BBA 65920

PURIFICATION AND PROPERTIES OF AN ABNORMAL GLUTATHIONE REDUCTASE FROM HUMAN ERYTHROCYTES

G. E. J. STAAL, P. W. HELLEMAN, J. DE WAEL AND C. VEEGER

Haematological and Biochemical Department, The State University Hospital, Utrecht (The Netherlands) and Department of Biochemistry, Agricultural University, Wageningen (The Netherlands)

(Received January 30th, 1969)

SUMMARY

1. Glutathione reductase (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) from the erythrocytes of a patient with a decreased activity of the enzyme was purified 10 000 times (specific activity, 20 μmoles NADPH oxidized per min per mg protein) by column chromatography; estimated purity, 10%.

2. The K_m values for GSSG and NADPH were practically the same as those for the normal enzyme. The influence of ions on the reaction velocity and the pH optimum were identical with the normal enzyme.

3. The K_{ass} value for FAD binding to the apoprotein was diminished.

4. Consequently FAD protected the enzyme against denaturation during heating at 55° and diminished the losses during purification. Incubation of the holoenzyme with FMN decreased the activity.

INTRODUCTION

CARSON *et al.*[1] were first to report a decreased activity of glutathione reductase in red blood cells. LÖHR AND WALLER[2] reported on glutathione reductase (NAD(P)H: oxidized glutathione oxidoreductase, EC 1.6.4.2) deficiency associated with non-spherocytic hemolytic anemia. At this moment several reports on glutathione reductase deficiencies have been published[3–5].

In order to explain the decreased activity of the enzyme from patients with glutathione reductase "deficiency", WALLER[6] purified the enzyme and investigated several enzymatic properties. With the partially purified enzyme, WALLER[6] found that the enzyme isolated from the erythrocytes of the patients, had a different pH optimum, a different K_m for GSSG and a normal K_m for NADPH with respect to the values obtained with the normal enzyme. LÖFFLER *et al.*[3] was not able to demonstrate such differences. As pointed out by KÜBLER *et al.*[7], the differences found by WALLER[6] are not significant, and he concluded that no differences could be detected between the normal enzyme and the enzyme of patients.

In the foregoing papers[8,9] we have reported on the normal human erythrocyte

glutathione reductase and the enzyme characterized with respect to physical and enzymatic properties. This paper deals with the enzyme obtained from a patient with a decreased glutathione reductase activity in the erythrocytes (see ref. 10).

MATERIALS AND METHODS

Chemicals

NADPH, GSSG, FAD and FMN were obtained from Boehringer and Soehne. DEAE-Sephadex A-50, capacity 3.5 ± 0.5 mequiv/g, particle size 40–120 μ; and CM-Sephadex C-50, capacity 4.5 ± 0.5 mequiv/g, particle size 40–120 μ, were purchased from Pharmacia, Uppsala. Calcium phosphate gel columns were prepared according to MASSEY[11].

Determination of activity

The assays were carried out as previously described[8]. The protein content was determined by the method of Lowry–Folin[12]. The apoenzyme was prepared according to the method of WARBURG AND CHRISTIAN[13]. For the hematological data of the patient see ref. 10.

RESULTS

The activity of the patient's enzyme in either the hemolysate or the purified state can be increased when the enzyme is preincubated with FAD in the reaction mixture prior to the assay. The reaction is started by the addition of NADPH. Therefore the whole purification procedure was carried out in the presence of 0.1 μM FAD. It is remarkable that 0.1 μM FAD protects the enzyme so well, since it will be shown later that $K_D = 4\ \mu$M for the purified enzyme. An influence of other proteins and ions on this process cannot be excluded.

The same purification method was used as previously[8] described except that the last two steps of the purification were omitted. Starting from 330 ml of erythrocytes with a specific activity in the hemolysate of $2 \cdot 10^{-3}$ μmoles NADPH oxidized per min per mg protein, an enzyme solution with a specific activity of 20 μmoles NADPH

TABLE I

PURIFICATION OF ABNORMAL GLUTATHIONE REDUCTASE

The purification steps correspond with those given in ref. 8.

Steps of purification	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Accumulative purification (-fold)*
1	700	$98 \cdot 10^3$	200	$2 \cdot 10^{-3}$	100	1
2	900	$1.9 \cdot 10^3$	194	$1 \cdot 10^{-1}$	98	50
3	250	260	131	$5 \cdot 10^{-1}$	65	250
4	300	123	100	$8.2 \cdot 10^{-1}$	50	410
5	50	46	92	2	46	1 000
6	125	12.5	54	4	27	2 000
7	6.5	1.5	30	20	15	10 000

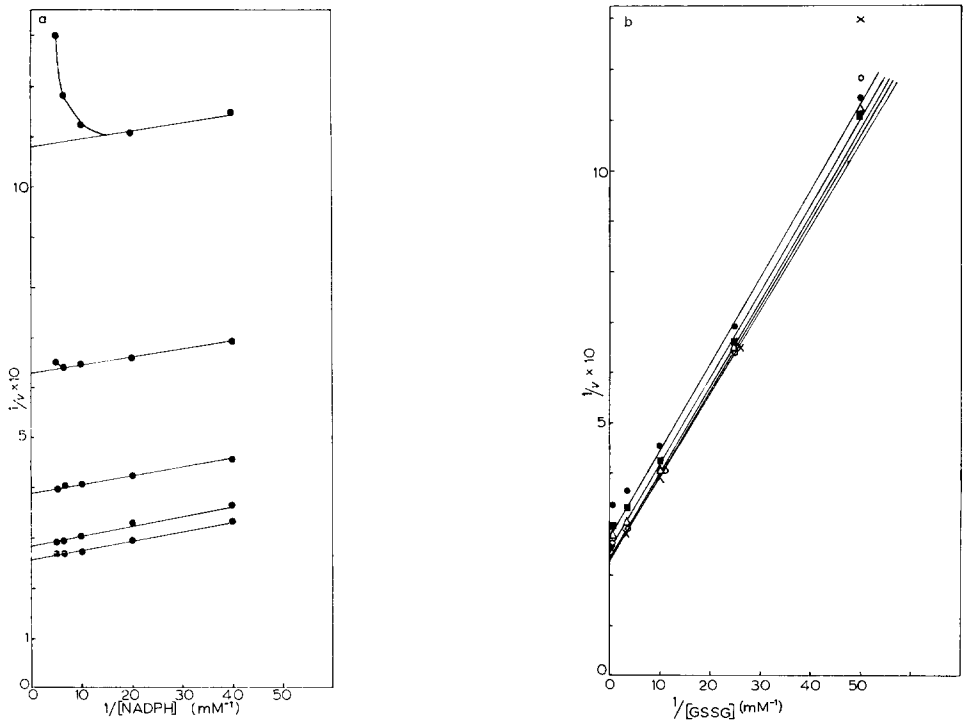

Fig. 1. (a) $1/v$ *vs.* 1/[NADPH] plots at different GSSG concentrations. Top to bottom: 20, 40, 100, 300 and 1500 μM GSSG. Buffer: 0.3 M phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADPH concentrations. ●—●, 25; ■—■, 50; △—△, 100; ○—○, 150 μM; and ×—×, 200 μM NADPH. Buffer: 0.3 M phosphate (pH 7.0). v in μmoles NADPH oxidized per min per mg.

oxidized per min per mg protein was obtained which means that the enzyme preparation was purified 10 000 times (Table I).

Kinetics of the enzyme

Fig. 1 shows the effects 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 substrate inhibition by NADPH is found. The noninhibitory parts of the plots are parallel. By plotting $1/v$ *vs.* 1/[GSSG] only at the lower NADPH concentrations the plots are parallel. From these results a K_m for GSSG of 82 μM

TABLE II

K_m VALUES FOR THE NORMAL AND ABNORMAL ENZYME AT TWO DIFFERENT SODIUM PHOSPHATE CONCENTRATIONS

Sodium phosphate (M)	*K_m (GSSG) (μM)*		*K_m (NADPH) (μM)*	
	Normal	*Abnormal*	*Normal*	*Abnormal*
0.3	125	82	13.3	8
0.03	19	22	9.5	5.0

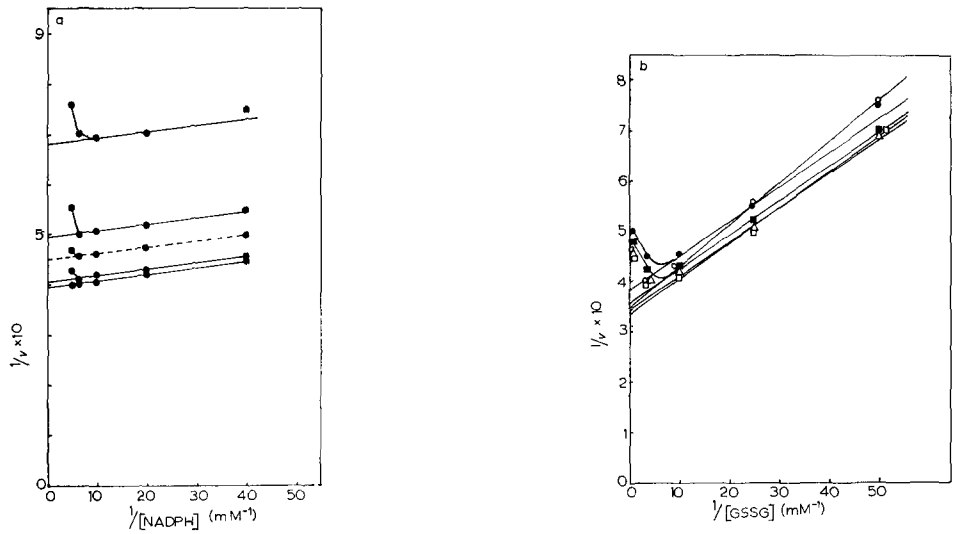

Fig. 2. (a) $1/v$ *vs.* 1/[NADPH] plots at different GSSG concentrations. Top to bottom: 20, 40, 1500, 100 and 300 μM GSSG. Buffer: 0.03 M phosphate (pH 7.0). (b) $1/v$ *vs.* 1/[GSSG] plots at different NADPH concentrations. ●—●, 25; ■—■, 50; △—△, 100; □—□, 150; and ○—○, 200 μM NADPH. Buffer: 0.03 M phosphate pH (7.0). v in μmoles NADPH oxidized per min per mg.

(Fig. 3a) and a K_m for NADPH of 8 μM (Fig. 3b) were calculated. These values differ only slightly from the values obtained with the normal enzyme (Table II).

The same investigations were carried out in 0.03 M of sodium phosphate buffer (pH 7.0). Fig. 2 shows the effect of varying the concentrations of both GSSG and NADPH. From these results an apparent K_m for GSSG of 22 μM (Fig. 3a) and a K_m for NADPH of 5.0 μM (Fig. 3b) were calculated; again these values differ only slightly from the values obtained with the normal enzyme (Table II). Furthermore the

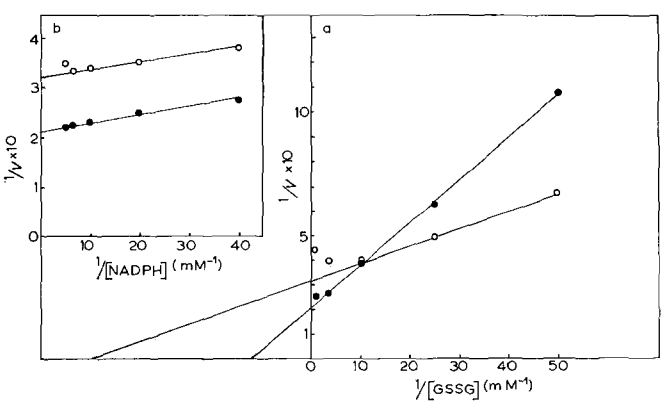

Fig. 3. (a) The plot $1/V$ (NADPH) *vs.* 1/[GSSG]. These values are calculated from Fig. 1a (●—●) and Fig. 2a (○—○). (b) The plot $1/V$ (GSSG) *vs.* 1/[NADPH]. These values are calculated from Fig. 1b (●—●) and Fig. 2b (○—○). V in μmoles NADPH oxidized per min per mg.

influence of Na^+ on the reaction velocity and the inhibition by GSSG and NADPH at high concentrations are identical as those found for the normal enzyme. Therefore we conclude that the kinetic behavior of the patient's enzyme is identical with the normal enzyme which is in agreement with the results reported by KÜBLER *et al.*[7] and LÖFFLER *et al.*[3] but differs from the results obtained by WALLER[6].

Stability of the enzyme

Glutathione reductase is very heat stabile even at low concentrations[8]. Heating the normal enzyme for 1 h at 60° in 0.1 M sodium phosphate buffer (pH 7.2) does not decrease activity; however, when the patient's enzyme (protein concn., 3.8 μg/ml) was heated for 30 min at 55°, about 30% of the activity was lost; in the presence of 10 μM FAD no loss of activity could be demonstrated.

pH optimum

WALLER[6] found with the enzyme from his patients a pH 6.4 optimum for glutathione reductase which differs from the value for the normal enzyme (6.8). Therefore we have studied the pH optimum in the pH range from 5.0 to 8.6. The pH optimum was found to be 6.8 which is identical with the value obtained for the normal enzyme. This result is in agreement with the results reported by LÖFFLER *et al.*[3] and KÜBLER *et al.*[7].

Recombination of the apoenzyme with FAD and the influence of FMN

3 ml of an enzyme solution were 55% saturated with $(NH_4)_2SO_4$ and brought to pH 3.1 with 1 M HCl (ref. 13). After standing for 10 min in an ice-bath, the apoenzyme was collected by centrifugation and was dissolved in 1 ml of 0.2 M phosphate buffer (pH 7.0) (concentration of the partially purified apoenzyme, 0.33 mg/ml). Fig. 4 shows the effect of the FAD concentration on the reactivation of the apoenzyme by FAD. From these data, an apparent K_m for FAD of 3.7 μM (normal 0.55 μM) and a K_{ass} for FAD of $2.7 \cdot 10^5$ M^{-1} (normal enzyme $1.8 \cdot 10^6$ M^{-1}) were calculated. From these data it can be concluded that the FAD of the abnormal enzyme shows less affinity for the apoenzyme.

Though FMN is not capable of restoring the catalytic activity upon incubation

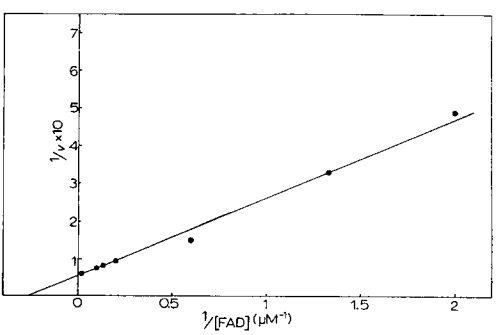

Fig. 4. Effect of FAD concentration on the reactivation of the apoenzyme by FAD. The apoenzyme was prepared as described in the text. After a 5-min incubation (33 μg protein in 0.2 ml buffer) with the concentration of FAD indicated, samples were withdrawn and activities measured in the standard assay medium without added extra FAD .v in μmoles NADPH oxidized per min per mg.

with the apoenzyme, we have demonstrated that the apoenzyme has affinity for this flavin compound[8]. When the holoenzyme of the patient was incubated for 12 h in the presence of 10 μM FMN, there was about 30% loss of activity, while enzyme incubated without added FMN showed no decrease in activity. The normal holoenzyme shows, under identical conditions, no diminished activity after incubation with FMN.

DISCUSSION

The glutathione reductase purified from the patient's erythrocytes has a specific activity of 20 μmoles NADPH oxidized per min per mg protein, corresponding to a purification of 10 000 times. WALLER[6] obtained a specific activity of 3.8 μmoles NADPH oxidized per min per mg protein for the normal enzyme, but he gives no values for the abnormal enzyme. Assuming an identical activity of the pure enzymes, our preparation is about 10% pure.

WALLER[6] obtained different K_m values for GSSG but normal values for NADPH. Our K_m values show no significant difference with respect to those of the normal enzyme (Table II), a result also obtained by KÜBLER *et al.*[7]. Furthermore WALLER[6] estimated the K_m by varying the concentration of one of the substrates; however for a two-substrate reaction, the K_m value for one substrate is dependent on the concentration of the other substrate and, therefore, the concentrations of both substrates must be varied to determine the actual K_m values. Furthermore the K_m values given in the literature are determined at different buffer concentrations with or without other additions (see refs. 3–6). Since the K_m values are dependent on the Na^+, K^+ and NH_4^+ concentrations used[9], it is difficult to compare the K_m values given by the different investigators.

Several explanations are possible for the observation that the dissociation constant of the abnormal apoenzyme–FAD complex (3.7 μM) is so much larger than the FAD concentration (0.1 μM) which protects fully the corresponding holoenzyme. The most likely one is that the abnormal apoenzyme–FAD complex differs slightly from the corresponding holoenzyme a difference also observed between the normal apoenzyme–FAD complex and the normal holoenzyme[8]. The normal apoenzyme shows a diminished affinity for FAD, as can be concluded from the inactivation following the addition of FMN to the reactivated enzyme, an effect which does not occur with the holoenzyme as isolated. The abnormal holoenzyme is inactivated to some extent by FMN and needs FAD during purification for stabilization which allows the conclusion that here the flavin moiety is much less tightly bound than in the normal holoenzyme. About the nature of the different affinities for FAD one can only speculate. In view of the stimulating effect of FAD on the abnormal enzyme, we administered a flavin preparation as a "therapeutic" agent to the patient (10 mg FMN per day). After a few weeks the enzyme activity had increased to an almost normal level. When this treatment was stopped, the glutathione reductase activity started to decline again. These experiments which will be published elsewhere will also include the effect of administered FAD.

ACKNOWLEDGMENTS

The authors are indebted to Dr. M. C. Verloop for support and advice in this

investigation. The authors are also grateful to Mrs. L. v. Milligen-Boersma for technical assistance.

REFERENCES

1 P. E. CARSON, G. J. BREWER AND C. ICKES, *J. Lab. Clin. Med.*, 58 (1961) 804.
2 G. W. LÖHR AND H. D. WALLER, *Med. Klin.*, 57 (1962) 1521.
3 G. LÖFFLER, G. BRITTINGER AND E. KÖNIG, *Klin. Wochschr.*, 44 (1966) 571.
4 H. D. WALLER, G. W. LÖHR, E. ZYSNO, W. GEROK, D. VOSS AND G. STRAUSS, *Klin. Wochschr.*, 43 (1965) 413.
5 K. MOSER, M. FISCHER, P. KREPLER AND K. LECHNER, *Klin. Wochschr.*, 46 (1968) 995.
6 H. D. WALLER, *Klin. Wochschr.*, 45 (1967) 827.
7 W. KÜBLER, W. SCHNEIDER AND R. GROSS, *Arch. Klin. Med.*, 215 (1968) 67.
8 G. E. J. STAAL, J. VISSER AND C. VEEGER, *Biochim. Biophys. Acta*, 185 (1969) 39.
9 G. E. J. STAAL, AND C. VEEGER, *Biochim. Biophys. Acta*, 185 (1969) 49.
10 K. PUNT, P. W. HELLEMAN, G. E. J. STAAL AND M. C. VERLOOP, *Proc. 10th Congr. European Soc. Haematol., Strasbourgh, 1965*, Karger, Basel, 1967.
11 V. MASSEY, *Biochim. Biophys. Acta*, 37 (1960) 310.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 295 (1938) 261.