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A novel dinitrophenylglutathione-stimulated ATPase is present in human erythrocyte membranes

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Vesicles prepared from human erythrocyte membranes were found to catalyze ATP hydrolysis that was stimulated by dinitrophenylglutathione (Dnp-SG). This activity was dependent on temperature and Mg^{2+} and independent of ion pump ATPases present in erythrocyte membranes. The activity was a linear function of protein and time up to 60 min. The K_m values of ATPase for Dnp-SG and ATP were found to be 49 μM and 1.67 mM, respectively. This suggests that in erythrocytes, the transport of Dnp-SG requires direct enzymatic hydrolysis of ATP and both Dnp-SG-stimulated ATPase activity and the ATP-dependent efflux of Dnp-SG from erythrocytes represent different activities of the same protein.

Erythrocyte; Membrane; Dinitrophenylglutathione; Transport

1. INTRODUCTION

A transport system has been described in erythrocyte membranes that is responsible for the active, ATP-dependent efflux of xenobiotic conjugates of glutathione (GSH), such as dinitrophenylglutathione (Dnp-SG) [1-4]. This transport system most likely plays a role in the detoxification of hydrophobic xenobiotics in erythrocytes. The transport system for Dnp-SG from erythrocytes has been suggested to be a primary active, ATP-dependent transport system completely independent of ion gradients established by either Na^+, K^+-ATPase or the Ca^{2+}-ATPase [4]. This system also appears to be different from the transport system that stimulates removal of oxidized glutathione (GSSG) from erythrocytes [5]. In order to prove that the Dnp-SG transport from erythrocytes is indeed primary transport, it is essential to demonstrate the existence of an ATPase in erythrocyte membrane that is stimulated by Dnp-SG. Here, we have characterized such a Dnp-SG ATPase activity in erythrocyte vesicles.

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2. MATERIALS AND METHODS

All studies were performed using freshly drawn blood in heparin from normal healthy donors. Dnp-SG was synthesized enzymatically according to our previous method [4]. [^{32}P]ATP was obtained from Dupont-NEN (Boston, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

2.1. *Preparation of vesicles from human erythrocytes*

Membrane vesicles were formed from human erythrocytes by a slight modification of the procedure of Kasahara and Hinkle [6]. Erythrocyte ghosts were prepared from human blood by a modification of the procedure of Dodge et al. [7]. The erythrocytes were washed initially with 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl instead of phosphate buffer used by Dodge et al. [7], and the cells were hemolyzed in 10 mM Tris-HCl, pH 7.4, and washed several times with this buffer at 22000 $\times g$ for 10 min. The centrifuge brake was not used during the centrifugation. The purified erythrocyte ghosts were stored at 4°C and extracted with EDTA and NaCl as described by Kasahara and Hinkle [6], except that the centrifugation was carried out at 48400 $\times g$. The final vesicle preparation was stored for up to 2 weeks at 4°C. The vesicle protein concentration was determined by the method of Bradford [8].

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2.2. *Measurement of ATPase activity*

The ATPase activity of erythrocyte vesicles was determined by a modification of the method of Knowles and Leng [9]. The vesicles were incubated with [^{32}P]ATP and other compounds in a final volume of 0.5 ml at the desired temperature and then the reaction was terminated by the addition of 2.5 ml of a 1:4 mixture of 5% (w/v) ammonium molybdate and 1.25 M perchloric acid (kept at 4°C). After vigorous mixing, the reaction mixture was treated with 2.5 ml isobutanol:benzene (1:1) and vortex-mixed further for 1 min. The mixture was then centrifuged at $800 \times g$ for 5 min. In order to separate the two phases. The radioactivity of the $^{32}P_i$-molybdate complex in the upper phase was determined using a Beckman LS-100 liquid scintillation counter.

3. RESULTS AND DISCUSSION

Human erythrocyte vesicles contained an ATPase activity that was stimulated by Dnp-SG (table 1). Dnp-SG dependent ATP hydrolysis by the vesicles was a linear function of protein (fig.1) and time up to 60 min. (not shown). This activity was dependent on temperature and Mg^{2+}. When the temperature of the incubation mixture was raised from 20 to 37°C, the Dnp-SG dependent ATP hydrolysis increased by a factor of 2.6. This activity was stimulated approx. 3.5-fold at 37°C by 10 mM Mg^{2+} (table 1). When Dnp-SG-stimulated ATP hydrolysis was measured as a function of increasing concentration of Dnp-SG, a linear double-reciprocal plot of the data was observed (fig.2). Analysis of the double-reciprocal plot

Table 1

Dependence of Dnp-SG-stimulated ATPase activity on temperature and Mg^{2+}

Temperature (°C)	$MgCl_2$ (10 mM)	ATPase activity (nmol/min per mg protein)	
		− Dnp-SG	+ Dnp-SG (0.11 mM)
20	+	0.75 ± 0.01 (n = 3)	0.96 ± 0.01 (n = 3)
37	−	0.52 ± 0.02 (n = 4)	0.70 ± 0.03 (n = 4)
37	+	1.76 ± 0.04 (n = 5)	2.48 ± 0.07 (n = 5)

Aliquots of vesicle formed from erythrocyte ghosts were incubated for 60 min with radiolabelled ATP (0.05 μCi, 0.6 mM), 50 mM Tris-HCl (pH 7.4), 2 mM EGTA, 0.6 mM ouabain, 0.75 mM sodium phosphate and the compounds shown above at the indicated temperatures in a total volume of 0.5 ml. The incubation mixtures were mixed with ammonium molybdate-perchloric acid solution and extracted with isobutanol-benzene as described in section 2. The radioactivity in the upper phase was determined. Values represent means ± SE.

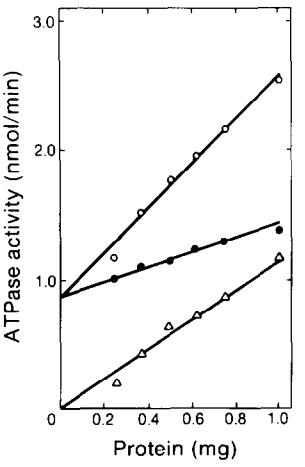

Fig.1. Dependence of Dnp-SG-stimulated ATPase activity on protein. The enzymatic breakdown of [^{32}P]ATP to form $^{32}P_i$ was catalyzed by increasing aliquots of erythrocyte vesicles during a 60 min incubation, either with (○) or without (●) 0.25 mM Dnp-SG. Aliquots of erythrocyte vesicles were incubated for the indicated periods of time with [^{32}P]ATP (0.05 μCi, 0.4 mM), Tris-HCl (50 mM, pH 7.4), EGTA (2 mM), ouabain (0.6 mM), sodium phosphate (0.75 mM) and $MgCl_2$ (10 mM) at 37°C in a total volume of 0.5 ml. The production of $^{32}P_i$ from [^{32}P]ATP was determined as described in section 2. The difference between ATP hydrolysis measured in the presence and absence of Dnp-SG is also shown (△).

revealed an apparent K_m value of 49 μM for Dnp-SG, with a V_{max} value of 2.7 nmol/min per mg protein. The affinity for ATP was tested using increasing concentrations of ATP and a linear double-reciprocal plot was observed (fig.3). The K_m value for ATP was found to be 1.67 mM with a V_{max} value of 1.89 nmol/min per mg protein.

Since the erythrocytes are capable of primary active, ATP-dependent efflux of Dnp-SG [3,4], it is logical to postulate the presence of a Dnp-SG-stimulated ATPase in erythrocyte membranes. The results of this study clearly demonstrate the presence of such a novel Dnp-SG-stimulated ATPase activity in erythrocyte membranes. This activity could be demonstrated in ghosts prepared by the procedure of Dodge et al. [7] as well as in the vesicles prepared according to Kasahara and Hinkle [6]. However, in the present study erythrocyte membrane vesicles were used because the eventual isolation of this ATPase from erythrocytes would depend upon detergent extraction procedures that work best with vesicles and poorly with ghosts (Awasthi and LaBelle, un-

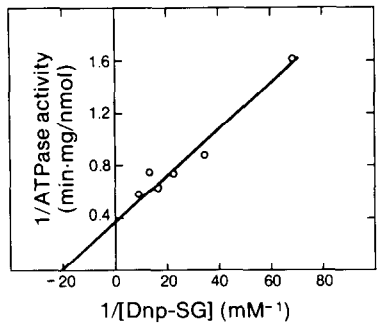

Fig.2. Dependence of Dnp-SG-stimulated ATPase activity on the concentration of Dnp-SG. ATPase activity of erythrocyte vesicles was determined as described in the legend to fig.1, with an incubation period of 60 min and an ATP concentration of 0.6 mM. ATP hydrolysis was measured in both the absence and presence of increasing amounts of Dnp-SG. A double-reciprocal plot of the data is shown.

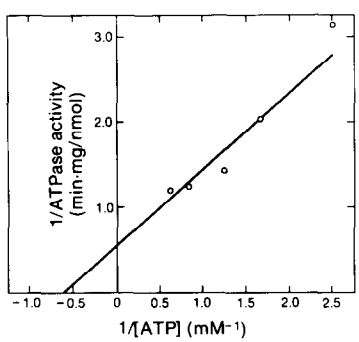

Fig.3. Dependence of Dnp-SG-stimulated ATPase activity on the concentration of ATP. ATPase activity of erythrocyte vesicles was determined as described in the legend to fig.1, with an incubation period of 60 min and increasing amounts of [^{32}P]ATP. ATP hydrolysis was measured in the presence (0.11 mM) and absence of Dnp-SG. A double-reciprocal plot of the data is shown.

published). Since ouabain and EGTA, which were always included in the assay medium, did not inhibit the Dnp-SG-stimulated ATPase activity, this ATPase is most likely independent of Na^+,K^+-ATPase or Ca^{2+}-ATPase. Sodium phosphate was included in our incubation mixtures to prevent the occurrence of specific phosphate binding by the vesicles which interferes with the ATPase measurements (Awasthi and LaBelle, unpublished). This ATPase activity is significantly stimulated by added Mg^{2+} and by an increase in temperature. The dependence of Dnp-SG-stimulated ATPase activity on added Mg^{2+} and on physiological temperature is similar to that of the Dnp-SG transport system on Mg^{2+} and temperature [4]. This suggests that both Dnp-SG-stimulated ATPase activity and ATP-dependent Dnp-SG transport probably represent different activities of the same protein.

Likewise, the rate of Dnp-SG-stimulated ATPase activity is linear with time up to 60 min and is also a linear function of the vesicle protein concentration. These linear relationships observed during this study are similar to those demonstrated previously in the transport experiments [4]. The K_m value for Dnp-SG determined here is comparable to that reported by us previously [4] during the ATP-dependent transport of Dnp-SG into inside-out vesicles prepared from human erythrocytes [4]. The K_m value determined for ATP during the ATPase measurements was 1.67 mM, which was slightly higher than that observed for ATP (0.93 mM) during the transport studies. The similarities in K_m values observed during the present study and in transport experiments support our contention that the Dnp-SG transport system and Dnp-SG-stimulated ATPase activity both rely on the same protein.

Nicotera et al. [10] have reported a Dnp-SG-stimulated ATPase activity in plasma membrane from rat liver but it is not known if Dnp-SG transport into liver membrane vesicles is dependent on ATP. The results of the present studies in conjunction with our earlier observations [4] provide strong evidence that in erythrocytes the active transport of Dnp-SG requires direct enzymatic hydrolysis of ATP. Characterization of this ATPase in erythrocyte membranes will be very helpful in monitoring this activity during its purification. The isolation and characterization of this ATPase from erythrocytes will provide insight into the interrelationships among the transport systems reported for GSH and its derivatives in mammalian tissues [11-16].

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