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ATP-stimulated uptake of *S*-(2,4-dinitrophenyl)glutathione by plasma membrane vesicles from rat liver

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ATP-stimulated uptake of *S*-(2,4-dinitrophenyl)glutathione with a high activity of 0.35 nmol/min per mg protein is found in a rat liver plasma membrane vesicle preparation enriched in sinusoidal marker enzymes. Transport takes place into an osmotically active space. Vanadate and *S*-(azidophenacyl)glutathione inhibit transport, whereas Ca^{2+}, EGTA and ouabain are without effect.

Glutathione disulfide (GSSG) and glutathione conjugates are exported from a variety of cells (see Ref. 1). Studies with inside-out erythrocyte plasma membranes [2–4] have established the presence of ATP-dependent transport systems for glutathione disulfide and *S*-(2,4-dinitrophenyl)glutathione (DNPSG) and the existence of GSSG- and DNPSG-stimulated ATPase [5,6]. Evidence was also presented that transport of GSSG and DNPSG is ATP-dependent in heart [7,8]. In liver, GSSG and DNPSG are transported preferentially into bile. Studies with canalicular membrane vesicles indicated that the transport of the glutathione conjugate is stimulated by a valinomycin-induced K^+ diffusion potential [9]. Stimulation of ATP hydrolysis by DNPSG was shown in hepatocyte plasma membrane fragments [10]. Recently, an ATP-dependent DNPSG uptake into liver plasma membrane vesicles was reported [11]. However, the uptake rate reported in [11] was extremely low and a distinction between the different domains of the plasma membrane was not made. Here, we investigate DNPSG transport with sinusoidal and canalicular rat liver plasma membrane vesicles.

Plasma membrane vesicles enriched in sinusoidal and canalicular marker enzyme activities, designated as SMV and CMV, were isolated as described in Refs. 12–14. The vesicles were suspended in a medium containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$, frozen in liquid nitrogen and stored for maximal 7 weeks at −70°C until use. Prior to transport experiments, the membrane preparations were vesiculated and preincubated at 37°C in the presence of 100 μM AT-125 (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) (The Upjohn Co, Kalamazoo, MI) for 30 min to inactivate γ-glutamyltranspeptidase, as described in Ref. 9. With this precaution > 90% of DNPSG was recovered after 60 min of transport as determined with an Isomess TLC analyzer (Isomess GmbH, Straubenhardt, F.R.G.) after TLC on silica gel plates using 1-propanol/water/acetic acid (10:5:1, v/v).

Nonradioactive DNPSG and *S*-(azidophenacyl)-glutathione (APASG) were prepared according to Ref. 15. [^{3}H]DNPSG was synthesized with glutathione *S*-transferase (GST) as in Ref. 16. The radiochemical purity analyzed by TLC (see above) was > 98%.

Uptake studies were performed at 37°C in a medium containing 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 100 μM [^{3}H]DNPSG diluted with unlabeled DNPSG to a specific radioactivity of 200 Ci/mol, 0.8 mg of vesicle protein/ml and 100 μM AT-125. Further added compounds were neutralized with Tris. Uptake was started by the addition of vesicles, the process was stopped by pipetting 20 μl of the incubation mixture into 1 ml of ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4)), immediate filtration through a 0.65 μm nitrocellulose filter (Type SM 11205-25-N, Sartorius GmbH, Göttingen, F.R.G.) and rapid washing with 2 × 2 ml ice-cold stop solution. Radioactivity remaining on the

Abbreviations: GSSG, glutathione disulfide; DNPSG, *S*-(2,4-dinitrophenyl)glutathione; APASG, *S*-(azidophenacyl)glutathione; SMV and CMV, plasma membrane vesicles enriched in, respectively, sinusoidal and canalicular marker enzyme activities.

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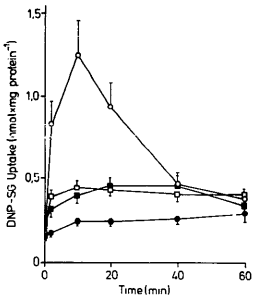

Fig. 1. Time-dependent uptake of [^{3}H]DNPSG (100 μM) by SMV (●, ○) and CMV (■, □) in the presence (open symbols) and absence (solid symbols) of an ATP-regenerating system (creatine phosphokinase 100 μg/ml, creatine phosphate (10 mM), ATP (1 mM). Final volume 165 μl. Data represent means ± S.E. of six experiments (three vesicle preparations).

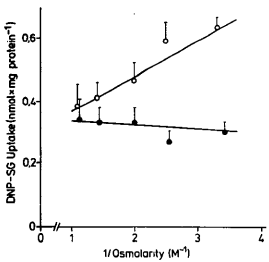

Fig. 2. Effect of osmolarity on [^{3}H]DNPSG import in the presence (○) or absence (●) of an ATP regenerating system (see Fig. 1). SMV were preincubated at 37 °C for 20 min at different sucrose concentrations. Transport was started by the addition of [^{3}H]DNPSG (final volume 60 μl) and was stopped after 20 min incubation. Data are given as means ± S.E. of 10 experiments (three vesicle preparations).

filter was counted in Quickszint Flow 303 (Zinsser Analytic, Frankfurt, F.R.G.). ATP was determined as in Ref. 17, ATPases as in Ref. 12. Chemicals were from Boehringer (Mannheim, F.R.G.), Sigma (St. Louis, MO) and E. Merck (Darmstadt, F.R.G.).

Fig. 1 shows the time dependence of the uptake of [^{3}H]DNPSG into SMV and CMV in the absence or the presence of ATP. To prevent rapid depletion of ATP due to the presence of ATPases [12], creatine phosphate and creatine kinase were added. The uptake into SMV is substantially increased by the presence of ATP with a rapid initial uptake phase and an overshoot. A maximum is reached after 10 min, followed by a decrease to a value at 60 min which is not significantly different from the value observed without ATP. The decline may be explained by the disappearance of ATP, 97% remaining after 5 min, 71% after 10 min and 32% after 15 min. Indeed, an extra addition of 10 mM creatine phosphate at $t = 20$ min prolonged the overshoot to 40 min. With CMV no significant difference of the transport with or without ATP was found.

The effect of variation of the external osmolarity on the uptake of [^{3}H]DNPSG into SMV is shown in Fig. 2. In the presence of an ATP-regenerating system (open symbols), the amount of vesicle-associated DNPSG is linearly related to the inverse osmolarity, indicating uptake into an osmotically active space. Without ATP (closed symbols), the amount of radioactivity associated with the SMV does not change significantly and probably represents binding.

With 5 mM ATP in the absence of an ATP-regenerating system the ATP dependent uptake observed during the first min amounts to 0.35 nmol/min per mg protein (Table I) which is similar to the rate observed in the presence of creatine phosphate and creatine kinase (Fig. 1). Neither the Ca^{2+} chelator EGTA (10 mM) nor the addition of Ca^{2+} show a significant effect on ATP-dependent or ATP-independent uptake (Table I). The glutathione conjugate APASG (1 mM), a potent photolabel for glutathione conjugate binding proteins [18], inhibits both ATP-dependent and ATP-independent uptake of [^{3}H]DNPSG. Ouabain, an inhibitor of Na^+/K^+-ATPase, shows no effect. Vanadate (1 mM), an inhibitor of many ATP-dependent ion transport systems [19], inhibits the ATP-dependent uptake but not the ATP-independent component.

The initial rate of ATP-stimulated DNPSG uptake in Table I (control) is 0.35 nmol/min per mg protein.

TABLE I

Uptake of [^{3}H]DNPSG by SMV

Transport was carried out in the presence of 5 mM ATP (no ATP regenerating system) and the additions indicated. Final volume 60 μl. Transport was stopped after 1 min. Data represent means ± S.E. of six experiments (three vesicle preparations).

Additions	DNPSG uptake rate (nmol/min per mg protein)	
	ATP (5 mM)	no ATP
None (control)	0.58 ± 0.06	0.23 ± 0.02
EGTA 10 mM	0.52 ± 0.02	0.16 ± 0.02
Ca^{2+} 1 mM [a]	0.44 ± 0.06	0.17 ± 0.03
APASG 1 mM	0.15 ± 0.03	0.11 ± 0.02
Ouabain 1 mM	0.54 ± 0.07	0.22 ± 0.02
Vanadate 1 mM	0.32 ± 0.05	0.22 ± 0.02

[a] $n = 4$, two vesicle preparations.

Recently, a V_{max} of 21 pmol/60 min per mg protein was reported [11]. However, this value was obtained from 60 min time points. As shown in Fig. 1, ATP-stimulated DNPSG transport is hardly detectable after one hour, even in the presence of an ATP-regenerating system. In the absence of an ATP-regenerating system as in Ref. 11, 5 mM ATP is hydrolyzed to 23% after 1 min and to 98% after 10 min. The rapid loss of ATP is possibly due to a Ca^{2+}-independent Mg^{2+}-ATPase activity of 0.28 U/mg protein present in our vesicles.

In summary, the following points emerge: (1) Transport of DNPSG is stimulated by ATP and occurs at a rate of 0.35 nmol/min per mg protein, higher than previously described [11], (2) transport shows an overshoot in the first 10 min and after 60 min there is no significant difference between transport with or without ATP, and (3) ATP-dependent transport is found only with the SMV. Canalicular rather than sinusoidal release was described for the intact organ, perfused rat liver [20]. At high infusion rates of 1-chloro-2,4-dinitrobenzene, an increasing amount of excretion of DNPSG into the perfusate was observed. Thus, the sinusoidal membrane may function as a transport site under conditions where the biliary transport capacity is exceeded. Upon infusion of DNPSG into the portal vein, no conjugate was found in bile [20]. This indicates that uptake of DNPSG does not take place across the sinusoidal membrane in intact liver and that the observed ATP-dependent import into SMV is likely to be due to the inside-out oriented SMV fraction reported to be present to at least 30% [21]. In the CMV, we could not find ATP-dependent transport. Whether this is due to the reported dominant right-side out configuration [13,22] or to the presence of a transport system different from the one observed with SMV, is under current investigation.

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