Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 155-161

PURIFICATION AND CHARACTERIZATION OF DINITROPHENYLGLUTATHIONE ATPase OF HUMAN ERYTHROCYTES AND ITS EXPRESSION IN OTHER TISSUES

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Received July 13, 1990

S-(2,4-dinitrophenyl)glutathione (Dnp-SG) ATPase of human erythrocytes has been purified to apparent homogeneity by affinity chromatography. In reduced denaturing gels, the subunit M_r value of Dnp-SG ATPase was found to be 38,000. Dinitrophenyl glutathione (Dnp-SG) stimulated the hydrolysis of ATP by the purified enzyme whereas oxidized glutathione (GSSG) did not, indicating that Dnp-SG and GSSG are transported from the erythrocytes by different transporters. Results of Western blot analysis using the antibodies against Dnp-SG ATPase subunits indicated that the enzyme was expressed in human liver, lung, placenta and pancreas.

Conjugates of glutathione (GSH) and electrophilic xenobiotics are transported out of human erythrocytes through an ATP dependent, primary active transport process (1-8). We have previously demonstrated that the GSH conjugate of 1-chloro-2,4 dinitrobenzene, S-dinitrophenylglutathione (abbreviated as Dnp-SG) stimulates ATP hydrolysis by an erythrocyte membrane ATPase designated as Dnp-SG ATPase (6,7). Transport of oxidized glutathione (GSSG) is also mediated by an ATP dependent transport process. Although several mechanisms for transport of GSH, GSSG and GSH-conjugates across biological membranes have been described in human and other mammalian tissues (9-14), the interrelationship among these transporters is not clearly understood. Further characterization of these transporter proteins is therefore needed. In the present communication we report the purification and characterization of human erythrocyte Dnp-SG ATPase by affinity chromatography using a Dnp-SG sepharose affinity matrix. We have also raised antibodies against the denatured subunits of Dnp-SG ATPase and have examined the expression of Dnp-SG ATPase in human tissues besides erythrocytes.

0006-291X/90 $1.50

MATERIALS AND METHODS

All studies were performed using less than one week old blood collected from normal subjects at the University of Texas Medical Branch (UTMB) Blood Bank. Human liver,pancreas,lung,and placenta were obtained from autopsy at UTMB, Galveston. Samples were only used when subjects had no diagnosed tissue specific disorder and the autopsy was performed within 24 hours of death. Samples were cleaned of the connective tissue, washed thoroughly with phosphate buffered saline (PBS) and stored at -20°C until used.

Dnp-SG was synthesized enzymatically using purified human erythrocyte glutathione S-transferase. Details of the method have been described by us previously (15). Dnp-SG was linked to CNBr-activated Sepharose 4B according to the method described by Porath et al. (16). (γ^{32}P) ATP was purchased from Du-Pont NEN, Boston, MA. and the sources of other chemicals were the same as those described by us previously (17).

Purification of Dnp-SG ATPase from human erythrocytes: Erythrocyte ghosts were prepared essentially according to the method of Dodge et al. (18) with a slight modification in which 10mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl was used instead of PBS. For better extraction of the enzyme, the ghosts were converted to membrane vesicles according to the method of Kasahara and Hinkle (19) by extracting them with 0.1mM EDTA and 0.5mM NaCl followed by centrifugation at 43,400 x g for 20 min in a Sorvall RC-5C centrifuge without applying brakes. Membrane vesicles were mixed with 0.5% lubrol PX in 20mM Tris-HCl, pH 7.4, containing 2.0mM EDTA and 2.8mM 2-mercaptoethanol. The mixture was incubated for 20 min at 4°C with occasional shaking and was then centrifuged at 48,400 x g in a Beckman J2-21 centrifuge for 2 hours. The supernatant was applied to a 1 x 4cm affinity chromatography column in which Dnp-SG was linked as affinity ligand to CNBr-activated Sepharose 4B. The column was pre-equilibrated with 10mM Tris-HCl, pH 7.4, containing 2.8mM 2-mercaptoethanol and 0.25% lubrol (buffer A). After applying the sample, the column was washed thoroughly with buffer A to remove unbound proteins, and the enzyme was eluted with 10mM Tri-HCl, pH 7.4, containing 0.4mM Dnp-SG, 2.0mM ATP, 2.0mM $MgCl_2$, 2.8mM 2-mercaptoethanol and 0.25% lubrol. The enzyme was dialyzed extensively against 10mM Tris-HCl, pH 7.4, to remove Dnp-SG, ATP and detergent. ATPase activity during purification was monitored by using the method of Knowles and Leng (20), details of which have been described by us previously (6). Enzyme activity unit was defined as one n mol of ATP hydrolysed/min at 37°C.

Immunological studies: Antibodies against the denatured subunits of Dnp-SG ATPase were raised in New Zealand albino rabbits by the method of Diano et al. (21). Dnp-SG ATPase peptide band from SDS-gels was transblotted onto nitrocellulose membranes according to the method of Burnette et al. (22) and stained with Ponceau red. The centers of the bands were carefully cut, suspended in phosphate buffered saline and sonicated to convert the cellulose strips to a fine suspension which could pass through an 18 gauge hypodermic needle. The suspension, without Freund's adjuvant or DMSO, was injected subcutaneously on the back of the rabbit. Two booster injections were given at intervals of 2 weeks and after an additional 2 weeks, the rabbit was bled and the serum prepared. Pre-immune serum was prepared from the blood of the same rabbit prior to injection.

For immunoprecipitation of Dnp-SG ATPase, 300μl of crude erythrocyte vesicle extract prepared as described above, was incubated with 2-20μl of serum at 37°C for 45min. The reaction mixture was then incubated with appropriate amount of protein A at 4°C for 15 min. and centrifuged at 22,000 x g for 10 min. ATPase activity was

determined in the supernatant. For controls, the enzyme was incubated with equivalent amounts of pre-immune serum.

For evaluating the expression of Dnp-SG ATPase in other human tissues; liver, lung, placenta and pancreas specimens obtained from autopsy were thoroughly perfused with phosphate buffered saline to remove as much blood as possible and 10% homogenates of each were prepared in 10mM phosphate buffer, pH 7.0. The homogenate was centrifuged at 28,000 x g for 30 min. The supernatant was collected and centrifuged at 100,000 x g for 60 min. The membrane pellet was washed with 20mM Tris-HCl buffer, pH 7.4, and solubilized in 0.5% lubrol in 20 mM Tris-HCl, pH 7.4, containing 2.0mM EDTA and 2.8mM 2-mercaptoethanol. The extracts were subjected to Western blot analysis according to the slightly modified method of Towbin et al. (23) as described by us previously (24).

RESULTS AND DISCUSSION

An apparently homogenous preparation of Dnp-SG ATPase was obtained from human erythrocyte membranes using affinity chromatography. Results of the purification over an affinity column having Dnp-SG as an affinity ligand are presented in Table 1. Approximately, a 13 fold purification was obtained with a 5% overall yield. It must be pointed out that on the basis of protein recovery in the affinity chromatography fraction (Table 1), about 250 fold purification of the enzyme is expected. The low yield and lower than expected specific activity of the purified enzyme appears to be due to inactivation of the enzyme in the presence of lubrol. This is indicated by the almost complete loss of enzyme activity in the purified preparation within 24 hours when stored

TABLE 1

Purification of Dnp-SG ATPase from human erythrocytes

	volume	activity units*/ml	Total units	protein (mg/ml)	specific activity (u/mg)	yield (%)	purif. fold
Membrane vesicles	20.00	1.32	26.4	1.5	0.88	100	--
Lubrol extract	15.00	0.33	4.95	--	--	18.75	--
Dnp-SG affinity chromato-graphy	6.00	0.22	1.32	0.02	11.00	5.0	12.5

* nmoles ATP hydrolyzed/min at 37°C.
protein determination in the lubrol extract was not possible because of the presence of excess detergent.

at 4°C. The instability of the enzyme made the analysis of kinetic parameters difficult. In several determinations from different batches of purified enzyme, the Km of the enzyme for Dnp-SG was found to be in the range of 62-128 μM. This value is in the same range as that of the previously reported value (6) for crude Dnp-SG ATPase from erythrocyte membrane vesicles. The pH optimum of the enzyme was found to be between 7.5 - 8.0 which is also in the same range as that for the crude enzyme. The purified preparation of Dnp-SG ATPase did not stimulate hydrolysis of ATP upon addition of GSSG, indicating it to be a different protein from the erythrocyte membrane ATPase described by Kondo et al. (8). This is further substantiated by the results of SDS-PAGE analysis which show a single peptide band corresponding to an Mr value of 38,000 (Fig. 1). The ATPase involved in the transport of GSSG has been shown to be a heterodimer of 85K and 62K Mr subunits by Kondo et al. (8). It is not known if the Dnp-SG ATPase is a monomer or an oligomer of 38K subunits because the molecular weight of the holoenzyme could not be determined in the present studies. Antibodies raised against the denatured 38K peptide according to the method of Diano et al. (21) showed a band at Mr 38K in Western blot analysis of crude lubrol extract of erythrocyte membrane (Fig. 2). Furthermore, these antibodies immunoprecipitated Dnp-SG ATPase activity from the lubrol extract of erythrocyte ghosts (Fig. 3) indicating that the antibodies against the 38K subunits were indeed against Dnp-SG ATPase.

The antibodies against Dnp-SG ATPase were used to examine whether or not this enzyme was expressed in other tissues. Tissues were perfused to free them from contaminating blood. Lubrol extracts of the membrane fractions, prepared as described in the method section were subjected to Western blot analysis. The results of the

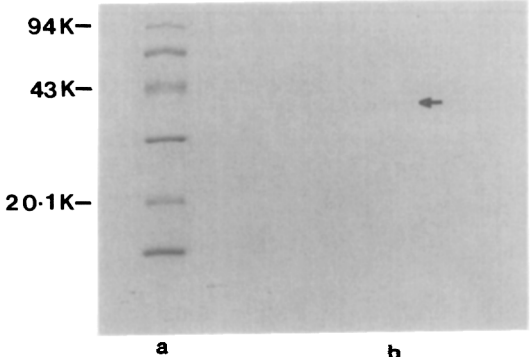
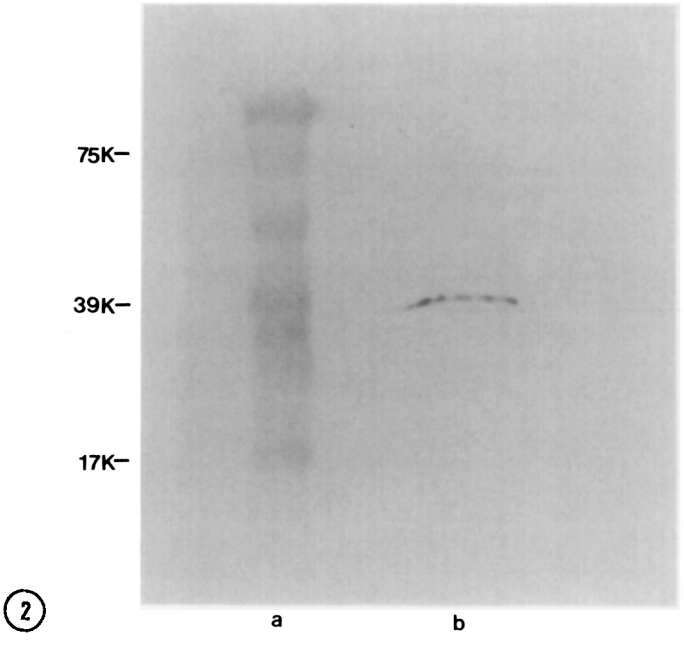


Figure 1. SDS-polyacrylamide gel electrophoresis of the purified Dnp-SG ATPase. Lane a, standard marker proteins, lane b, fraction eluted from Dnp-SG affinity column.



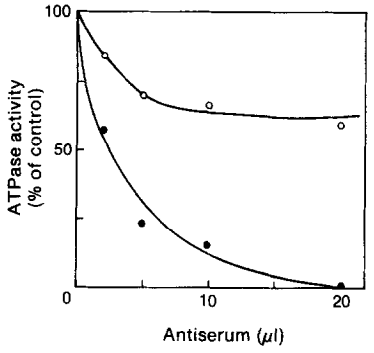


Figure 2. Western blot analysis of erythrocyte membrane extract with antibodies raised against 38K peptide. Lane a, prestained standard marker proteins, lane b, lubrol extract of erythrocyte membrane proteins.

Figure 3. Immunotitration of erythrocyte membrane Dnp-SG ATPase with the antibodies raised against 38K peptide (•) and the preimmune serum (o).

Western blot analysis of membrane fractions of human liver, pancreas,lung and placenta (Fig.4) indicated Dnp-SG ATPase was expressed in all these tissues. Since mature erythrocytes do not have mRNA, this finding is significant because now the primary structure of erythrocyte Dnp-SG ATPase can be determined by cloning its cDNA from cDNA libraries prepared from any of these tissues. This will be important in understanding the structural and functional interrelationships among various ATP dependent transport systems described for the transport of GSH, GSSG and GSH-conjugates from human and other mammalian tissues (8,10,13). Another important ATP hydrolyzing transporter is the multidrug resistance conferring protein, P-glycoprotein, which is the product of mdr-1 gene (25) and is overexpressed in a number of multidrug resistant cancer cell lines. P-glycoprotein mediated drug efflux is believed to be at least one of the mechanisms for the development of resistance towards structurally unrelated drugs during chemotherapy (26). Dnp-SG ATPase pumps the GSH-conjugates of hydrophobic xenobiotics out of cells, whereas P-glycoprotein transports hydrophobic chemotherapeutic drugs such as adriamycin or their amphiphatic conjugates out of cells.

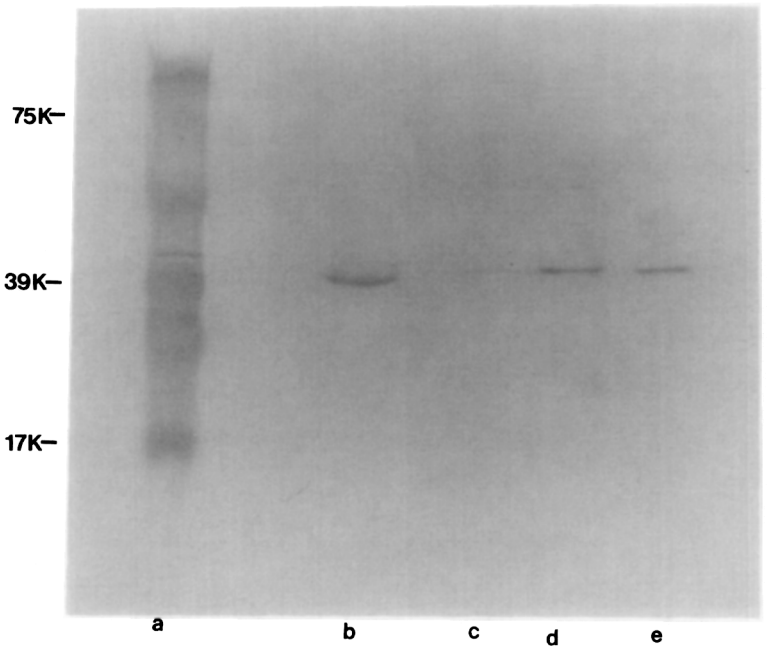


Figure 4. Western blot analysis of crude membrane extracts from different human tissues. Lane a, prestained standard marker proteins, lanes b, c, d, and e lubrol extract of the membrane proteins from human liver, pancreas, lung and placenta, respectively.

Because of their similar physiological roles of pumping toxic compounds out of the cells, an interrelationship between these two transporters has been suggested (27). Even though P-glycoprotein has been shown to be a 170 KD monomeric protein, and the subunit M_r of Dnp-SG ATPase was found to be 38K in these studies, the possibilities of structural similarities within their functional domains or ATP and hydrophobic substrate binding regions can not be ruled out. This question is currently being addressed in our laboratory by kinetic and structural studies, including the cloning of the Dnp-SG ATPase cDNA from a human liver cDNA library, using the antibodies raised in the present study.

ACKNOWLEDGMENT

This investigation was supported by US PHS grant GM 32304, awarded by the National Institute for General Medical Sciences.

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