Biochimica et Biophysica Acta, 645 (1981) 132–136
Elsevier/North-Holland Biomedical Press

BBA 79282

STUDIES ON GLUTATHIONE TRANSPORT UTILIZING INSIDE-OUT VESICLES PREPARED FROM HUMAN ERYTHROCYTES

TAKAHITO KONDO, GEORGE L. DALE and ERNEST BEUTLER *

Department of Clinical Research, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037 (U.S.A.)

(Received November 18th, 1980)
(Revised manuscript received March 6th, 1981)

Key words: Glutathione transport; Inside-out vesicle; (Human erythrocyte)

Adenosine triphosphate-dependent glutathione transport was characterized using inside-out vesicles made from human erythrocytes. Kinetic analysis of the glutathione disulfide (GSSG) transport showed a biphasic Lineweaver-Burk plot as a function of GSSG concentration suggesting the operation of two different processes. One phase had a high affinity for GSSG and a low transport velocity. Most active at acidic pH and at 25°C, this transport activity was easily lost during the storage of vesicles at 4°C. The K_m for Mg-ATP was 0.63 mM; guanosine triphosphate (GTP) substituted for ATP gave a 340% stimulation of transport activity. Neither dithiothreitol nor thiol reagents affected this transport process. The other phase had a low affinity for GSSG and a high transport velocity. Most active at pH 7.2 and 37°C, this transport activity was stable during storage of vesicles at 4°C for several days. The K_m for Mg-ATP was 1.25 mM; GTP substituted with no change in activity. Dithiothreitol increased the V but did not alter the K_m, and thiol reagents inhibited the transport. These findings suggest that there are two independent transfer processes for GSSG in human erythrocytes.

Introduction

Reduced glutathione (GSH) protects erythrocytes against oxidative damage by its reaction with free radicals and peroxides. In human erythrocytes glutathione is synthesized and degraded with a half-life of 4 days [1]; the degradation step is actually transport of oxidized glutathione (GSSG) out of the cell.

The active transport of GSSG was first reported from this laboratory using erythrocytes which were subjected to oxidative reagents and, therefore, had artificially high levels of GSSG [2–4]. Isotopic labeling of red cell GSH made possible demonstration of GSSG transport from erythrocytes under physiological conditions [5].

Recently, we have utilized inside-out vesicles from human erythrocytes to further characterize GSSG transport. The kinetics of this transport showed a biphasic Lineweaver-Burk plot which suggested that two different transport processes existed [6]. The present report describes the characteristics of these two GSSG transport systems.

Materials and Methods

Chemicals. L-[*glycine*-2-^{3}H]glutathione was purchased from New England Nuclear (Boston, MA). Concanavalin A, α-cellulose, ATP, GTP, GSSG, dithiothreitol, *p*-chloromercuribenzoic acid, *N*-ethylmaleimide and iodoacetamide were from Sigma Chemical Company (St. Louis, MO).

Preparation of oxidized glutathione. Oxidized glutathione solutions were prepared from [^{3}H]GSH as described previously [7].

* To whom correspondence should be sent.
Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

Purification of sealed inside-out vesicles. Fresh blood from normal donors was collected in 1 mg ethylenediaminetetraacetic acid (EDTA) per ml blood and was freed of leukocytes and most platelets by passing through a column of α-cellulose and microcrystalline cellulose [8]. Erythrocyte membranes were prepared according to the method of Dodge et al. [9] with 10 mM Tris-HCl, pH 7.4. Sealed vesicles were prepared from erythrocyte membranes as described by Steck et al. [10]; inside-out vesicles were purified using concanavalin A-cellulose as described elsewhere [11].

Transport experiments with inside-out vesicles. Unless otherwise indicated, the assay system for glutathione transport consisted of 2 mM ATP, 10 mM $MgCl_2$, 20 μM or 5 mM [^{3}H]GSSG, and 50 μl of inside-out vesicles in a final volume of 250 μl phosphate buffered saline, pH 7.4 (1 part 0.1 M KH_2PO_4/K_2HPO_4, pH 7.4, 9 parts 0.154 M NaCl). Other conditions for the GSSG transport experiments were the same as described previously [6].

In order to determine the effect of thiols or thiol reagents, inside-out vesicles were incubated with the effector in phosphate buffered saline, pH 7.4, for 30 min at 37°C, and were then washed three times with 30 vol. of phosphate buffered saline, pH 7.4.

Analytical methods. Protein concentrations were estimated by the method of Lowry et al. [12] with bovine serum albumin as a standard. GSSG was determined as previously reported [13].

Results

A Lineweaver-Burk analysis of GSSG transport as a function of GSSG concentration resulted in a biphasic plot as shown in Fig. 1, with apparent K_m values of 0.1 mM and 7.3 mM GSSG. In order to characterize the putative separate transport mechanisms, 20 μM and 5 mM GSSG concentrations were used to represent the low K_m and high K_m phases of the transport, respectively. The characteristics of the two active GSSG transport systems in purified inside-out vesicles from human erythrocytes were analyzed as outlined below and are summarized in Table I.

Substrate specificity. We previously demonstrated that GSSG transport is dependent on Mg-ATP with a K_m of 0.63 mM at 20 μM GSSG and 1.25 mM at 5 mM GSSG [6]. Guanosine triphosphate (GTP) substituted for ATP as an energy source for GSSG transport; in fact, at 20 μM GSSG 340% of the activity observed with ATP occurred with GTP while at 5 mM GSSG GTP manifested an activity which was 118% of that observed with ATP (Table I).

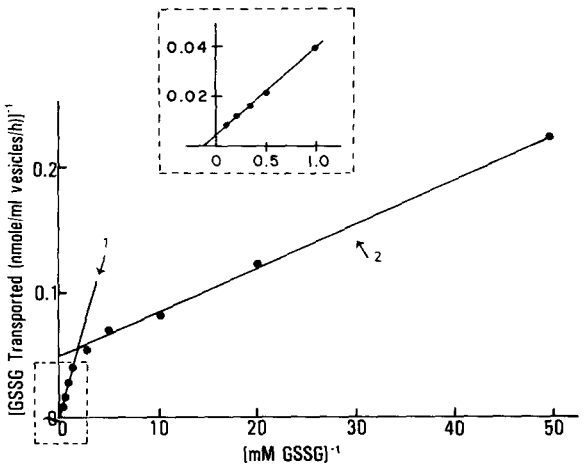

Fig. 1. A Lineweaver-Burk plot of GSSG transport. Vesicles were incubated with various concentrations of GSSG. 2 mM ATP, and 10 mM $MgCl_2$ in 250 μl phosphate-buffered saline, pH 7.4, for 2 h at 37°C. Vesicles without ATP served as a blank. The insert is an expanded scale drawing to better illustrate the high K_m process. This insert corresponds to the boxed area centered around the origin of the large graph. Line 1: K_m = 7.3 mM and V = 210 nmol/ml per h. Line 2: K_m = 0.1 mM and V = 20 nmol/ml per h.

Effect of pH. The effect of pH on the transport was examined in the range from pH 6.5 to pH 8.2 in phosphate-buffered saline (Fig. 2). At 20 μM GSSG the transport was most active at an acidic pH and at 5 mM GSSG the pH profile showed a symmetrical curve with a maximal activity at pH 7.2.

Effect of temperature. The effect of temperature on transport was studied from 0°C to 37°C. At 5 mM GSSG the transport rate increased linearly with the increase of temperature (Fig. 2). However, at 20 μM GSSG the transport rate increased up to 25°C where it reached a plateau.

Effect of storage. Purified inside-out vesicles were stored with phosphate-buffered saline at 4°C for various periods to analyze the stability of the transport activities. The transport activity at 20 μM GSSG was decreased by 62% during 5 days of storage; however, the transport activity at 5 mM GSSG was stable to

TABLE I

COMPARISON OF TWO DIFFERENT GSSG TRANSPORT PROCESSES IN HUMAN ERYTHROCYTES

The low K_m process and the high K_m process were monitored with 20 μM GSSG and 5 mM GSSG, respectively. See Methods for details of individual reactions.

Property or assay condition	Unit	Low K_m process	High K_m process
Apparent K_m (GSSG)	mM	0.1	7.3
V	nmol/ml vesicles/h	20	210
K_m (Mg ATP)	mM	0.63	1.25
Transport rate	nmol/ml/h	3.90 ± 1.20 (20) *	71.0 ± 9.70 (20)
pH optimum		≤6.5	pH 7.2
Temperature		Plateau at 25°C–37°C	Maximal activity at 37°C
Erythrocyte age			
Top fraction (young cells)	nmol/ml/h	3.82 ± 0.84 (4)	70.8 ± 1.8 (4)
Bottom fraction (old cells)	nmol/ml/h	2.84 ± 0.37 (4)	73.5 ± 2.5 (4)
Nucleotide requirement			
2 mM ATP	nmol/ml/h	3.1 ± 1.1 (4)	75.1 ± 6.3 (4)
2 mM GTP	nmol/ml/h	10.5 ± 3.3 (4)	88.6 ± 7.4 (4)
Stability			
0 day	nmol/ml/h	3.89 ± 0.17 (4)	71.2 ± 1.4 (4)
2 days	nmol/ml/h	2.50 ± 0.76 (4)	73.1 ± 3.3 (4)
5 days	nmol/ml/h	1.51 ± 0.05 (4)	67.5 ± 3.8 (4)
Effect of thiols and thiol reagents (%)			
1 mM dithiothreitol		94 **	170
0.1 mM *p*-chloromercuribenzoate		101	49
1 mM *N*-ethylmaleimide		94	75
1 mM iodoacetamide		81	66
Control		100	100

* These values are mean ± 1 S.D. (number of determinations).

** Averages of two experiments.

storage (Table I). Addition of bovine serum albumin as a stabilizer had no effect on the loss in transport activity for 20 μM GSSG (data not shown).

Effect of magnesium. When vesicles were incubated with 2 mM ATP without the addition of Mg^{2+}, about 40% residual transport activity was observed. To prevent the effect of contaminating cations, 2 mM EDTA was added to the incubation mixture; this resulted in a total loss of the transport activity.

Changes in cell aging. Erythrocytes were separated into age groups on the basis of specific gravity according to the method described by Murphy [14]. Inside-out vesicles of young cells were prepared from the top 20% of the fractionated cells and the bottom 20% were used to make vesicles from old cells. A slight decrease in GSSG transport was observed at 20 μM GSSG during cell aging, but the transport at 5 mM GSSG did not change during cell aging (Table I).

Effect of thiols and thiol reagents. Preincubation of vesicles with 1 mM dithiothreitol followed by washing three times with 30 vol. of phosphate-buffered saline, pH 7.4 resulted in an activation of the transport rate at 5 mM GSSG but had no effect at 20 μM GSSG. This increase changed maximal transport velocity from 190 nmol/ml vesicles per h to 320 nmol/ml vesicles per h but did not change the K_m.

Effects of thiol reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide or iodoacetamide were examined. They showed an inhibition of transport at 5 mM GSSG but no effect was seen on the transport

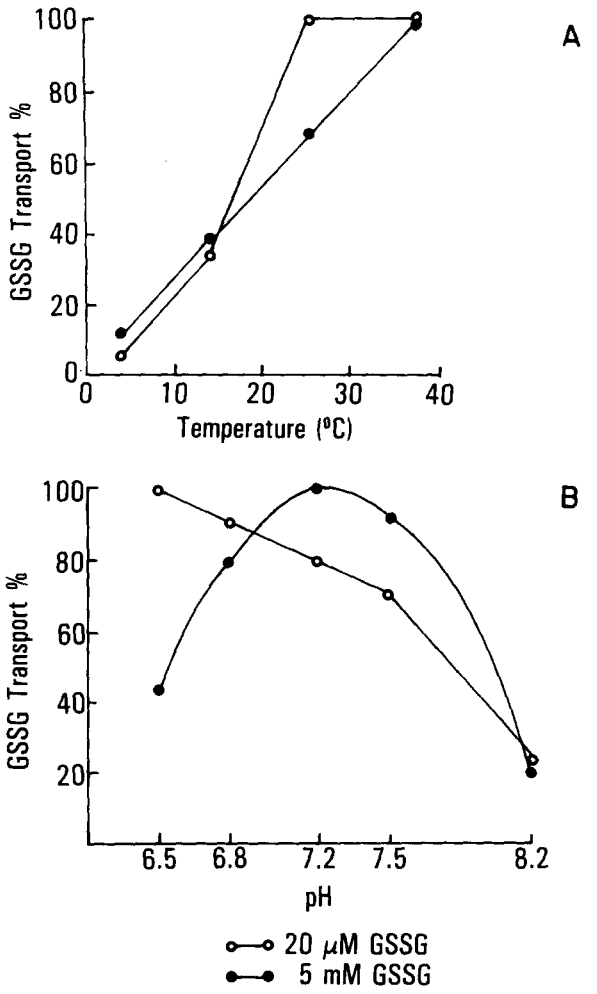

Fig. 2. A. Effect of temperature on GSSG transport. The incubation mixture was the same as for panel B, but the pH was fixed at pH 7.4. B. Effect of pH on GSSG transport. Vesicles were incubated with 5 mM or 20 μM GSSG. 2 mM ATP and 10 mM $MgCl_2$ in 250 μl phosphate-buffered saline at different pHs, for 2 h at 37°C.

at 20 μM (Table I). These results suggest that a sulfydryl group may be involved in the high K_m transport process.

Discussion

Glutathione transport out of erythrocytes is the sole process by which these cells can turn over glutathione; the γ-glutamyl cycle [15] which may play a role in glutathione catabolism in some tissues does not appear to operate in red blood cells [16–18]. Glutathione transport, which requires ATP, has been difficult to study in whole red cells or resealed erythrocytes due to the technical problems of manipulating the internal environment of these cells. However, the introduction of inside-out vesicles prepared from erythrocytes as a means of studying glutathione transport has facilitated the analysis of this transport process. We have previously reported that a Lineweaver-Burk analysis of the transport rate as a function of GSSG concentration shows a biphasic plot [6]. One interpretation of this kinetic plot is that two different transport processes are functioning. We have now further characterized GSSG transport by selecting two concentrations of GSSG which fall into different regions of this biphasic plot and have examined the properties of the transport processes represented by these two extreme GSSG concentrations.

The high K_m transport process, as represented by a GSSG concentration of 5 mM, as well as the low K_m process, as represented by 20 μM GSSG, both require a nucleoside triphosphate. A dramatic difference in the nucleotide requirement for these two transport processes is demonstrated when GTP is substituted for ATP in the transport. At 5 mM GSSG, GTP substitutes with little effect on the transport rate. However, at 20 μM GSSG GTP causes transport to occur at a rate 3.4 times as great as is observed with ATP.

A further difference in the two transport processes is the pH profile. At 5 mM GSSG the pH optimum is 7.2 while at 20 μM GSSG the optimum is more acidic. In addition, the transport system active at 20 μM GSSG seems to be considerably more labile than that at 5 mM GSSG. Over a 5-day storage period at 4°C transport with 5 mM GSSG was stable; however, the 20 μM GSSG transport process lost 62% of its activity during this same storage. To investigate the possibility that the low K_m transport process was more labile, in vivo, and to explore the idea that perhaps the high K_m systems represented a degraded form of the low K_m system, erythrocytes were fractionated as a function of age and inside-out vesicles were prepared from young cells and old cells. As shown in Table I, the specific activity for 5 mM GSSG transport was the same in the young cells as in the old cells; the transport process as represented by 20 μM GSSG was considerably decreased in the older cell population. Since the loss of capability to trans-

port at 20μM GSSG was not associated with increase of the transport rate at 5 mM GSSG, it seems unlikely that the 5 mM transport system is a degradation product of the low K_m system.

A difference was also noted between the susceptibility of these two transport processes to thiols or thiol reagents. Preincubation of the inside-out vesicles with 1 mM dithiothreitol caused a 70% stimulation of the transport rate at 5 mM GSSG while incubation of the inside-out vesicles with thiol reagents such as *p*-chloromercuribenzoate or iodoacetamide caused inactivation up to 50% of the transport rate. However, with 20 μM GSSG neither thiols or thiol reagents had any appreciable effect on the transport activity.

These data support rather strongly the possibility that two separate GSSG transport processes exist in the human erythrocyte. While we have no direct evidence concerning the role of these two different processes, it seems clear that only the transport rate with the low K_m value would be active under physiological concentrations of GSSG, which are approx. 4 μM [19] and this transport may be responsible for the turnover of glutathione observed under physiological conditions. On the other hand, the GSSG transport process with the high K_m value may not function under normal conditions but may be an emergency mechanism for the elimination of very high levels of GSSG which might develop during oxidative stress of the erythrocyte. The deleterious effects of high GSSG concentrations are well documented and the need for an emergency transport process with a high velocity rate is quite feasible.

Acknowledgments

This work was supported by Grant HL 07449 and HL 25552 from the National Institutes of Health.

References

1 Dimant, E., Landberg, E. and London, I.M. (1955) J. Biol. Chem. 213, 769–776
2 Srivastava, S.K. and Beutler, E. (1969) J. Biol. Chem. 244, 9–16.
3 Beutler, E. and Srivastava, S.K. (1968) in Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes (Deutsch, E., Gerlach, E. and Moser, K., eds.), pp. 91–95, Georg Thieme, Stuttgart
4 Srivastava, S.K. and Beutler, E. (1967) Biochem. Biophys. Res. Commun. 28, 659–664
5 Lunn, G., Dale, G.L. and Beutler, E. (1979) Blood 54, 238–244
6 Kondo, T., Dale, G.L. and Beutler, E. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6359–6362
7 Prchal, J., Srivastava, S.K. and Beutler, E. (1975) Blood 46, 111–117
8 Beutler, E., West, C. and Blume, K.G. (1976) J. Lab. Clin. Med. 88, 328–333
9 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
10 Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255–257
11 Kondo, T., Dale, G.L. and Beutler, E. (1980) Biochim. Biophys. Acta 602, 127–130
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Srivastava, S.K. and Beutler, E. (1968) Anal. Biochem. 25, 70–76
14 Murphy, J.R. (1973) J. Lab. Clin. Med. 82, 334–341
15 Meister, A. (1973) Science 180, 33–39
16 Board, P.G. and Smith, J.E. (1977) Blood 49, 667–668
17 Srivastava, S.K., Awasthi, Y.C., Miller, S.P., Yoshida, A. and Beutler, E. (1976) Blood 47, 645–650
18 Young, J.D., Ellory, J.C. and Wright, P.C. (1975) Biochem. J. 152, 713–715
19 Beutler, E. (1975) A Manual of Biochemical Methods, 2nd edn., Grune and Stratton, New York