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Glutathione transport by inside-out vesicles from human erythrocytes

(cytidine monophosphate/inhibition/kinetics)

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ABSTRACT **Purified inside-out vesicles from human erythrocytes were used to investigate the active transport of oxidized glutathione (GSSG). Incubation of vesicles and GSSG in the presence of ATP resulted in the transport of GSSG into the vesicles. When vesicles were incubated with reduced glutathione (GSH), no transport was observed. At GSSG concentrations of less than 5 mM, transport was linear up to 4 hr at 37°C. A Lineweaver–Burk plot of the transport rate as a function of GSSG concentration was biphasic and gave apparent K_m values of 0.1 and 7.1 mM. The K_m for ATP·Mg in this transport process was 0.63 mM at a GSSG concentration of 20 μM and 1.25 mM at a GSSG concentration of 5 mM. The transport rate at low GSSG concentrations was inhibited by CTP or UTP, which acted as competitive inhibitors of ATP; K_i = 0.51 mM. This inhibition may account for the high erythrocyte GSH levels observed in pyrimidine-5′-nucleotidase deficiency, a disorder in which erythrocytic levels of CTP and UTP are elevated.**

Glutathione (γ-glutamylcysteinylglycine) in the erythrocyte exists principally in the reduced form (GSH) and is believed to act to protect the cell from oxidative damage. Normally, the concentration of GSH in human erythrocytes is ≈2 mM (1) and that of the oxidized form (GSSG) is ≈4 μM (2). Active transport of GSSG from erythrocytes was initially demonstrated after increasing the intracellular GSSG concentrations by oxidative stress (3); the rate of this transport has been shown to depend on the intracellular GSSG levels (4). Recent studies have extended the sensitivity of GSSG transport measurements and demonstrated that the rate of GSSG transport out of erythrocytes under physiological conditions is sufficient to account for the entire *in vivo* turnover rate of glutathione (5).

This paper describes the use of inside-out vesicles prepared from erythrocytes to study the transport of GSSG. Because the GSSG transport complex is presumably located on the outside of these vesicles, the effects of substrates, activators, and inhibitors on GSSG transport are easily studied in this system.

MATERIALS AND METHODS

Chemicals. L-[*glycine*-2-^{3}H]glutathione was purchased from New England Nuclear, and concanavalin A (Con A) and α-cellulose were from Sigma.

Preparation of Oxidized Glutathione. Solutions of oxidized glutathione were prepared by the incubation of [^{3}H]GSH with a 10 M excess of H_2O_2 at pH 8.0 in a Dubnoff shaker for 20 min at 37°C as described (4). Catalase, 18 units per ml, was added to destroy any residual H_2O_2.

Preparation of Erythrocyte Membrane. Fresh blood from normal donors was collected in EDTA (1 mg/ml of blood) and freed of leukocytes and most platelets by passage through a small column of α-cellulose and microcrystalline cellulose (1). The erythrocytes were washed three times with 0.12 M KCl containing 0.02 M K_2HPO_4/KH_2PO_4, pH 7.3, and then lysed in 25 vol of ice-cold 10 mM Tris·HCl, pH 7.0. Membranes were rinsed and washed with the same buffer until white.

Preparation of Sealed Inside-Out Vesicles. Sealed vesicles were prepared from red cell membranes as described by Steck and Kant (6). The washed membranes were suspended in 40 vol of ice-cold 0.5 mM Tris·HCl, pH 8.0, for 2 hr, washed with the same buffer 2 times, and centrifuged at 28,000 × *g* for 30 min. The pellet was vesiculated by passing it five times through a 15-mm-long 27-gauge needle.

Purification of Inside-Out Vesicles. Sealed inside-out vesicles were purified by using Con-A-α-cellulose as described (7). Homogenized vesicles, which are a mixture of inside-out and right-side-out vesicles, were mixed batchwise with con-A-cellulose for 10 min. The Con-A-cellulose was then collected on a coarse sintered glass funnel and washed with 0.5 mM Tris·HCl, pH 8.0. Inside-out vesicles do not have external carbohydrate chains and therefore do not bind to the Con-A-cellulose, while right-side-out vesicles do have external glycoprotein that binds to the Con-A-cellulose. The vesicles that washed through the funnel were collected and washed 2 times with 0.5 mM Tris·HCl, pH 8.0.

Transport Experiments Using Inside-Out Vesicles. Unless otherwise indicated, the assay system for glutathione transport consisted of 50 μl of inside-out vesicles/2 mM ATP/10 mM $MgCl_2$/20 μM or 5 mM [^{3}H]GSSG, in a final volume of 250 μl phosphate-buffered saline, pH 7.4 (P_i/NaCl; 1 part 0.1 M KH_2PO_4/K_2HPO_4, pH 7.4 plus 9 parts 0.154 M NaCl). A blank assay mixture contained no ATP. After 2 hr incubation at 37°C, the reaction was stopped by the addition of 40 vol of ice-cold 30 mM NaF in 0.5 mM Tris·HCl, pH 8.0. The vesicles were washed 2 times with the same solution by centrifuging at 25,000 × *g* for 20 min. One milliliter of water was added to the vesicles, and the mixture was then frozen and thawed. The lysed vesicles were centrifuged to remove membrane fragments, and the radioactivity in the supernatant was determined. The lysed membrane fragments were removed because variable amounts of [^{3}H]glutathione were bound to them; this probably represents the reaction of sulfhydryl groups on the membrane surface with [^{3}H]GSSG disulfide.

Transport Experiment with Reconstituted Erythrocyte Membranes. Resealed erythrocyte membranes were prepared as described (4). The resealed membranes contained 2 mM ATP/10 mM $MgCl_2$/20 μM or 5 mM [^{3}H]GSSG, with or without CTP or UTP at various concentrations; [^{14}C] sucrose was used as a marker of lysis. The outward transport of GSSG from resealed membranes was estimated as described (4).

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Abbreviations: GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; Con A, concanavalin A; P_i/NaCl, phosphate-buffered saline.

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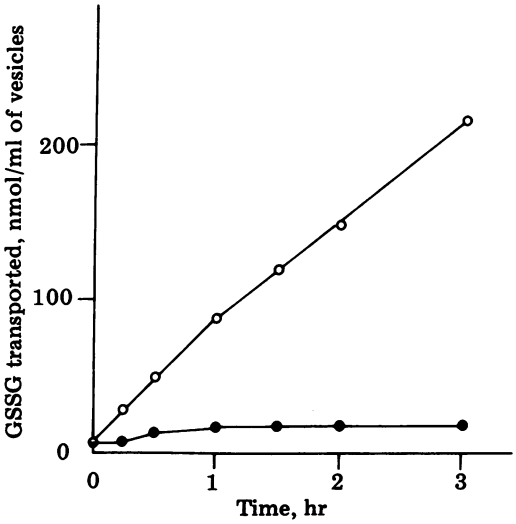

FIG. 1. Effect of ATP on GSSG transport into inside-out vesicles. Vesicles were incubated with GSSG in the presence (○) or absence (●) of ATP. GSSG levels transported were estimated on lysed solutions of vesicles.

Analytical Methods. Protein concentrations were estimated by the method of Lowry *et al.* (8) with bovine serum albumin as a standard. GSSG and GSH were determined as reported (1).

RESULTS

Active Transport of GSSG. Inside-out vesicles were incubated with 5 mM GSSG in the presence or absence of 2 mM ATP at 37°C. No GSSG was transported into the vesicles in the absence of ATP, but in its presence, GSSG was transported at a rate linear with time (Fig. 1). The transport rate increased as the concentration of GSSG increased (Fig. 2) and was linear with time for GSSG concentrations of 5 mM or less.

Transport of GSH. To determine whether GSH is transported into the inside-out vesicles, the GSSG in the incubation mixture was replaced by 5 mM [^{3}H]GSH. Glutathione reductase and NADPH were also added to the mixture to reduce any GSSG that might be formed by autooxidation of GSH. No uptake of [^{3}H]GSH was observed after 2 hr incubation (Table 1).

Table 1. Glutathione transport

	Concentration	Additive	Transport, (nmol/ml of vesicles)/hr
GSH	5 mM	Glutathione reductase plus NADPH	0
GSSG	5 mM		68.4
GSSG	5 mM	CTP (2 mM)	64.3
GSSG	5 mM	UTP (2 mM)	64.3
GSSG	20 μM		3.3
GSSG	20 μM	CTP (2 mM)	1.9
GSSG	20 μM	UTP (2 mM)	2.7

Glutathione transport was estimated by using inside-out vesicles. The incubation mixture was composed of 50 μl of vesicles/ATP (2 mM)/$MgCl_2$ (10 mM)/presence or absence of inhibitors/[^{3}H]GSH or [^{3}H]GSSG in 250 μl of P_i/NaCl, pH 7.4. GSH transport was estimated in the presence of an excess of glutathione reductase plus NADPH.

Kinetic Analysis of GSSG Transport. The dependence of GSSG transport on GSSG concentration was measured over a 10 μM–10 mM range of initial extravesicular GSSG concentrations. A Lineweaver–Burk analysis of these data gave a biphasic plot (Fig. 3). At low GSSG concentrations, the transport rates observed extrapolate to an apparent K_m of 0.1 mM GSSG having an apparent V_{max} of 20 (nmol of GSSG/ml of vesicles)/hr; at high GSSG concentrations, the apparent K_m is 7.1 mM GSSG and the apparent V_{max} is 190 (nmol of GSSG/ml of vesicles)/hr.

This discontinuity in the Lineweaver–Burk plot of the GSSG concentration data may reflect either the existence of two independent transport systems that have different kinetic parameters or a negative cooperativity in a single transport system. To decide between these two possibilities, the effect of modifiers on the two phases of the transport curve was studied. Two GSSG concentrations were chosen to represent the two phases of the transport—20 μM GSSG for the low K_m system and 5 mM GSSG for the high K_m system.

The K_m for ATP·Mg in the transport of GSSG was 0.63 mM when the GSSG concentration was 20 μM and 1.25 mM when the GSSG concentration was 5 mM.

Inhibition by Nucleosides. The nucleoside triphosphates CTP and UTP showed competitive inhibition (versus ATP) of GSSG transport when the GSSG concentration was 20 μM

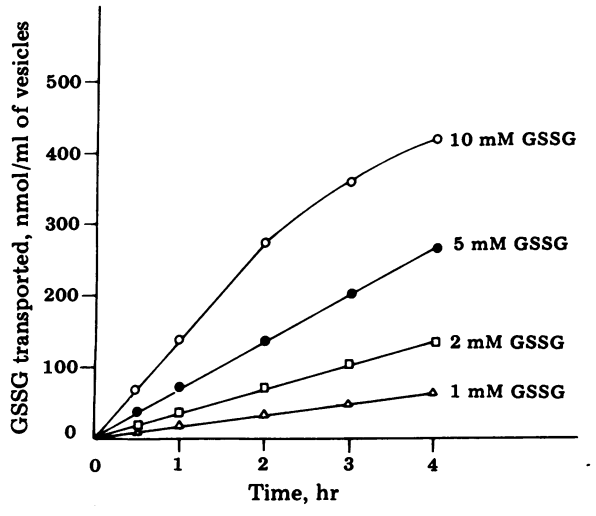

FIG. 2. Effect of concentration of GSSG on active GSSG transport. Approximately 50 μl of vesicles were incubated with various concentrations of GSSG/2 mM ATP/10 mM $MgCl_2$ in 250 μl of P_i/NaCl, pH 7.4, at 37°C. Vesicles incubated without ATP served as blanks.

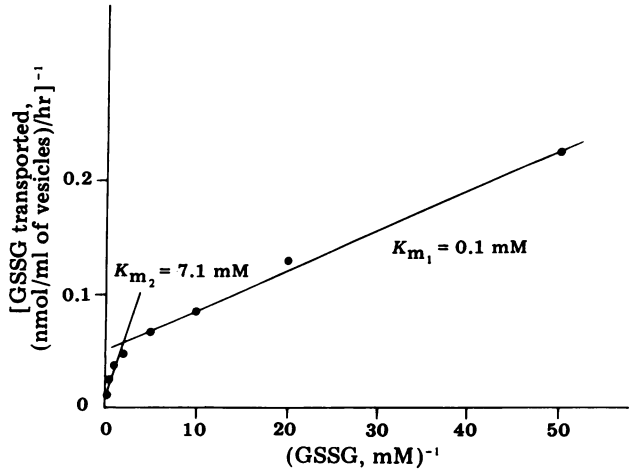

FIG. 3. Lineweaver–Burk plot of GSSG transport. Vesicles were incubated with various concentrations of GSSG/2 mM ATP/10 mM $MgCl_2$ in 250 μl of P_i/NaCl, pH 7.4, for 2 hr at 37°C. Vesicles incubated without ATP served as blanks.

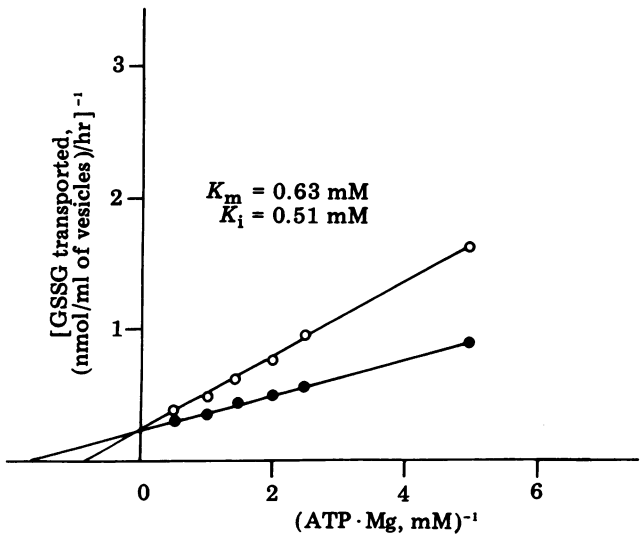

FIG. 4. Inhibition of GSSG transport by CTP. The incubation mixture was the same as for Fig. 3, but the ATP·Mg concentration was 0.1–2.0 mM, and the GSSG concentration was fixed at 20 μM. Presence (○) and absence (●) of CTP.

(Table 1, Fig. 4); the K_i for CTP was 0.51 mM. However, when vesicles were incubated with 5 mM GSSG, neither UTP or CTP inhibited transport. However, neither the nucleoside diphosphates (CDP and UDP) nor the monophosphates (CMP and UMP) inhibited transport at either GSSG concentration.

The effect of CTP and UTP on GSSG transport was also studied in resealed erythrocyte membranes. [^{3}H]GSSG was loaded into membranes with or without the inclusion of the nucleoside triphosphates, and the rate of transport of GSSG out of the resealed erythrocyte membranes was measured. When 20 μM GSSG was entrapped in the resealed erythrocytes, CTP and UTP inhibited transport by 41% and 76%, respectively, but, when the glutathione level was 5 mM, no inhibition of transport was observed (Table 2). These results agree with those obtained by using inside-out vesicles.

DISCUSSION

Inside-out vesicles from human erythrocytes have been described by Steck *et al.* (9) and found to be useful in the study of transport systems (10, 11). In this way, intracellular membrane proteins can be exposed to the external environment and thereby be monitored and modified. In this study, transport of GSSG was demonstrated in inside-out vesicles, and the effects of various inhibitors and modifiers were easily characterized.

We have reported that human erythrocytes transport GSSG at high levels of intracellular GSSG [e.g., after treatment with oxidative reagents (3)], and it has also been found that resealed erythrocyte ghosts (4), lens (12), and perfused liver cells (13) transport GSSG. Efflux of GSH has been reported to occur from perfused liver (14), lymphoid cells (15), and diploid fibroblasts (16). The relationship of the transport of GSH to GSSG transport is not known. However, it is clear that red cell membranes transport GSSG but not GSH.

The rate of GSSG transport observed for inside-out vesicles is not as rapid as that observed for intact erythrocytes or for resealed erythrocyte membranes. One milliliter of membranes produced about 0.5 ml of vesicles. At a GSSG concentration of 5 mM, the transport rate for inside-out vesicles is 68 (nmol/ml of vesicles)/hr, while, at the same concentration of GSSG, the transport rate for reconstituted ghosts is 290 (nmol/ml of ghosts)/hr. The decrease in transport rate for the inside-out vesicle may result from one of several possible causes. A part of the GSSG transport mechanism may have been lost from the membrane during the process of forming the inside-out vesicles. Alternatively, the GSSG transport process may require a cofactor that is normally present in the hemolysate but is lost during the vesicularization procedure. In either event, it is obvious that inside-out vesicles serve as a useful tool for the investigation of GSSG transport.

In the kinetic studies described in this report, two apparent half-saturation constants for GSSG were found. The high component had a K_m of 7.1 mM for GSSG, a value that is in good agreement with previous data taken by using reconstituted ghosts (4). This high-rate transport phase may serve as an emergency pathway for the elimination of GSSG during oxidative stress. However, because the levels of GSSG in normal erythrocytes are quite low (2), the low-K_m GSSG transport phase is probably more important under physiological conditions. Recently, the basal level of GSSG transport in intact erythrocytes was shown to be 6.7 (nmol/ml of red cells)/hr. This rate is relatively close to the rate observed here for transport of GSSG in reconstituted erythrocyte ghosts.

Our experiments also suggest that nucleoside triphosphates such as UTP and CTP may inhibit ATP-driven GSSG transport in erythrocytes. This is of particular interest because hereditary hemolytic anemia associated with pyrimidine-5′-nucleotidase deficiency is invariably associated with increased amounts of pyrimidine nucleotides and high levels of intracellular GSH (17–19). Torrance and Whittaker (20) have reported that, in this disorder, the red cell levels of CTP, UTP, and ATP are ≈0.8, 0.2, and 1.1 mM, respectively. The kinetic constant reported here for CTP inhibition of GSSG transport indicates that, in pyrimidine-5′-nucleotidase deficiency, the intracellular CTP levels are sufficient to substantially inhibit GSSG transport. Such inhibition of GSSG transport, along with a normal synthesis rate for glutathione, could explain the increased glutathione levels observed in the erythrocytes of these patients. The high levels of GSH found in pyrimidine-5′-nucleotidase deficiency therefore are consonant with the view that outward transport of GSSG is one of the factors that determine the steady-state level of glutathione in erythrocytes.

Table 2. Active GSSG transport in reconstituted membranes

GSSG concentration	Additive	Transport, (nmol/ml of membranes)/hr
5 mM		289
5 mM	CTP (2 mM)	326
5 mM	UTP (2 mM)	270
20 μM		12.3
20 μM	CTP (2 mM)	5.0
20 μM	UTP (2 mM)	9.4

Effect of pyrimide nucleoside triphosphates on active GSSG transport was studied by using reconstituted membranes. Membranes were reconstituted in the presence of [^{3}H]GSSG/ATP (2 mM)/$MgCl_2$ (10 mM) and the indicated nucleotides. Ghosts were suspended in 2 vol of P_i/NaCl, pH 7.4 and incubated for 2 hr at 37°C, and GSSG transport to the medium was estimated.

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