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# PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE DISULFIDE-STIMULATED $Mg^{2+}$-ATPase FROM HUMAN ERYTHROCYTES

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**SUMMARY** We have previously shown the presence of two different forms of glutathione disulfide(GSSG)-stimulated $Mg^{2+}$-ATPases in human erythrocytes. We have now investigated a low-Km form of the enzyme from human erythrocytes. Purification of the enzyme was performed to apparent homogeneity involving procedures of affinity chromatography and gel filtration. The enzyme was composed of two non-identical subunits of Mr=82K and 62K. The enzyme reconstituted into phospholipid vesicles showed both GSSG-stimulated $Mg^{2+}$-ATPase activity (285 nmol Pi released/mg protein /min) and active GSSG transport activity (320 nmol GSSG/mg protein /min). The amino acid composition of the enzyme was similar to that of the enzyme purified from cytoplasmic membranes of human hepatocytes. These enzymes were immunologically cross reactive. These results indicate that this enzyme functions in the active transport of GSSG as it possibly does in hepatocytes.


The importance of GSH is recognized in the maintenance of normal erythrocyte function and stability; GSH protects the erythrocyte membranes and other cell constitutions from oxidative stress(1). GSSG efflux is thought to be important on maintaining the high ratio of GSH/GSSG (2). The ATP dependent transport system for GSSG was described by Srivastava and Beutler using erythrocytes exposed to oxidative stress(3). We previously reported the presence of two ATP dependent GSSG transport systems using

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ABBREVIATIONS: GSSG, glutathione disulfide; GSH, reduced form of glutathione; SDS: sodium dodecyl sulfate.

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inside-out vesicles of human erythrocytes (4,5). However, the precise mechanism of the transporter, for which ATP is required, has not been clear. Quite recently, we have found the presence of two GSSG-stimulated $Mg^{2+}$-ATPases in human erythrocytes; a low-Km form and a high-Km form(6). The presence of GSSG efflux(7), and the presence of GSSG-stimulated ATPase(8) or GSH S-conjugate-stimulated ATPase(9) were also found in plasma membranes of rat hepatocytes. In the present study, we purified and characterized the low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase which functions in the active transport of GSSG.

## MATERIALS AND METHODS

Preparation of Erythrocytes. Erythrocytes were prepared from fresh human venous blood of normal donors, using a small column of α-cellulose-microcrystalline cellulose(10).

Purification of GSSG-Stimulated $Mg^{2+}$-ATPase. The low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase was purified according to the method described(6) with a slight modification. Briefly, white ghosts (100 ml) were suspended in 20 vol. of 0.5 mM Tris-HCl (pH 8.0) for 12 h in order to remove most of the extrinsic proteins (11). After centrifugation, the ghosts were solubilized in 10 mM imidazole-HCl, pH 7.4, 0.2 mM EDTA, 50 μM 2-mercaptoethanol (buffer A) containing 0.5% Triton X-100(wt/vol). They were centrifuged, and the extract was diluted with buffer A to adjust the concentration of Triton X-100 at 0.25% and was then applied to a column of S-hexylglutathione-Sepharose 6B. A bound fraction was eluted with a linear gradient of 0-2 mM S-hexylglutathione. Triton X-100 in the enzyme fractions was removed using Bio-Beads SM-2(Bio-Rad). The enzyme was resolubilized in 200 μl of 0.1% Lubrol PX in buffer A and was applied to a Sephacryl S-400 column(1x50 cm). GSSG-stimulated $Mg^{2+}$-ATPase was also purified from cytoplasmic membranes of human hepatocytes. Liver was obtained by autopsy. Cytoplasmic membranes were prepared as described(12). Purification procedure for the hepatocyte enzyme was as same as that for the erythrocyte enzyme.

GSSG-Stimulated $Mg^{2+}$-ATPase Activity. GSSG-Stimulated $Mg^{2+}$-ATPase activity was measured as described(6) at 37°C in a 250-μl system containing 100 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM [$\gamma$-$^{32}$P]ATP with a specific activity of 1.5 pmol/cpm, 50 μM GSSG, and 150 μl of enzyme solution. As a blank system, no GSSG was added to the assay mixture. The activity was expressed as the amount of Pi released by the addition of GSSG.

Protein Determination. Protein concentration was determined according to the method of Redinbaugh and Turley(13), with bovine serum albumin as a standard.

Electrophoresis. Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis was performed according to the method of Laemmli(14), in a 12% gel. Polyacrylamide gel electrophoresis was performed in a 7.5% gel in the presence of 0.1% Triton X-100. Protein bands were stained with Coomassie Blue.

Reconstitution of The GSSG Transport System. The fraction of the enzyme from the gel permeation was freed of Lubrol PX using Ectracti-gel D(Pierce), and was reconstituted in phospholipid vesicles according to the method of Kasahara and Hinkle(15). Briefly, the enzyme fraction (9-40 μg of protein) was added to

the sonicated liposomes (30 μmol of phospholipids) in a final volume of 3 ml. The mixture was rapidly frozen in a test tube using liquid nitrogen, and was then thawed at room temperature. The liposome fraction was washed with 20 ml of 10 mM Tris-HCl (pH 7.4), and was then centrifuged at 10,000 x g for 20 min at 4°C. Reconstituted liposomes were flushed with nitrogen and were used for the experiments within an hour.

Transport Assay of GSSG. Unless otherwise indicated, GSSG transport was measured as described(4) at 37°C in a 500-μl system containing 100 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 50 μM [$^3$H]-GSSG with a specific activity of 0.2 pmol/dpm, and 250 μl liposomes. A blank assay mixture containing no ATP was used. The reaction was terminated after 5 min by the addition of 5 ml of an ice-cold stopping solution (10 mM Tris-HCl, pH 7.4). The mixture was then applied to a membrane filter (0.3 μ, type TM-3, Toyo) and was washed twice with 5 ml of the ice-cold stopping solution. The radioactivity in liposomes was estimated using a liquid scintillation counter.

Amino Acid Analysis. Amino acid analysis was carried out on acid hydrolysates (6 N HCl for 20 and 40 h at 110°C) by a one-column procedure using a Nihondenshi model JLC-6AH amino acid analyzer.

Immunological Study. Specific antisera to GSSG-stimulated ATPase was obtained by immunization of individual rabbits, with purified antigens. The precipitin reactions of the radial diffusion assay method of Mancini et al.(16).

## RESULTS AND DISCUSSION

Purification of The Low-Km Form of GSSG-Stimulated $Mg^{2+}$-ATPase. The low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase was purified from human erythrocyte membranes. Solubilization of membrane proteins, affinity chromatography on S-hexylglutathione-Sepharose 6B, and gel chromatography on Sephacryl S-400 resulted in a purification of the enzyme as shown in Fig.1. The enzyme ac-

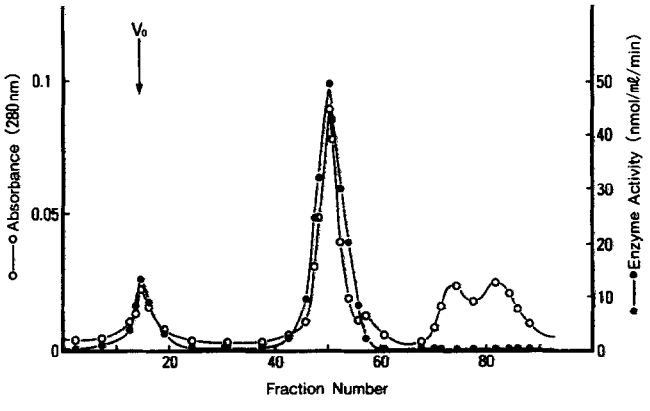


Figure 1. Gel permeation on Sephacryl S-400. The low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase was purified by application to Sephacryl S-400 gel chromatography. The activity of GSSG-stimulated $Mg^{2+}$-stimulated ATPase (•) and the protein concentration (o) were determined. Vo denotes the void volume fraction.

tivity was detected both at the void fractions and at the peak protein fractions. The enzyme proteins at the void fractions were thought to be an aggregated form. The peak fraction of the enzyme, with a specific activity of 1250 nmol Pi released/mg protein/min at 1 mM ATP and 50 uM GSSG at 37°C, was used for the following experiments. The apparent molecular weight of the enzyme determined by the gel permeation was 150K in the presence of 0.1% Lubrol PX(Fig.2). Polyacrylamide gel electrophoresis (Fig.3) demonstrated a main single band when stained with Coomassie blue. Analysis of a similarly treated gel gave a peak activity at the same location. The electrophoretic pattern on SDS-polyacrylamide gel electrophoresis showed the presence of two subunits, with molecular weights of 82K and 62K, respectively (Fig.4).

Comparison with the Enzyme of Hepatocytes. Amino acid analysis of the enzyme from erythrocytes was compared with that of the enzyme from cytoplasmic membranes of hepatocytes. As shown in Table I, there is a similarity in the amino acid composition be-

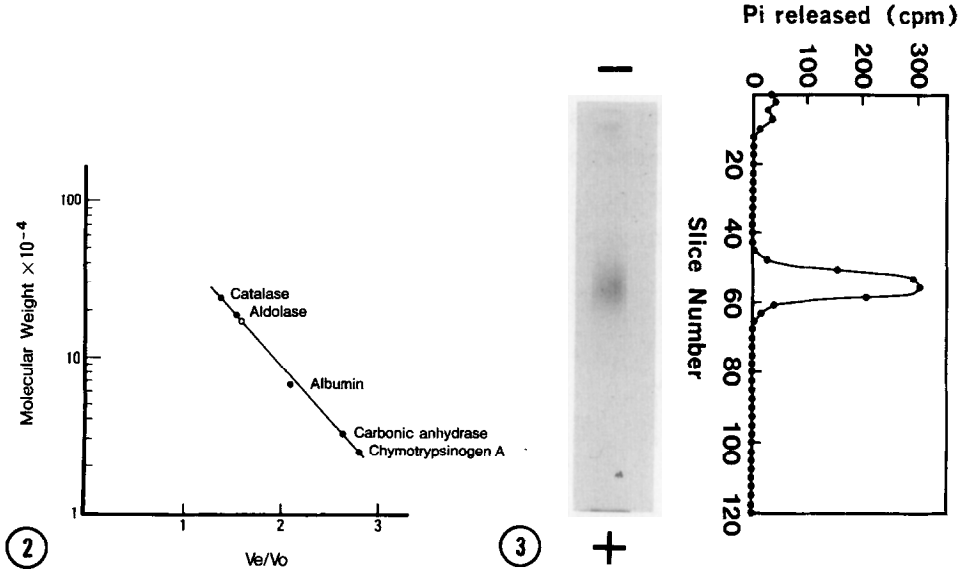


Figure 2. Molecular weight determination. The apparent molecular weight of the low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase (o) was estimated using the data shown in Fig. 1. The molecular weight standards used were catalase (220K), aldolase (150K), albumin (68K), carbonic anhydrase (31K), and chymotrypsinogen A (25K).

Figure 3. Correlation of enzymatic activity and localization of the enzyme protein in a 7% acrylamide gel with 0.1% Triton X-100. Electrophoresis was performed until the marker comes to the anodal edge. Slices of the gel (2mm) were used for enzymatic assay while the gel analyzed simultaneously was stained with Coomassie blue.

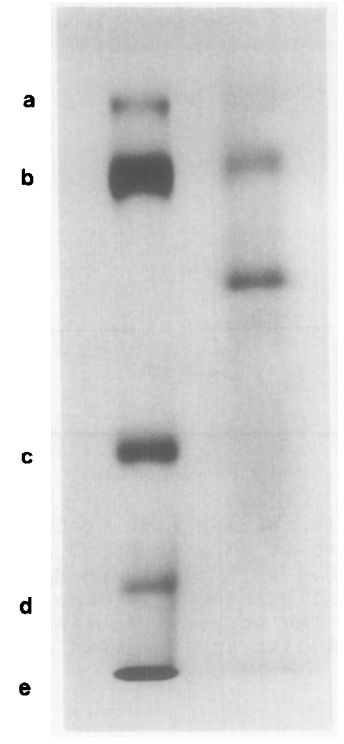


Figure 4. Electrophoresis on SDS-polyacrylamide gel. SDS-polyacrylamide gel electrophoresis was performed in a 12% gel. In lane 1, Mr markers: a, phosphorylase b (97.4K); b, bovine serum albumin (66.2K); c, ovalbumin (42.7K); d, bovine carbonic anhydrase (31K); e, soy bean trypsin inhibitor (21.5K). In lane 2, the low-Km form of GSSG-stimulated $Mg^{2+}$-ATPase. Protein bands were stained with Coomassie blue.

**Table I.** Comparison of amino acid residues of the low-Km form of GSSG-stimulated ATPase from erythrocytes and hepatocytes

| Amino acid | Erythrocyte enzyme (residues/100 amino acids) | Liver enzyme (residues/100 amino acids) |
|---|---|---|
| Asp | 7.6 | 7.7 |
| Thr | 5.0 | 4.5 |
| Ser | 16.9 | 15.5 |
| Glu | 15.1 | 17.8 |
| Gly | 21.0 | 18.6 |
| Ala | 8.3 | 7.9 |
| Val | 4.3 | 4.4 |
| Met | 0.7 | 0.7 |
| Ile | 2.7 | 2.6 |
| Leu | 5.7 | 5.4 |
| Tyr | 2.3 | 1.9 |
| Phe | 0.7 | 1.8 |
| Lys | 3.7 | 3.9 |
| His | 2.4 | 2.9 |
| Arg | 2.7 | 2.6 |
| Cys1/2 | 0.9 | 1.8 |

Values are means of duplicate analyses.

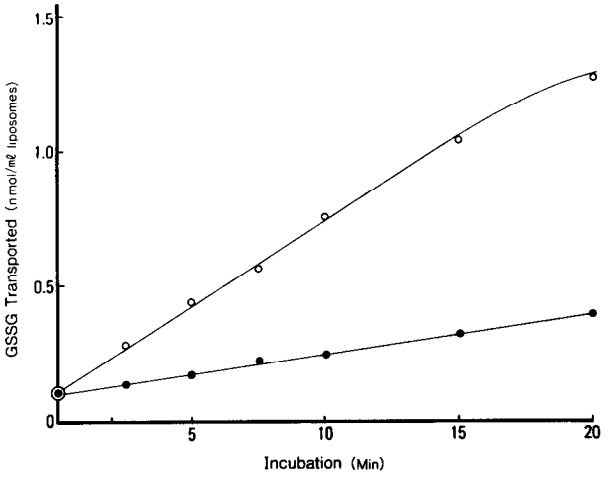


Figure 5. Effect of incubation time on GSSG transport. GSSG transport was estimated at 37°C in a 500 µl system containing 100 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 50 µM [$^3$H]-GSSG, and 250 ul liposomes (o). One ml liposomes contained approximately 3 µg of the enzyme. A blank assay mixture contained no ATP (•). Results are the mean of duplicate assays for each point.

tween the enzymes from both tissues. Antisera raised against erythrocyte GSSG-stimualted $Mg^{2+}$-ATPase showed a cross reactivity to hepatocyte GSSG-stimulated $Mg^{2+}$-ATPase (Fig.5).

GSSG Transport Activity of Reconstituted liposomes. In order to study the physiological significance of this enzyme, the enzyme was reconstituted into phospholipid vesicles. The isolation of membrane bound enzymes and their subsequent reconstitution into a well defined artificial membrane are indispensable for the study of the physiological significance of the enzymes at the molecular level. The GSSG-stimulated $Mg^{2+}$ATPase activity and GSSG transport were estimated in these reconstituted liposomes. The active and specific transport of GSSG was defined as the difference of GSSG transport in the presence and absence of ATP. The incubation of reconstituted liposomes in the presence of ATP at 37 °C resulted in a transport of GSSG. Neither incubation of liposomes without enzyme proteins, nor that of liposomes with protein fractions having no GSSG-stimulated ATPase activity, showed the GSSG transport. Fig.6 shows the effect of incubation time on GSSG transport into the liposomes. The active GSSG transport was dependent on the incubation time and was linear for up to 20 min at 37 °C. The GSSG transport was dependent on the protein concentration in the liposomes.

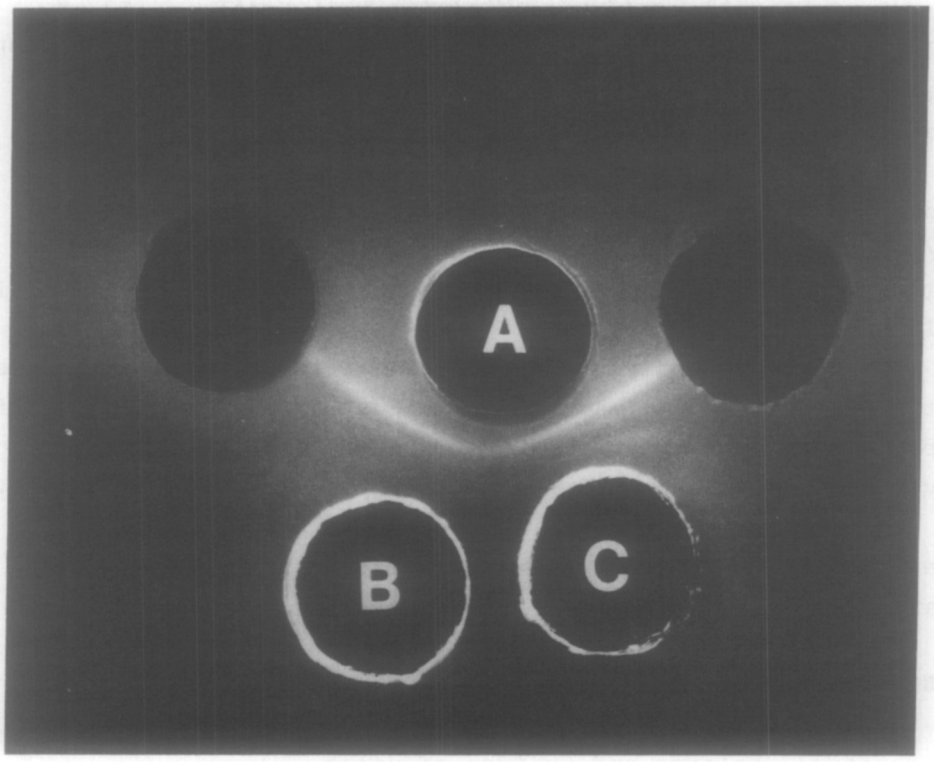


Figure 6. Immunological characteristics. Immunological activity of the enzymes was estimated using antisera raised against erythrocyte GSSG-stimulated $Mg^{2+}$-ATPase. (A) anti-erythrocyte GSSG-stimulated $Mg^{2+}$-ATPase, (B) GSSG-stimulated $Mg^{2+}$-ATPase of erythrocytes, (C) GSSG-stimulated $Mg^{2+}$-ATPase of hepatocytes.

After incubation, the liposomes were lysed by repeated freezing and thawing, followed by centrifugation at 100,000 x g for 30 min. The supernatant was applied to a column of Dowex-1(formate form) and was eluted by a linear gradient of 0 - 4 N formate. A single peak of radioactivity was observed in the elution fraction which was identical to the GSSG fraction.

The reconstituted GSSG-stimulated $Mg^{2+}$-ATPase in liposomes showed the GSSG transport with a mean value of 320 ± 11 nmol GSSG/mg protein/min (mean ± SD), and the enzyme activity of 285 ±

**Table II.** Enzyme activity and GSSG transport activity of reconstituted GSSG-stimulated $Mg^{2+}$-ATPase

| Fraction | GSSG-stimulated $Mg^{2+}$-ATPase activity (nmol Pi released/mg protein/min) | GSSG transport activity (nmol/mg protein/min) |
|---|---|---|
| Gel permeation | 285 ± 14 | 320 ± 11 |

Values are expressed as means ± SD of four experiments.

14 nmol of Pi released/mg protein/min at 1 mM ATP and 50 μM GSSG(Table II). No GSSG was transported by the addition of ADP, AMP, or adenosine 5'-[β,γ-imido]triphosphate(Sigma) in the absence of ATP. The activity observed for reconstituted enzyme was not as high as that observed for the purified enzyme. The specific activity of the enzyme was 1200 nmol of Pi released/min/mg of protein, while, that of reconstituted enzyme was 285 nmol of Pi released/min/mg protein. The decrease in the activity could be due to the fact that the direction of enzyme proteins in the liposomes may not be the same as that in natural membranes, or the enzyme activity may have been lost during the process of preparing the liposomes. However, the reconstituted enzyme provided a suitable method for the study of transport activity.

In conclusion, GSSG-stimulated $Mg^{2+}$-ATPase plays a major role in GSSG transport in human erythrocytes and possibly in human hepatocytes.

## ACKNOWLEDGMENT

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