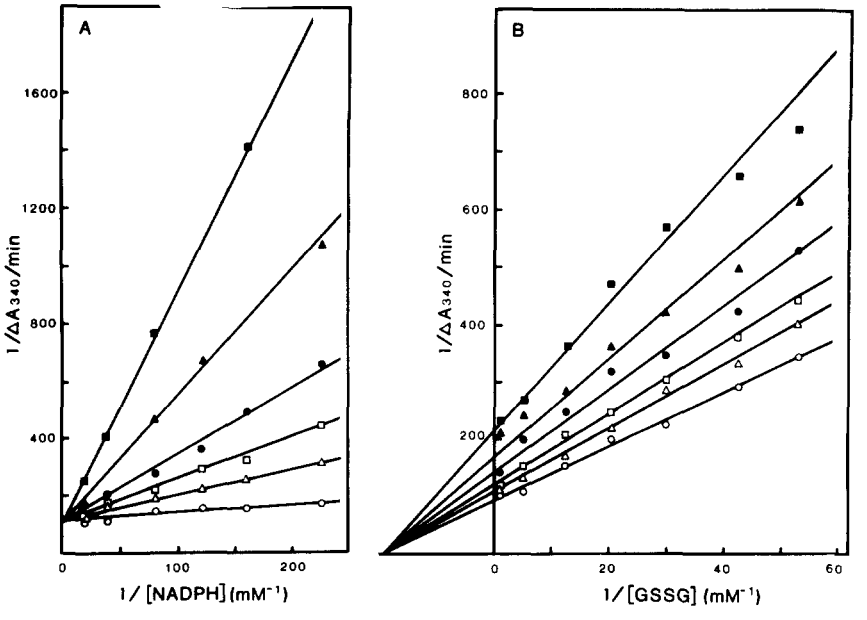

Fig. 4. $NADP^+$ inhibition in the presence of 100 mM sodium phosphate. General conditions were as described in Fig. 3. (A) NADPH was the varied substrate. Oxidized glutathione (GSSG) concentration was 2 mM. $NADP^+$ concentration was: (○) no $NADP^+$; (△) 0.05 mM; (□) 0.125 mM; (●) 0.25 mM; (▲) 0.5 mM; (■) 1.0 mM. (B) Oxidized glutathione was the varied substrate. NADPH concentration was 80 μM. $NADP^+$ concentration was: (○) no $NADP^+$; (△) 0.125 mM; (□) 0.25 mM; (●) 0.5 mM; (▲) 1.0 mM; (■) 2.0 mM.

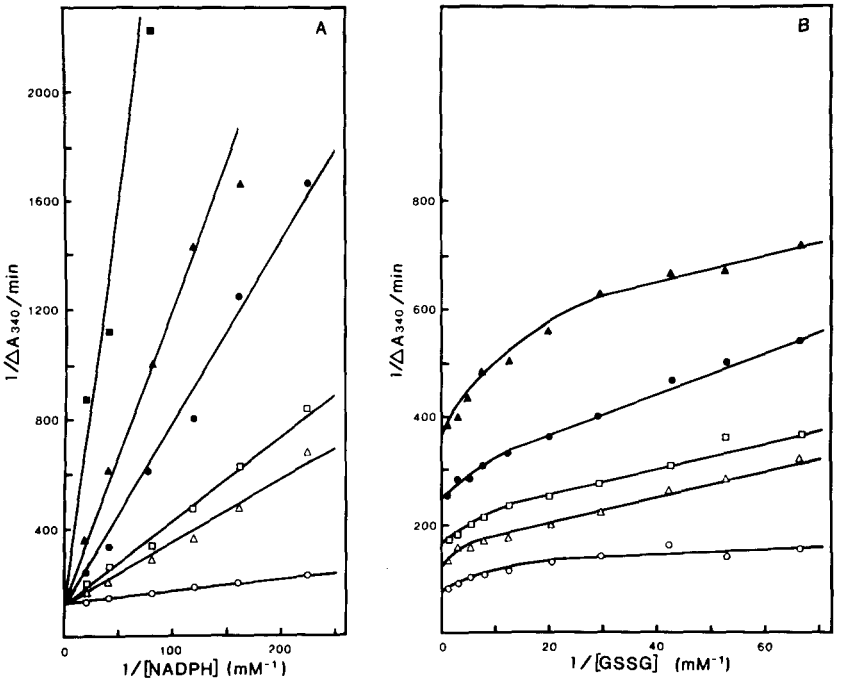

Fig. 5. $NADP^+$ inhibition in the presence of 10 mM sodium phosphate. The reaction mixture contained 1.0 mM EDTA and $4.8 \cdot 10^{-3}$ units of enzyme. (A) NADPH was the varied substrate. Oxidized glutathione (GSSG) concentration was 80 μM. $NADP^+$ concentration was: (○) none; (△) 0.05 mM; (□) 0.125 mM; (●) 0.25 mM; (▲) 0.5 mM; (■) 1.0 mM. (B) Oxidized glutathione was the varied substrate. NADPH concentration was 80 μM. $NADP^+$ concentration was: (○) none; (△) 0.25 mM; (□) 0.5 mM; (●) 1.0 mM; (▲) 2.0 mM.

K_{NADPH} (apparent) nor the $K_{oxidized\ glutathione}$ (apparent) are altered by FMN. Similar results were obtained for riboflavin and FAD. K_i values for FAD, FMN and riboflavin, calculated from replots of the slopes of $1/v$ vs. 1/[NADPH] plots are given in Table I. Non-enzymatic reduction of the flavins [27] by NADPH was negligible. Quinacrine dihydrochloride, a flavin analog, does not inhibit glutathione reductase at a concentration of 0.3 mM. This is not due to non-enzymatic reduction of quinacrine dihydrochloride by NADPH.

TABLE I

INHIBITION CONSTANTS

Inhibitor	Sodium phosphate (mM)	K_I (μM)
$NADP^+$	100	60
$NADP^+$	10	28
FMN	100	4
FAD	100	50
Riboflavin	100	20
Nitrofurantoin	100	1

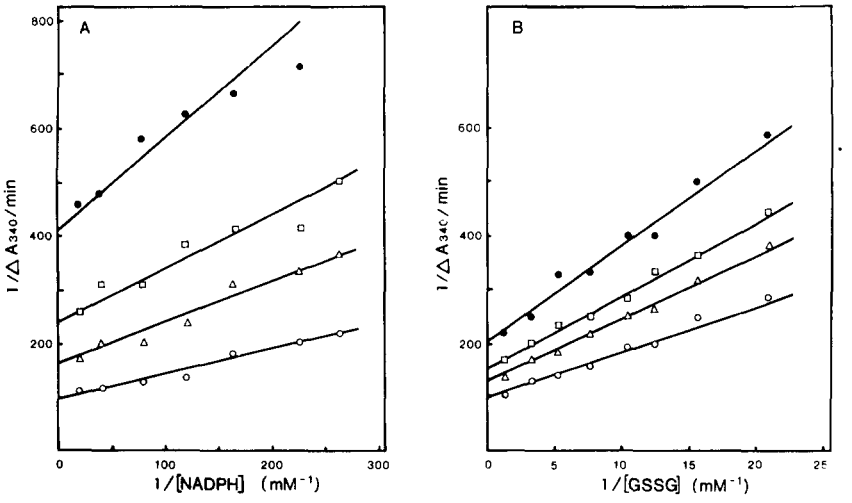

Fig. 6. Inhibition by FMN. The reaction mixture contained: 100 mM sodium phosphate (pH 7.4, 1 mM EDTA and $1.6 \cdot 10^{-3}$ units of enzyme. (A) NADPH was the varied substrate. Oxidized glutatione (GSSG) concentration was 2 mM. FMN concentration was: (○) none; (△) 1.4 μM; (□) 6.9 μM; (●) 13.8 μM. (B) Oxidized glutatione was the varied substrate. NADPH concentration was 40 μM. FMN concentration was: (○) none; (△) 3.45 μM; (□) 6.9 μM; (●) 13.8 μM.

Inhibition by nitrofurantoin

Nitrofurantoin is a non-competitive inhibitor with respect to both oxidized glutathione and NADPH (Fig. 7) and does not alter the K_{NADPH} (apparent). Replots of the slopes and intercepts obtained from the $1/v$ vs. 1/[NADPH] plots are hyperbolic. The $K_{oxidized\ glutathione}$ (apparent) is not affected at nitrofurantoin concentrations of 1 μM and below but is significantly increased

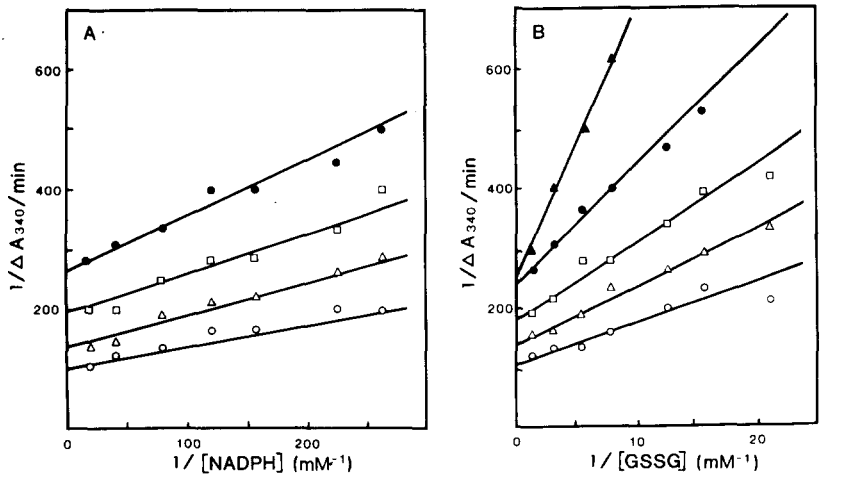

Fig. 7. Inhibition by nitrofurantoin. General assay conditions were as described in Fig. 1. (A) NADPH was the varied substrate. Oxidized glutathione (GSSG) concentration was 2.0 mM. Nitrofurantoin concentration was: (○) none; (△) 0.27 μM; (□) 1 μM; (●) 4 μM. (B) Oxidized glutathione was the varied substrate. NADPH concentration was 80 μM. Nitrofurantoin concentration was: (○) none; (△) 0.27 μM; (□) 1 μM; (●) 2 μM; (▲) 4.3 μM.

at concentrations above 1 μM. Replots of the intercepts obtained from the $1/v$ vs. 1/[oxidized glutathione] data are hyperbolic while the corresponding linear slope replot gives a K_i of 1 μM (Table I). Nitrofurantoin is a more effective inhibitor at 10 mM than at 100 mM sodium phosphate. The hyperbolic inhibition persists at the lower phosphate concentration. Nitrofurantoin was not reduced enzymatically or non-enzymatically.

Discussion

The properties of glutathione reductase isolated from human blood platelets have been studied. This enzyme appears to have similar characteristics as glutathione reductase isolated from other sources. These similarities include a flavin prosthetic group [1,2,4,5,7,11,15], NADPH being preferred over NADH [1—3,6,7,12,15], activation by inorganic salts [2,3,7,9,15], and non-linear $NADP^+$ inhibition [1,13,14]. Furthermore, the values for $K_{\text{oxidized glutathione}}$ (66 μM) and K_{NADPH} (4 μM) under saturating salt conditions, are similar to the kinetic parameters determined for other glutathione reductase enzymes. pH optimum data are also similar [1,2,7,8,11].

The platelet enzyme is activated by sodium citrate. Monovalent salt and sodium citrate activation appear to involve multiple sites, as judged by the curvature in Fig. 1. Neither of these observations have been reported previously for any form of glutathione reductase.

The activation by salts appears to be due to the anion component and not just to changes in ionic strength, since the activation by NaBr is greater than the activation by NaCl. The activation by sodium citrate and sodium phosphate do not follow identical ionic strength profiles.

The activation of glutathione reductase by oxidized glutathione is seen only at sub-saturating sodium phosphate concentrations. This phenomenon may reflect negative cooperativity or two non-interacting oxidized glutathione binding sites.

Qualitatively, salt concentration does not influence the general kinetic characteristics of the platelet enzyme. The non-linear $NADP^+$ inhibition indicates that the mechanism by which platelet glutathione reductase catalyzes the reduction of oxidized glutathione appears to be of a complex nature. The simplest explanation for the hyperbolic inhibition is that the reaction flux proceeds via two pathways [26] which differ in terms of their maximum velocity. Mannervik and co-workers [1,13,14] observed non-linear $NADP^+$ inhibition with yeast and liver glutathione reductase and suggested an alternate pathway mechanism consisting of one ping-pong loop and one sequential loop. Further experimentation is needed to elucidate the details of the mechanism of the platelet glutathione reductase.

Platelet glutathione reductase is inhibited by purified flavin compounds and nitrofurantoin. Inhibition of glutathione reductase by FAD in platelet lysates [28] and erythrocyte lysates [29,30] has been previously reported. Nitrofurantoin inhibition has also been observed with rat tissue [31] and yeast [32] glutathione reductase. The kinetics of those compounds, however, have not been previously delineated. The non-linear replots of the nitrofurantoin inhibi-

tion data indicate the existence of a complex pattern of interaction between glutathione reductase and this inhibitor.

Acknowledgements

Contribution No. 343 from the American Red Cross Blood Research Laboratory, Bethesda, Md. 20014, U.S.A. This investigation was supported in part by Research Grant No. HL 18502 and Biomedical Support Grant 5 S07 RR05737 from the National Institutes of Health.

References

1 Carlberg, I. and Mannervik, B. (1975) J. Biol. Chem. 250, 5475—5480
2 Scott, E.M., Duncan, I.W. and Ekstrand, V. (1963) J. Biol. Chem. 238, 3928—3933
3 Staal, G.E.J. and Veeger, C. (1969) Biochim. Biophys. Acta 185, 49—62
4 Staal, G.E.J., Visser, J. and Veeger, C. (1969) Biochim. Biophys. Acta 185, 39—48
5 Worthington, D.J. and Rosemeyer, M.A. (1974) Eur. J. Biochem. 48, 167—177
6 Worthington, D.J. and Rosemeyer, M.A. (1976) Eur. J. Biochem. 67, 231—238
7 Icén, A. (1967) Scand. J. Clin. Lab. Invest. 20, Suppl. 96
8 Moroff, G. and Brandt, K.G. (1975) Biochim. Biophys. Acta 410, 21—31
9 Moroff, G. and Brandt, K.G. (1973) Arch. Biochem. Biophys. 159, 468—474
10 Moroff, G., Ochs, R.S. and Brandt, K.G. (1976) Arch. Biochem. Biophys. 173, 42—49
11 Massey, V. and Williams, Jr., C.H. (1965) J. Biol. Chem. 240, 4470—4480
12 Bulger, J.E. and Brandt, K.G. (1971) J. Biol. Chem. 246, 5570—5577
13 Mannervik, B. (1974) in Glutathione (Flohé, L., Benöhr, H.Ch., Sies, H., Waller, H.D. and Wendell, A., eds.), pp. 114—120, Georg Thieme, Stuttgart
14 Mannervik, B. (1973) Biochem. Biophys. Res. Commun. 53, 1151—1158
15 Woodin, T.S. and Segal, I.H. (1968) Biochim. Biophys. Acta 167, 64—77
16 Horecker, B. and Kornberg, A. (1948) J. Biol. Chem. 175, 385—390
17 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
18 Benohr, H.Ch. and Waller, H.D. (1974) in Glutathione (Flohé, L., Benöhr, H.Ch., Sies, H., Waller, H.D. and Wendel, A., eds.), pp. 184—191, Georg Thieme, Stuttgart
19 Barber, A.J. and Jamieson, G.A. (1970) J. Biol. Chem. 245, 6357—6365
20 Davies, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
21 Ray, L.E. and Prescott, J.M. (1975) Proc. Soc. Exp. Biol. Med. 143, 402—409
22 Massey, V. and Swoboda, B.E.P. (1963) Biochem. Z. 338, 474—484
23 Beinert, H. (1960) in The Enzymes (Boyer, P.D., Lardy, H. and Myrback, K., eds.), pp. 339—416, Academic Press, New York
24 Beutler, E. (1974) in Glutathione (Flohé, L., Benöhr, H.Ch., Sies, H., Waller, H.D. and Wendel, A., eds.), pp. 109—114, Georg Thieme, Stuttgart
25 Tietze, F. (1969) Anal. Biochem. 27, 502—522
26 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 173—187
27 Singer, T.P. and Kearney, E.B. (1950) J. Biol. Chem. 183, 409—429
28 Karpatkin, S. and Strick, N. (1972) J. Clin. Invest. 51, 1235—1243
29 Glatzle, D., Korner, W.F., Christeller, S. and Wiss, O. (1970) Int. J. Vitam. Res. 40, 166—170
30 Hornbeck, C.L. and Bradley, D.W. (1974) Clin. Chem. 20, 512—514
31 Buzard, J.A. and Kopko, F. (1963) J. Biol. Chem. 238, 464—468
32 Ondarza, R.N. and Abney, R. (1970) FEBS Lett. 7, 227—230