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HUMAN ERYTHROCYTE GLUATHIONE REDUCTASE

I. PURIFICATION AND PROPERTIES

KOJI NAKASHIMA, SHIRO MIWA and KATSUYO YAMAUCHI

Third Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Yamaguchi 755 (Japan)

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Summary

1. Glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase, EC. 1.6.4.2) from human erythrocytes was purified 49 000-fold with an overall yield of 15% and a 280/460 nm absorbance ratio of 6.03. The procedure used was the method of Worthington and Rosemeyer modified by addition of heating and recrystallization.

2. It was concluded from the results of purification, electrofocusing and inhibition studies that glutathione reductase is a single enzyme which used both NADPH and NADH as hydrogen donors.

3. Apoenzyme cross-reacts with the antibody to the holoenzyme but has a slightly reduced affinity to the antibody. Apoenzyme can be removed from the hemolysate by heating and centrifugation without loss of holoenzyme.

4. Indirect immunological assay of the specific activity of the erythrocyte glutathione reductase is possible in the enzyme saturated with FAD.

Introduction

Genetic deficiency in glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase, EC. 1.6.4.2.) in red cells is associated with hemolytic anemia [1]. Secondary enzyme deficiency is also induced by an inadequate intake of riboflavin [2]. However, in certain deficient subjects often having aplastic anemia, the enzyme deficiency cannot be fully corrected by riboflavin administration [3].

Eifler and Wagenknecht [4] reported that the two reductase activities (i.e. one specific to NADPH and another specific to NADH) were separated by purification procedures, while Icén [5] reported that the utilization ratio of two substrates was not changed even after 30 000-fold purification. The enzyme has

been purified to various degrees by several investigators [5—9] and more recently, Worthington and Rosemeyer [10] prepared crystalline enzyme.

In order to study the mechanism of enzyme deficiency in aplastic anemia and other diseases, the properties of the purified crystalline glutathione reductase from the human erythrocyte were studied and the indirect immunological measurement of the enzyme protein in the red cell was devised in the present study.

Materials and Methods

ACD-treated human blood was obtained from the Yamaguchi Red Cross Blood Transfusion Service. NADPH, NADP, NADH, GSSG and FAD were purchased from Boehringer-Mannheim, Germany. DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-200 were products of Pharmacia, Sweden. Ampholine was obtained from LKB, Sweden. All other common reagents were of analytical grade.

Glutathione reductase activity was determined with NADPH or NADH as a hydrogen donor and GSSG as an acceptor using a Gilford 2400-S recording spectrophotometer according to Icén's method [5]. The protein content was determined by the method of Lowry et al. [11] with crystalline bovine serum albumin as a standard, and in Steps 9 and 10 by absorbance at 280 nm using an $E^{1\%}_{1\,cm}$ at 280 nm of 13.5 [10].

The enzyme was purified from 10 l of human blood (less than a week old, kept at 4° C) by a modified version of the method reported by Worthington and Rosemeyer [10]. The modifications are as follows: (1) Hemolysis was carried out by freezing/thawing; stroma was eliminated at pH 5.5 by centrifugation without toluene. After the centrifugation, the hemolysate was neutralized to pH 7.0 with 1 M KOH. (2) The enzyme was precipitated with 30—55% saturated ammonium sulfate. (3) After Sephadex G-200 gel-filtration, the enzyme solution was heated for 60 min at 60° C, and the precipitate was removed by centrifugation. (4) Recrystallization was carried out by washing the crude crystals with deionized water and dissolving them in a 0.2 M phosphate buffer, pH 7.0, containing 1% (v/v) mercaptoethanol and dialysing the enzyme solution against 30% saturated ammonium sulfate to crystallize it again. The crystals were washed and dissolved again. Thin layer polyacrylamide gel electrofocusing was performed by a modified version of the method of Jeppsson and Berglund [12]. The protein was stained with Coomassie Brilliant Blue R 250 and the detection of enzyme reaction was according to the method of Susor at al. [13]. The conditions were described in the figure legends.

NADPH-X and NADH-X * were prepared by incubating 5 mM NADPH and NADH in a 0.5 M phosphate buffer, pH 6.8, at 37° C for 12 h as described previously [14].

For preparation of the apoenzyme, FAD was removed from the purified enzyme by Icén's method [5]. FAD (about 0.1 mg) was added directly to 0.1

* NADPH-X and NADH-X are the structural modifications of NADPH and NADH respectively, which are converted in vitro at neutral pH and have no absorbance at 340 nm but exhibit an increase in absorbance in the region of 280—300 nm.

ml of the apoenzyme solution to make the holoenzyme by recombination with FAD.

In order to remove apoenzyme from hemolysate, the hemolysate was incubated at 57°C for 60 min, and was centrifuged at 26 800 × *g* for 15 min to remove the insoluble denatured protein. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, enolase, pyruvate kinase and lactate dehydrogenase were measured by Beutler's method [15] in the crude hemolysate, the hemolysate treated by heating and the hemolysate which was incubated with the antiserum as in neutralization test.

An anti-human erythrocyte glutathione reductase anti-serum was obtained by intradermally injecting a rabbit with 1.5 mg of the purified enzyme emulsified with an equal volume of complete Freund's adjuvant at multiple sites on the back. The procedure was repeated 10 and 20 days later. After 45 days, 2 mg of the enzyme without the adjuvant was injected intradermally as a booster. On the 50th day, the blood was collected.

For the separation of immunocomplexes by gel filtration, the hemolysate or the yeast glutathione reductase was mixed with an equal volume of the antiserum solution, and the mixture was applied to a Sephadex G-200 column. Elution was performed according to the conditions described in the figure legends.

For electroimmunodiffusion (Laurell [16]) of glutathione reductase, electrophoresis was carried out with agarose gel containing the antiserum and the buffer of Svendsen [17] as described in the figure legends.

Neutralization testing with the antiserum was performed by incubation of the enzyme with the antiserum and enzyme assay after incubation. The details of the conditions are in the figure legend.

Results

The results of purification are summarized in Table I. The heating procedure of Step 6 removed a large quantity of impurities without inactivating the enzyme, and produced the extra protein peaks in step 8 (Fig. 1). Recrystallization somewhat reduced the yield, but elevated the specific activity. the 280/460 nm absorbance ratio of the recrystallized enzyme was 6.03, lower than that of the previous report (6.7—9.0) [5,9,10]. The utilization ratio of NADPH to NADH was not changed through the purification steps until the crystalline enzyme.

Inhibition studies of NADH-dependent enzyme by NADPH-X, NADP and NADH-X at pH 6.05 (the optimum pH of NADH-dependent enzyme) and at pH 6.8 (the optimum pH of NADPH-dependent enzyme), and NADPH-dependent enzyme by NADPH-X and NADP at pH 6.8 were done. The modes of inhibition were competitive with NADH and NADPH. The inhibitor constants and Michaelis constants for NADH and NADPH are summarized in Table II. The inhibition of NADPH-dependent enzyme by NADH-X was too low to be measured.

As shown in Fig. 2, the gel slab was divided into two parts after focusing of the purified enzyme. One was stained by NADPH and the other by NADH as a hydrogen donor. No difference was observed in the patterns and the isoelectric points. In Fig. 3, after focusing, the gel slab was divided and different stainings

TABLE I

PURIFICATION OF ERYTHROCYTE GLUTATHIONE REDUCTASE

Step	Volume (ml)	Protein (mg)	NADPH-dependent glutathione reductase			
			Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield
1 Hemolysate plus FAD	23 300	1 400 000	5850	0.0042	1	100
2 DEAE-Sephadex	4 590	20 900	5270	0.25	60	90.1
3 CM-Sephadex	3 070	4 240	4830	1.14	270	82.6
4 Ammonium sulfate (30—55%)	122	2 080	4120	1.98	470	70.4
5 G-200 Sephadex	275	230	3250	13.9	3 310	55.6
6 Heating at 60° C	360	80	3250	40.6	9 670	55.6
7 CM-Sephadex column	110	28	2530	90.4	21 500	36.8
8 G-200 Sephadex	23	16	2000	125	29 800	29.0
9 Ammonium sulfate (30—55%)	1.25	8.9	1620	182	43 300	27.7
10 Recrystallization	1.7	4.3	890	207	49 300	15.2

* Ratio of activity, NADPH-dependent glutathione reductase/NADH-dependent glutathione reductase

of the enzyme and the protein were done. It revealed that 4 protein bands of holoenzyme had enzyme activity. The sample in Fig. 2 was used 1 day after recrystallization and showed 3 bands, but the sample in Fig. 3 was used 7 days after recrystallization. Storage of the enzyme with thymol at 4° C might cause slight molecular alteration, with different net charge producing an extra cathodal band with enzyme activity. The purified enzyme and the recombined

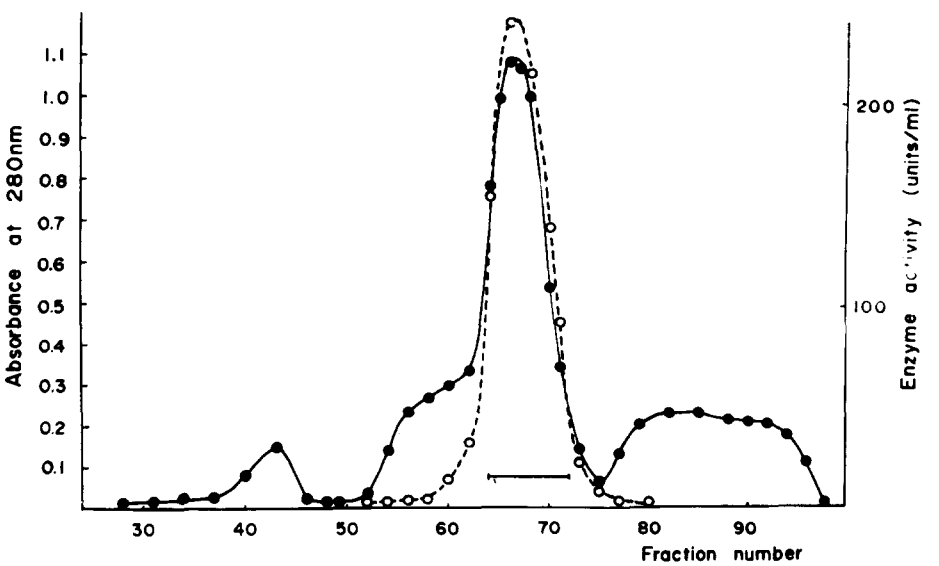


Fig. 1. Elution profile from gel-filtration on Sephadex G-200 column (1.5 X 200 cm) The condition was the same as that in Step 8 of ref. 10. Fractions of 2 ml began to collect when the orange-yellow came down to the lower part of the column. The horizontal line indicates the fractions pooled after elution. ●———●: Absorbance at 280 nm. ○- - - - -○: Glutathione reductase activity.

NADH-dependent glutathione reductase				Ratio of activity *	Absorbance ratio 280/460 nm
Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield		
920	0.00066	1	100	6.36	—
890	0.042	60	96.9	5.92	—
870	0.20	310	94.5	5.55	—
740	0.36	540	80.7	5.57	—
560	2.40	3 600	61.2	5.80	—
590	7.33	11 100	63.9	5.51	—
420	15.1	22 900	46.2	6.02	—
300	18.9	28 600	32.9	6.67	9.95
260	29.4	44 600	28.6	6.23	7.12
150	34.0	51 500	15.9	5.93	6.03

enzyme had the same protein but the apoenzyme had a broad additional cathodic band which disappeared upon recombination with FAD. The enzyme reaction also showed the same pattern although the concentration of each sample was quite different as seen in Fig. 3.

Results of the Ouchterlony method are in Fig. 4. The human sample showed precipitation against the antiserum but the yeast sample did not. The purified human enzyme, its apoenzyme and the recombined enzyme showed no difference in antigenicity in this method.

In the immunological study of crude hemolysate as shown in Fig. 5, the immunocomplex of the enzyme and the antibody was separated from the free enzyme. The immunocomplex still has enzyme activity. Because crude hemolysate without antiserum showed only one enzyme peak (Peak I) as seen in Fig. 5A, the running buffer contains 0.2% mercaptoethanol to prevent aggregation

TABLE II

EFECT OF NUCLEOTIDES ON GLUTATHIONE REDUCTASE

All concentrations are μM

	NADH-dependent glutathione reductase at pH 6.05	NADH-dependent glutathione reductase at pH 6.8	NADPH-dependent glutathione reductase at pH 6.8
K_m for NADH	250	670	
K_m for NADPH			9.0
K_i for NADPH-X	6.6	2.5	22
K_i for NADP	18	7.2	55
K_i for NADH-X	190	540	—

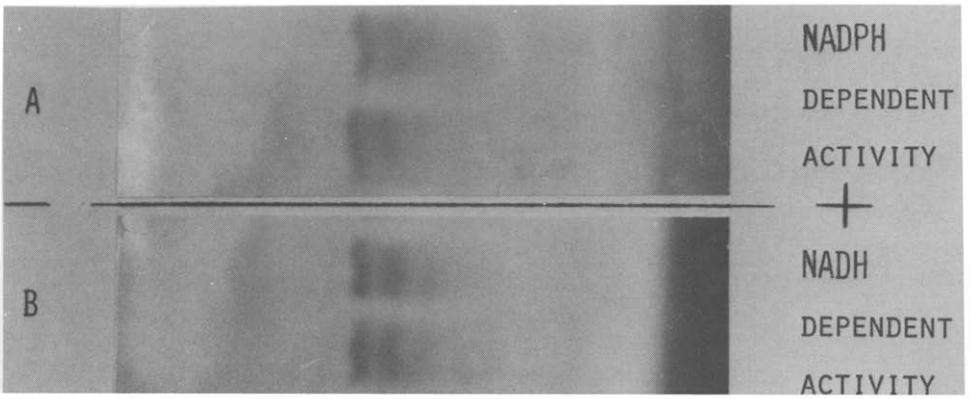


Fig. 2. Thin layer polyacrylamide gel elctrofocusing of purified crystalline glutathione reductase. Simultaneous focusing and different staining of NADPH-dependent enzyme for gel slab A and NADH-dependent enzyme for gel slab B. Polyacrylamide gel was prepared as the method of Jeppsson and Berglund [12] except for mercaptoethanol, 1 mM, Triton X, 0.02% and Ampholine pH 5—8, 0.6 ml; pH 7—9, 0.6 ml and pH 3.5—10, 0.2 ml. The purified glutathione reductase was diluted to adjust 0.20 units/ml of the NADPH-dependent activity for the gel slab A and of the NADH-dependent activity for the gel slab B. 5 μl of each sample was applied after prefocusing for 2 h at 12.5 V/cm, and electrofocusing was continued for 12 h at 25 V/cm. For the detection of the enzyme reaction, 20 mg NADPH and 30 mg GSSG were dissolved in 12 ml of the buffer for NADPH-dependent activity assay [5] for the gel slab A, and 20 mg NADH and 30 mg GSSG in 12 ml of the buffer for NADH-dependent enzyme assay [5] for the gel slab B, and 3 ml of 3% agar solution (55°C) were added to each reaction mixture, and the mixture was poured onto each gel slab.

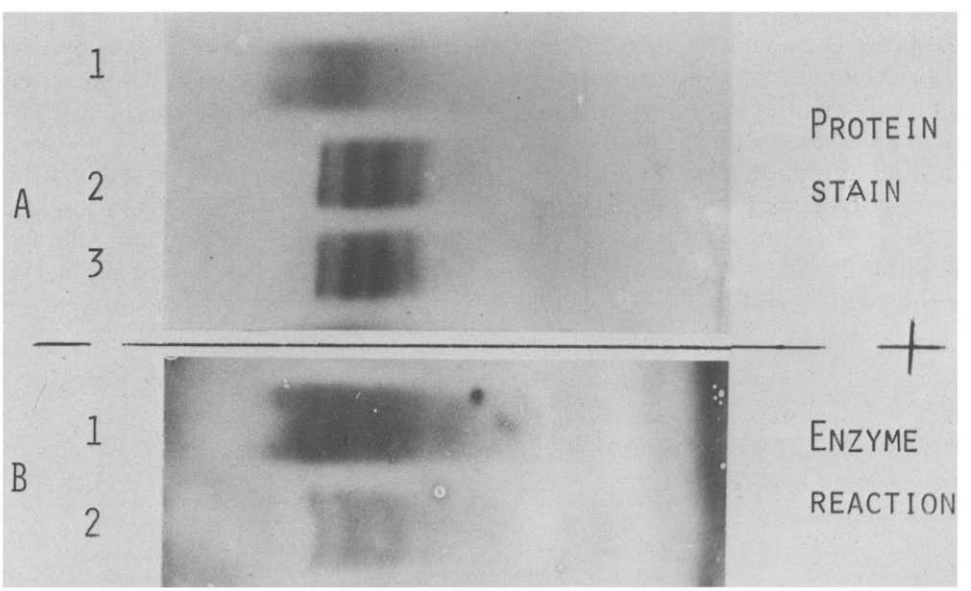


Fig. 3. Thin layer polyacrylamide gel electrofocusing of purified glutathione reductase. Simultaneous focusing and different staining of protein for gel slab A and enzyme for gel slab B. The conditions of the electrofocusing were the same as that in Fig. 2. The samples of gel slab A had 0.26 mg protein. Sample of B-2 was adjusted to 0.2 units/ml of the NADPH-dependent activity. Sample B-1 had 20 times higher concentration than sample B-2. The gel slab A was washed for 24 h with 10% 5% and 3% solutions of trichloroacetic acid and was stained with Coomasie Brilliant Blue R 250. The gel slab B was treated the same as the gel slab A in Fig. 2. 1. Dissociation of FAD, 2. Recombination with FAD and 3. Purified glutathione reductase.

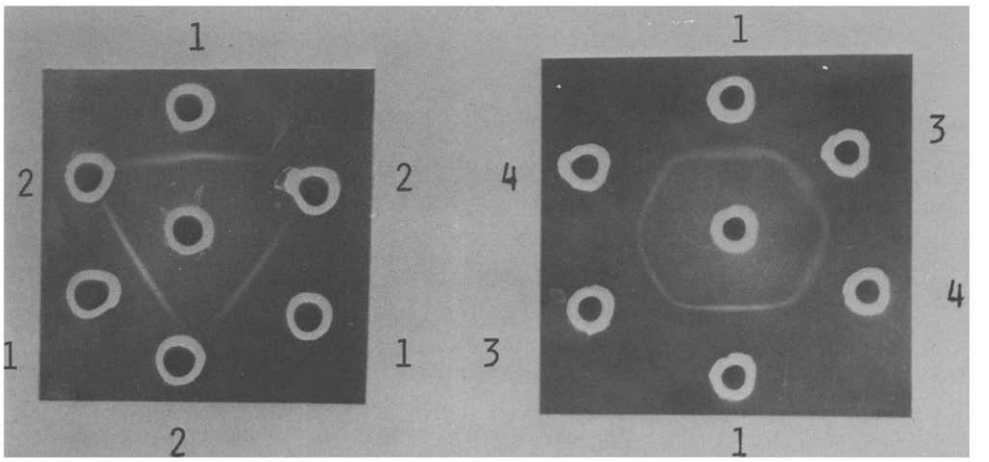


Fig. 4. Single immunodiffusion (Ouchterlony) of purified glutathione reductase against anti-human erythrocyte glutathione reductase anti-serum. The samples were dialysed against the buffer for the enzyme assay and diluted to the same enzyme concentration. 1. Purified human erythrocyte glutathione reductase. 2. Yeast glutathione reductase (Boehringer-Mannheim No. 15338). 3. Dissociation of FAD (human erythrocyte glutathione reductase). 4. Recombination with FAD (human erythrocyte glutathione reductase).

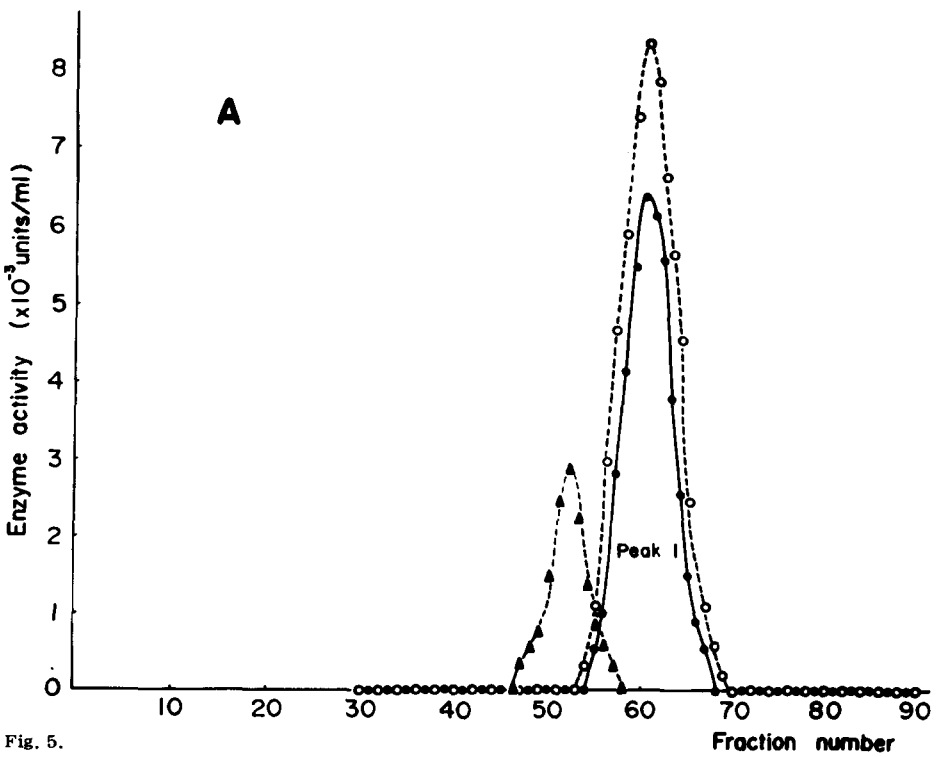
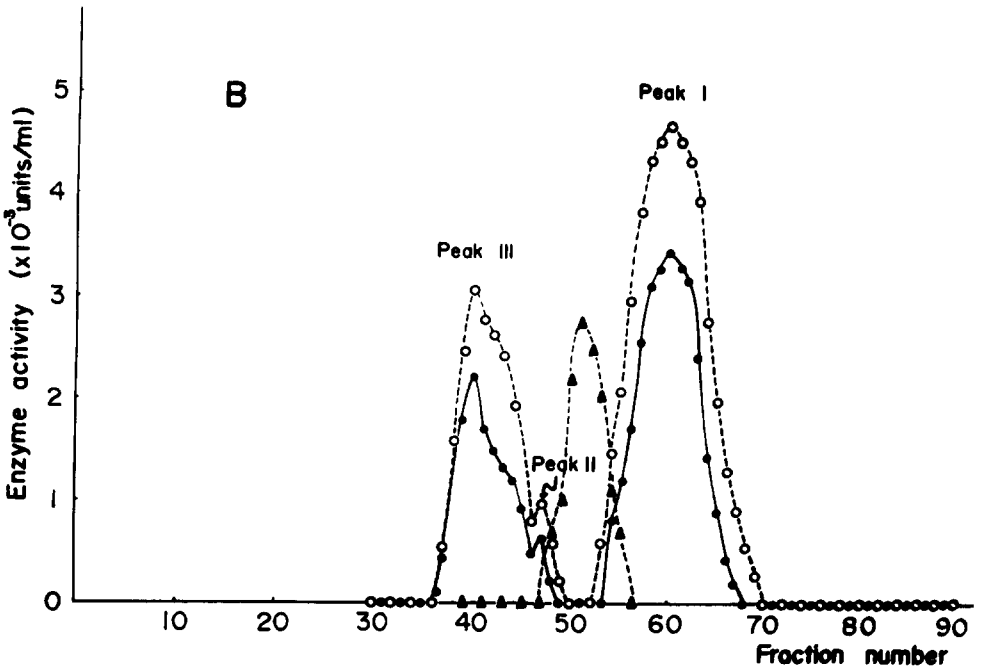


Fig. 5.


B
Peak I
Peak III
Peak II
Enzyme activity (x10⁻³units/ml)
Fraction number
0
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2
3
4
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10
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80
90

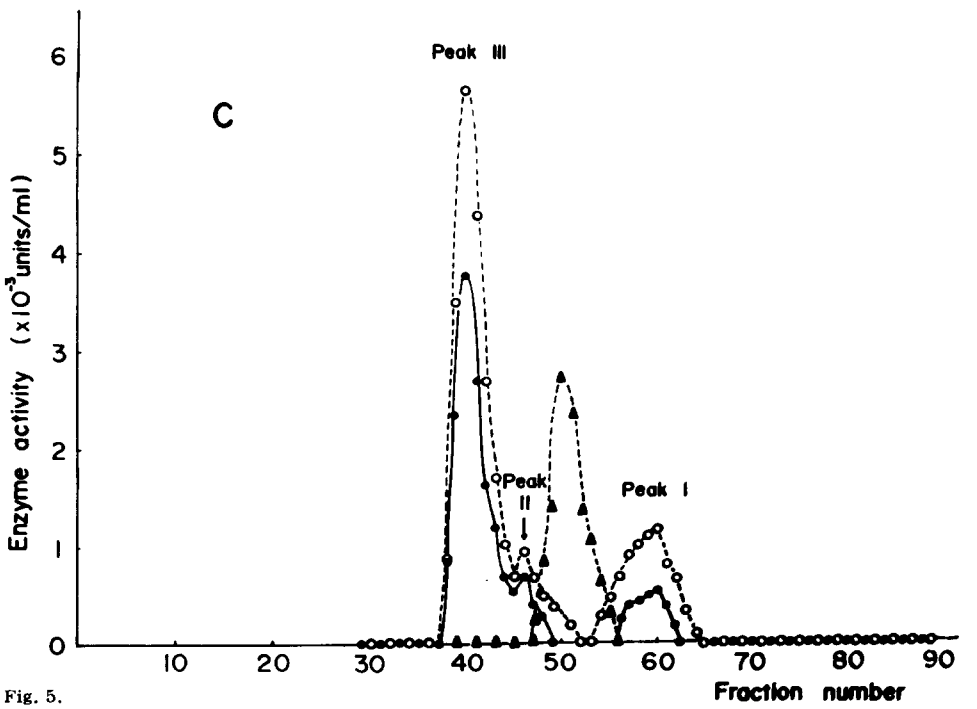
C
Peak III
Peak II
Peak I
Enzyme activity (x10⁻³units/ml)
Fraction number
0
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Fig. 5.

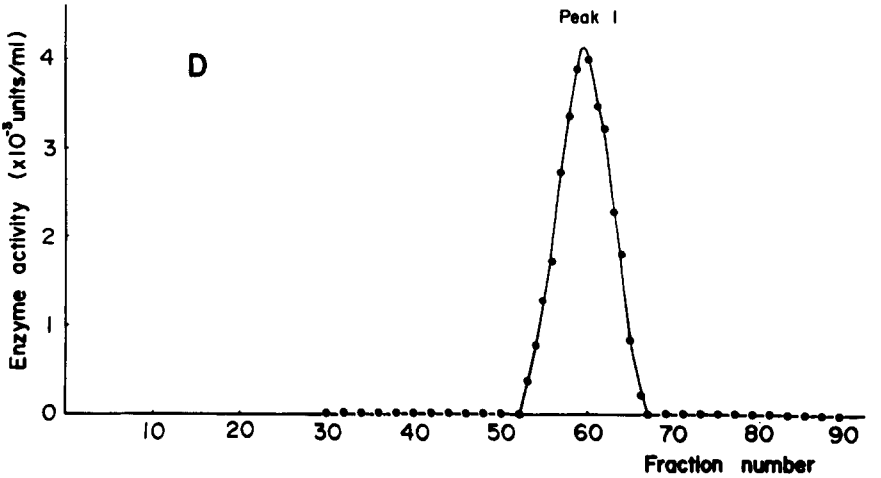

Fig. 5. Elution profile from gel-filtration on Sephadex G-200 (1.5 × 100 cm). The hemolysate or the yeast glutathione reductase solution was mixed with an equal volume of 0.5% bovine serum albumin without anti-serum or anti-serum solution diluted with 0.5% bovine serum albumin. The mixture was incubated at 25°C for 2 h and at 4°C overnight. 1 ml of the mixture was applied and gel-filtration was performed using a 0.05 M phosphate buffer, pH 7.0, containing 0.2 M KCl, 1 mM EDTA, 0.2 μM NADP and 0.2% mercaptoethanol at a flow rate of 9.5 ml/h and 1.7 ml fractionation. After the enzyme assay, 10 μM FAD was added to each fraction, and after incubation at 37°C for 20 min, glutathione reductase was measured again. A: The mixture of the hemolysate with 0.10 units/ml glutathione reductase and 0.5% bovine serum albumin without anti-serum. B: The mixture of the same hemolysate and 0.25 μl/ml anti-serum solution. C: The mixture of the same hemolysate and 0.75 μl/ml antiserum solution. D: The mixture of the yeast glutathione reductase of 0.08 units/ml and 0.75 μl/ml antiserum solution. ●———●: Glutathione reductase activity before addition of FAD. ○- - - - -○: Glutathione reductase activity after addition of FAD. ▲- - - - -▲: Pyruvate kinase activity.

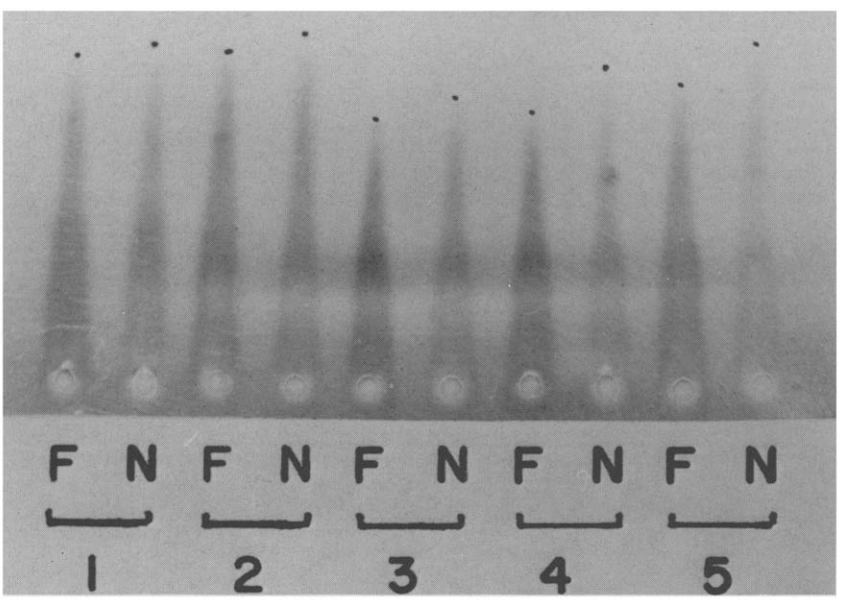

Fig. 6. Electroimmunodiffusion of glutathione reductase of 5 crude hemolysate from the normal subjects. Electrophoresis was carried out with 0.8% agarose (Dotite Agarose I, Japan) containing 0.013% (v/v) rabbit antiserum using a barbital/glycine/Tris buffer, pH 8.8, of Svendsen [17] at 18 V/cm for 6 h. After the electrophoresis, the peaks of the immunoprecipitate were developed in the same way as in the detection of glutathione reductase on the gel electrofocusing for the gel slab A in Fig. 2.

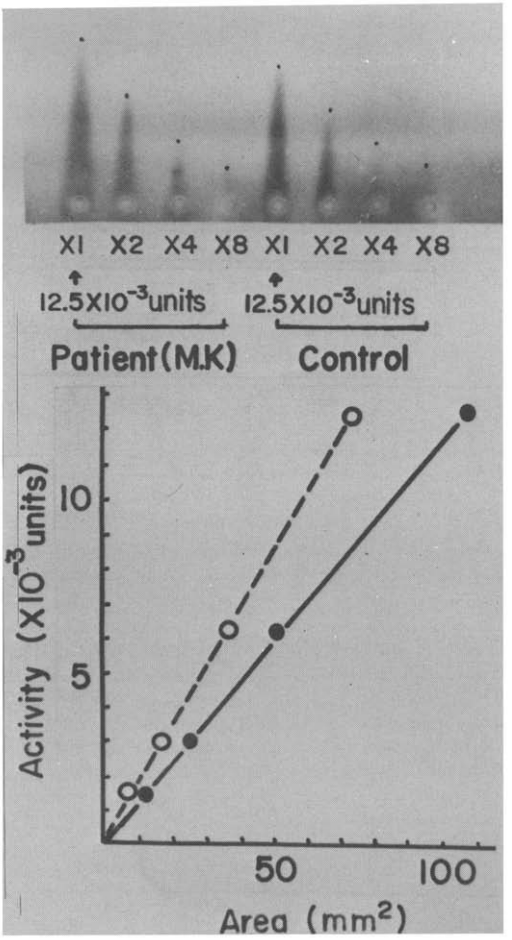

Fig. 7. Electroimmunodiffusion of erythrocyte glutathione reductase of the normal and the patient with aplastic anemia and the enzyme deficiency, and its graphic representation. Both hemolysates were saturated with FAD and adjusted the activities by dilution of the normal hemolysate to be the same. Both hemolysates were diluted 2, 4 and 8 times. 5 μl of each sample were applied. The condition and the method were the same as those in Fig. 6. ○- - - - -○: Normal control. ●———●: Aplastic anemia.

TABLE III

CHANGES IN ENZYME ACTIVITIES BY ANTI-HUMAN ERYTHROCYTE GLUTATHIONE REDUCTASE ANTI-SERUM AND UPON HEATING

The hemolysate was mixed with an equal volume of water or antiserum solution and the mixture was incubated as in the neutralization test in Fig. 9. The hemolysate mixed with water was treated by heating as described in the text. The activities of the enzymes (units/ml) and the concentration of the anti-serum (μl/ml) were those of the mixture.

	Hemolysate without anti-serum	Hemolysate with antiserum		Hemolysate with heating
		0.8 μl/ml	1.6 μl/ml	
Glutathione reductase	0.11	0.064	0.053	0.15
Glucose-6-phosphate dehydrogenase	0.14	0.13	0.13	0
6-Phosphogluconate dehydrogenase	0.13	0.13	0.14	0
Enolase	0.012	0.011	0.012	0
Pyruvate kinase	0.21	0.21	0.21	0
Lactate dehydrogenase	2.80	2.79	2.79	1.28

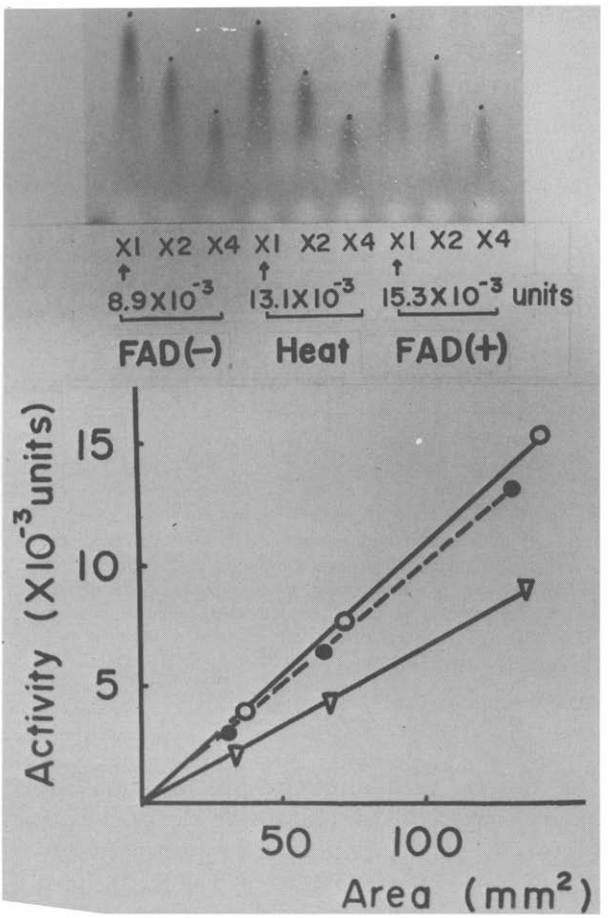

Fig. 8. Electroimmunodiffusion of glutathione reductase of the crude hemolysate, the hemolysate saturated with FAD and the hemolysate treated by heating, and its graphic representation. These hemolysates were diluted 2 and 4 times. 5 μl of each sample were applied. The conditions and the method were the same as those in Fig. 6. △———△: Crude hemolysate. ○———○: Hemolysate saturated with FAD. ●------●: Hemolysate treated by heating.

of the enzyme [10] and an increase of antiserum produced lower Peak I and higher Peak II and III as seen in Fig. 5B and 5C, Peak II and III are not aggregation but immunocomplexes. After the addition of FAD the apoenzyme was saturated but only the same fraction as in the original holoenzyme developed activity. Yeast glutathione reductase produced only one peak at the same position as Peak I in human enzyme in spite of the presence of antiserum.

In Fig. 6, the electroimmunodiffusions of the normal samples with and without 10 μM FAD are shown. The samples with FAD have a stronger staining of the precipitation area, but the samples without FAD have slightly taller precipitation peaks. A photograph of the result of the normal control and that for aplastic anemia with the enzyme deficiency saturated with FAD, and its graphic presentation are shown in Fig. 7. The area of the immunoprecipitate and its activity is perfectly linear in each sample.

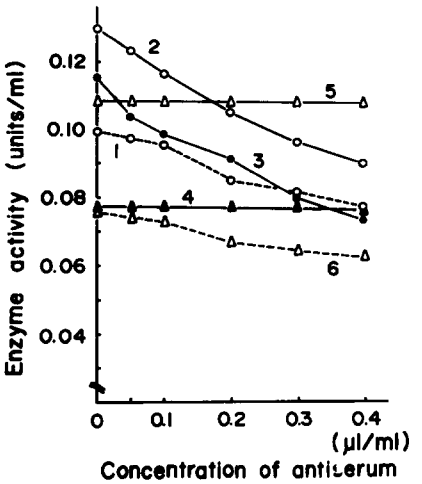

Fig. 9. Neutralization test with anti-human erythrocyte glutathione reductase anti-serum. The hemolysate was mixed with an equal volume of the anti-serum serially diluted with 0.5% bovine serum albumin and incubated at 25°C for 2 h and at 4°C overnight. Because anti-serum had no glutathione reductase activity and the enzyme activities of the mixture were not different before and after centrifugation, the activity was measured immediately after incubation without centrifugation. The final concentrations of anti-serum and enzyme activity of the mixture were numbered in the figure: 1: Human crude hemolysate without FAD. 2: Human hemolysate saturated with FAD. 3: Human hemolysate treated by heating. 4: Yeast glutathione reductase. 5: Rabbit hemolysate saturated with FAD. 6: Rat hemolysate saturated with FAD.

Upon heating 30% of the total volume of hemolysate precipitated. Supernatant lost enzyme activity except for those of glutathione reductase and lactate dehydrogenase and no elevation of glutathione reductase activity occurred upon addition of FAD (Table III). The hemolysate mixed with the antibody showed no inactivation of other enzymes than glutathione reductase (Table III).

In Fig. 8, electroimmunodiffusion and its graphic presentation for the crude hemolysate, the hemolysate treated by heating and the hemolysate saturated with FAD of a normal subject are shown. Molecular specific activity was lower in crude hemolysate but higher in the hemolysate treated by heating and the hemolysate saturated with FAD.

Neutralization by the antiserum are shown in Fig. 9. The slope of inhibition is related to the molecular specific activity and the antigenicity, and was steeper in the hemolysate saturated with FAD and the hemolysate treated by heating than in the crude hemolysate. Yeast and rabbit enzyme were not inhibited at all but rat enzyme was slightly neutralized.

Discussion

In the purification, by addition of a heating step after the Sephadex G-200 large column step, the protein, which cannot be separated from glutathione reductase by means of differences of net charge, solubility in ammonium sulfate solution or molecular weight in the previous steps, is denatured to form the insoluble precipitate which can be removed by centrifugation. The de-

natured soluble protein has differences in net charge, solubility in ammonium sulfate solution, and molecular weight from the natural protein and can be separated by the subsequent steps. Fig. 1 shows that the small column of Sephadex G-200 of Step 8 revealed the extra protein peaks which were colorless, except for the orange yellow enzyme peak. Without the heating step, the Sephadex G-200 small column revealed only one peak associated with the enzyme activity. 30% saturated ammonium sulfate also produced a white precipitation of impure protein, which was not observed without the heating step. Staal et al. [9] used heating at 60°C for 30 min in their purification procedure. By the inclusion of a heating step at an appropriate stage, Worthington's method [10] was improved.

The constant ratio of NADPH-dependent enzyme to NADH-dependent enzyme through the purification steps until the crystalline enzyme, as reported by Icén [5], suggests that both enzymes are a single enzyme. On electrofocusing, the crystalline enzyme showed 4 protein bands, all of which had enzyme activity (Fig. 3) and the pattern of NADPH-dependent enzyme was identical to that of NADH-dependent enzyme (Fig. 2). In the inhibition tests, inhibitions by NADPH-X, NADP and NADH-X were competitive to NADH-dependent enzyme. The inhibitions by NADPH-X and NADP were stronger at pH 6.8 than at pH 6.05 although the inhibition by NADH-X was stronger at pH 6.05 than at pH 6.8. NADPH-dependent enzyme has an optimum pH of 6.8, and NADH-dependent enzyme one of 6.05 [5]. This suggests that the inhibitors have optimum pH values similar to those of the original nucleotide-dependent reaction and that the reaction sites of NADPH and NADH are identical on the single enzyme. From the results of purification, electrofocusing and inhibition studies, it can finally be concluded that glutathione reductase is a single enzyme which uses both NADPH and NADH as hydrogen donors, although Eifler and Wagenknecht [4] reported that NADH-dependent enzyme was separated from NADPH-dependent enzyme.

Electrofocusing studies revealed that the apoenzyme had a broad additional cathodic band which disappeared upon recombination with FAD. This cathodic band in the apoenzyme of the partially purified enzyme was first observed by Rüdiger et al. [18] using column electofocusing and enzyme reaction. In the present study, the additional band with positive net charge in the apoenzyme of the crystalline enzyme was confirmed by gel electrofocusing and protein detection as well as enzyme reaction. The identical protein and enzyme stain patterns of the apoenzyme suggest that either the apoenzyme still has faint enzyme activity, contains a small amount of holoenzyme or contains an intermediate form between holoenzyme and apoenzyme, but this is not conclusive. In the separation of immunocomplexes of glutathione reductase of crude hemolysate, the saturation of each fraction by FAD showed the development of activity in the same fractions as holoenzyme. These results indicate that the apoenzyme exists in dimeric form as holoenzyme in red cells, that the apoenzyme cross-reacts with the antibody to holoenzyme and that the apoenzyme combined with an antibody can receive FAD to become holoenzyme.

Kahn et al. [19] reported the method of indirect immunological assay of the specific activity of glucose-6-phosphate dehydrogenase. In electoimmunodiffudion, an antigen-antibody reaction occurs between the migrating antigen and

the fixed antibody on electrophoresis. Harboe and Inglid [20] suggested that the amount of antibodies giving rise to precipitates in the immunoelectrophoresis is smaller than the "equivalent amount" of antibodies to be calculated by precipitation in tube experiments. Immunocomplexes in the precipitate should be relatively unsaturated with antibodies, which means that the antigen with lower affinity for the antibody produces a larger precipitation area than the normal antigen because such antigens run further with less antigen-antibody reaction or need more antibodies to produce precipitation. The crude hemolysate with both holoenzyme and apoenzyme showed a larger precipitation area than did the hemolysate saturated by FAD (Fig. 6). This means that the apoenzyme has a slightly lower affinity to the antibody than the holoenzyme although the Ouchterlony method with sufficient reaction time revealed no difference in antigenicity between the apoenzyme and the holoenzyme (Fig. 4). In Fig. 7, four peaks of activities and precipitation area are perfectly linear. It is possible to measure the relative specific activity of the glutathione reductase saturated with FAD, provided that the enzyme examined is completely identical immunologically to the normal enzyme. It is expected that the enzyme structure in the case of secondary deficiency is identical to (or at least more similar to) the normal structure than the unstable genetic variant enzyme is.

The hemolysate treated by heating had the same specific activity as the hemolysate saturated with FAD in electroimmunodiffusion (Fig. 8) as well as in neutralization tests (Fig. 9), and had lost other enzyme activities (Table III) and the ability to elevate the glutathione reductase activity by addition of FAD. These results indicate that the apoenzyme in the hemolysate can be removed by heat denaturation without inactivation of the holoenzyme. If there are some thermolabile proteins cross-reactive to the antibody, the molecular specific activity of the hemolysate treated by heating must be higher than that of the hemolysate simply saturated with FAD because thermolabile cross-reactive protein must be removed by heating as other enzymes but must coexist in the hemolysate saturated with FAD. The activities of other enzymes were not influenced by incubation with anti-human erythrocyte glutathione reductase anti-serum (Table III). On the other hand, this antibody obtained from the rabbit does not cross-react to the rabbit enzyme (Fig. 9) or to the yeast enzyme with more different species specificity (Figs. 4 5 and 9), but cross-reacts to the rat enzyme although to a lesser extent than to human enzyme (Fig. 9). These findings suggest that the anti-serum obtained by immunization with the crystalline enzyme is highly specific to human erythrocyte glutathione reductase. Even if there exists another antibody which causes another precipitation peak on the electroimmunodiffusion and causes another immunocomplex in the neutralization test, such immunoprecipitation peak or immunocomplex produced by another antibody cannot be detected by presented methods since the detections of the immunoprecipitation peak and immunoinactivation are done by the most sensitive and specific procedure, enzyme reaction. In fact, protein staining in this method of electroimmunodiffusion does not show any precipitation peak. Even if it exists, another such antibody is of negligible amount for the purpose in the present study.

The electroimmunodiffusion and the neutralization test of human erythrocyte glutathione reductase and the heating procedure to remove apoenzyme

from the hemolysate are useful tools in characterization studies of glutathione reductase deficiency. The evaluation of the deficiency in aplastic anemia and other diseases is in progress in our laboratory.

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