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Involvement of thiol groups in the function of the dipeptide/proton cotransport system in rabbit renal brush-border membrane vesicles

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The role of thiol groups in H^+-gradient-dependent dipeptide transport in rabbit renal brush-border membrane vesicles was investigated using glycylsarcosine as the substrate. Treatment of the membrane vesicles with a thiol-group-reducing agent, dimercaptopropanol, stimulated Gly-Sar transport. On the other hand, treatment with thiol group oxidants such as 5,5′-dithiobis(2-nitrobenzoic acid), plumbagin and phenazine methosulfate inhibited Gly-Sar transport. These effects were irreversible, because washing the membranes after treatment failed to reverse the effects. Incubation of the membrane vesicles with phenylarsine oxide, a reagent which interacts specifically with vicinal dithiols, significantly inhibited Gly-Sar transport. In all cases, the stimulation or the inhibition of the dipeptide transport was primarily due to changes in the maximal velocity of the transport system, the apparent affinity constant remaining unaltered. These results demonstrate the involvement of one or more vicinal dithiol groups in the function of the renal dipeptide transport system and that these thiol groups must exist in reduced form to maintain maximal transport activity. In addition, these data indirectly suggest that a dithiol–disulfide interchange may play a role in the function of the renal dipeptide transport system.

Introduction

Solute transport systems which are energized by a protonmotive force ($\Delta\mu H^+$) are common in microorganisms [1]. In many of these transport systems, thiol groups have been shown to play an essential role in the function of the transport protein [2–7]. Considerable evidence has accumulated in recent years that the transport of small peptides in the mammalian small intestine and kidney belongs to the category of $\Delta\mu H^+$-driven solute transport [8–12]. The transport system responsible for the peptide/H^+ cotransport in the intestine and kidney is unique because a majority of solute transport systems in these tissues are energized by a sodium motive force ($\Delta\mu Na^+$) rather than a protonmotive force ($\Delta\mu H^+$). The molecular mechanisms involved in the intestinal and renal peptide transport are currently being worked out. Peptide transport is electrogenic in nature [13–19] and the peptide:H^+ stoichiometry has been determined to be 1:2 [20]. Histidyl and thiol groups have been shown to be essential for the activity of the transport protein and these groups are located at or near the active site [21].

The present study was designed to investigate in greater detail the involvement of the essential thiol groups in the function of the peptide/H^+ cotransport system in rabbit renal brush-border membrane vesicles. The data presented here demonstrate the involvement of one or more vicinal dithiol groups in the function of the renal dipeptide transport system and that these thiol groups must exist in reduced form to maintain maximal transport activity.

Methods and Materials

Methods

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles from rabbit renal cortex were prepared by Mg^{2+}-precipitation in the pres-

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N′*-tetraacetic acid.

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ence of EGTA [16,17]. Cortical tissue (20 g) was homogenized in a Waring blender for 4 min in 100 ml 12 mM Tris-NaOH buffer (pH 7.5)/5 mM EGTA/300 mM mannitol. The homogenate was mixed with 120 ml of ice-cold deionized water and a 1 M solution of $MgCl_2$ was added to the homogenate so as to give a final concentration of 10 mM $MgCl_2$. The mixture was stirred for 1 min and left for 15 min at 4°C. It was then centrifuged at $4000 \times g$ for 10 min. The supernatant was centrifuged at $42000 \times g$ for 30 min. The pellets containing brush-border membranes were washed twice with the preloading buffer by dilution and centrifugation. The composition of the preloading buffer varied depending upon the individual experiment. Protein concentration of the final membrane suspension was adjusted to 10 mg/ml and stored in aliquots in liquid nitrogen until use.

Uptake measurements. Uptake measurements were made at room temperature (21–22°C), as described earlier [22], using a rapid filtration technique. Millipore membrane filters (type DAWP; pore size, 0.65 μm) were used for this purpose. Uptake of Gly-Sar into the membrane vesicles was measured in the absence of Na^+, but in the presence of an inward-directed H^+ gradient ($pH_i = 8.4$; $pH_o = 6.7$). In these experiments, the membrane vesicles were suspended in 50 mM Hepes/75 mM Tris buffer (pH 8.4)/100 mM K_2SO_4. Uptake was measured using a buffer whose composition was 50 mM Mes/50 mM Hepes/25 mM Tris/300 mM mannitol (pH 5.8). Uptake was initiated by mixing 40 μl of the membrane suspension with 160 μl of uptake buffer containing radiolabelled Gly-Sar. Under these conditions the extravesicular pH was 6.7. Uptake was terminated by adding 3 ml of ice-cold stop buffer (1 mM Hepes-Tris/210 mM KCl (pH 7.5)) followed by filtration under vacuum. The filter was washed three times with 5 ml of the stop buffer and then transferred to a counting vial. The radioactivity associated with the filter was counted in a liquid scintillation spectrometer.

Treatment of membrane vesicles with reagents

2,3-Dimercaptopropanol and DTNB. Brush-border membrane vesicles preloaded with 50 mM Hepes/75 mM Tris buffer (pH 8.4)/100 mM K_2SO_4 were treated with 2,3-dimercaptopropanol or DTNB at 37°C for 30 min. Stock solutions of 2,3-dimercaptopropanol and DTNB were made in the same buffer. Protein concentration during this treatment was 9 mg/ml. The treated membrane vesicles were used in uptake experiments either dir[illegible]ly (without washing the excess reagent) or after w[illegible]hing two times to remove the unreacted, excess reagent.

Plumbagin and phenazine methosulfate. Brush-border membrane vesicles preloaded with 50 mM Hepes/75 mM Tris buffer (pH 8.4) containing 100 mM K_2SO_4 were treated with 1 mM plumbagin or 1 mM phenazine methosulfate at 37°C for 30 min. Stock solutions of these reagents were made in ethanol. The concentration of ethanol during the treatment was 2.5%. Control membrane vesicles were treated in a similar way with an equal concentration of ethanol alone. The treated membrane vesicles were used in uptake experiments either directly (without washing the excess reagent) or after washing two times to remove the unreacted, excess reagent.

Phenylarsine oxide. Brush-border membrane vesicles preloaded with 50 mM Hepes/75 mM Tris buffer (pH 8.4) containing 100 mM K_2SO_4 were treated with phenylarsine oxide in the same buffer for 20 min at 37°C. Stock solutions of phenylarsine oxide were made in ethanol. The concentration of ethanol during the treatment was 2%. Control membrane vesicles were treated in a similar way with an equal concentration of ethanol alone. The treated membrane vesicles were washed twice with the same pH 8.4 buffer to remove the unreacted reagent and then used in uptake experiments.

Reversal of inhibition. In the experiments where the reversibility of the inhibition caused by plumbagin or phenylarsine oxide was investigated, the membrane vesicles were first treated with the reagent (1 mM plumbagin or 0.5 mM phenylarsine oxide) at 37°C and the excess, unreacted reagent was removed by washing once with the pH 8.4 buffer following the treatment. These membrane vesicles were again incubated with 10 mM 2,3-dimercaptopropanol in the same buffer at 37°C for 30 min. After the incubation, the vesicles were washed twice to remove the unreacted reagent and then used in uptake experiments.

Statistics. Uptake measurements were routinely done in duplicate or triplicate and the variation among the replicate values was always less than ±10% of the mean value. Most of the experiments were done with two or three different membrane preparations. Statistical analysis was performed with Student's *t* test and a *P* value of less than 0.05 was considered significant.

Materials

Glycylsarcosine was purchased from Bachem, and phenylarsine oxide and plumbagin were purchased from Aldrich. Valinomycin, 2,3-dimercaptopropanol, 5,5′-dithiobis(2-nitrobenzoic acid) and phenazine methosulfate were from Sigma. All other chemicals were of analytical grade.

[*glycyl*-U-^{14}C]Glycylsarcosine (specific radioactivity, 100 mCi/mmol) was obtained from Amersham International, U.K. [^{14}C]Formate (spec. act., 55 mCi/mmol) was obtained from DuPont-New England Nuclear, Boston, MA, U.S.A.

Results and Discussion

We have recently demonstrated that thiol groups are essential for the activity of the rabbit renal dipeptide

transporter [21]. In the present study, we have probed the nature of these thiol groups and their exact role in the function of the transport protein by using various thiol-group-reactive reagents. These reagents were chosen for their ability to oxidize or reduce thiol groups. First, the effects of 2,3-dimercaptopropanol, a thiol-group-reducing agent, and DTNB, a thiol-group-oxidizing agent, on the uptake of Gly-Sar in renal brush-border membrane vesicles were investigated. In these experiments, the membrane vesicles were treated with either 1 mM dimercaptopropanol or 1 mM DTNB at 37°C for 30 min. The treated membrane vesicles were then used directly in uptake experiments. The uptake of Gly-Sar (15 μM) was measured in these vesicles in the presence of an inward-directed H^+ gradient ($pH_i = 8.4$; $pH_o = 6.7$). Treatment of the membrane vesicles with dimercaptopropanol greatly enhanced Gly-Sar uptake. In contrast, treatment of the membrane vesicles with DTNB drastically reduced Gly-Sar uptake (Fig. 1). The dose–response of these effects was then studied over a concentration range of 0.2–1 mM of the reagents (Fig. 2). Stimulation of Gly-Sar uptake was noticeable even at the lowest concentration (0.2 mM) of dimercaptopropanol employed in the study. The uptake rate of Gly-Sar measured after 30 s incubation was almost double in membrane vesicles treated with 1 mM dimercaptopropanol, compared to control vesicles. On the other hand, treatment of membrane vesicles with 0.2 mM DTNB caused a 40% reduction in Gly-Sar uptake. The inhibition progressed with increasing concentrations of DTNB and the dipeptide uptake was almost totally abolished when the concentration of DTNB was above 0.5 mM.

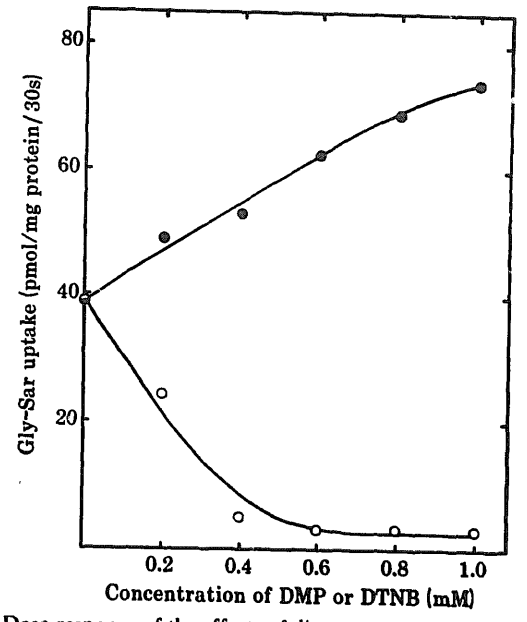

Fig. 2. Dose-response of the effects of dimercaptopropanol and DTNB on Gly-Sar uptake. Membrane vesicles were treated with varying concentrations of dimercaptopropanol (DMP) or DTNB as described in the legend to Fig. 1. Uptake of 15 μM Gly-Sar was measured in these vesicles with a 30 s incubation in the presence of an inward-directed H^+ gradient. ●, dimercaptopropanol; ○, DTNB.

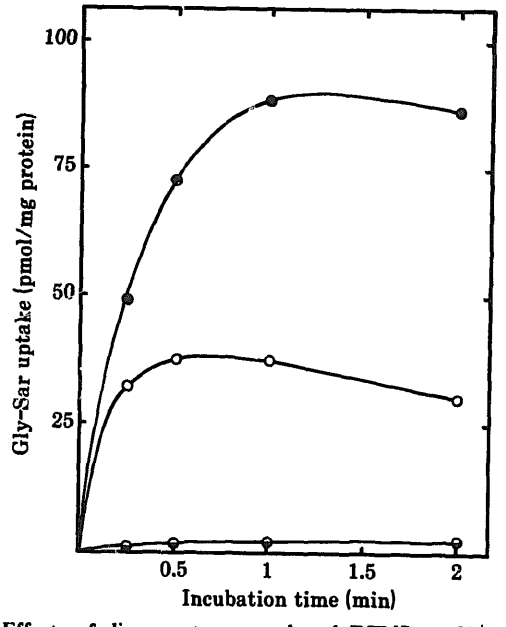

Fig. 1. Effects of dimercaptopropanol and DTNB on H^+ gradient-driven Gly-Sar uptake. Membrane vesicles were treated with 1 mM dimercaptopropanol or 1 mM DTNB at 37°C for 30 min in 50 mM Hepes/75 mM Tris buffer (pH 8.4) containing 100 mM K_2SO_4. The treated membrane vesicles were used directly in uptake measurements. Uptake of 15 μM Gly-Sar was measured in the presence of an inward-directed H^+ gradient ($pH_i = 8.4$; $pH_o = 6.7$). The uptake buffer contained 300 mM mannitol, buffered with 50 mM Hepes/50 mM Mes/25 mM Tris (pH 5.8). The extravesicular pH at the beginning of uptake measurement was 6.7, owing to the carry-over of the pH 8.4 buffer containing the membranes. ○, control; ●, dimercaptopropanol-treated; ⊖, DTNB-treated.

Fig. 3 shows the effects of dimercaptopropanol (1.0 mM) and DTNB (0.25 mM) on the kinetic parameters of H^+-gradient-dependent Gly-Sar uptake in brush-border membrane vesicles. Uptake rates of Gly-Sar were measured using a 15 s incubation over a Gly-Sar concentration range 0.04–0.40 mM in control vesicles and in vesicles which were treated with dimercaptopropanol or DTNB. The data given in Fig. 3 as Eadie-Hofstee plots (uptake rate/substrate concentration vs. uptake rate) show that the dimercaptopropanol-induced stimulation of Gly-Sar was primarily due to an increase in the maximal velocity. The apparent K_t value of the transporter for its substrate, Gly-Sar, was the same in control and treated vesicles (0.18 mM). Similarly, the DTNB-induced inhibition of Gly-Sar uptake was also due to a reduction in the maximal velocity, since the K_t remained unchanged in control and DTNB-treated vesicles.

It can be argued that the effects of dimercaptopropanol and DTNB on Gly-Sar uptake ob-

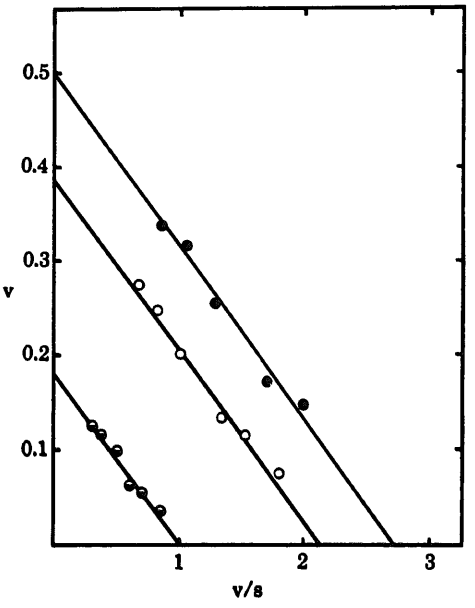

Fig. 3. Effects of dimercaptopropanol and DTNB on the kinetic parameters of Gly-Sar uptake. Uptake rates (15 s incubations) of Gly-Sar were measured in the presence of an inward-directed H^+ gradient over a Gly-Sar concentration range 0.04–0.40 mM in control vesicles and in vesicles which had been treated with 1 mM dimercaptopropanol or with 0.25 mM DTNB. The results are given as Eadie-Hofstee plots (v/s vs. v). v, nmol/mg protein per 15 s; s, Gly-Sar concentration (mM); ○, control; ●, dimercaptopropanol-treated; ⊖, DTNB-treated.

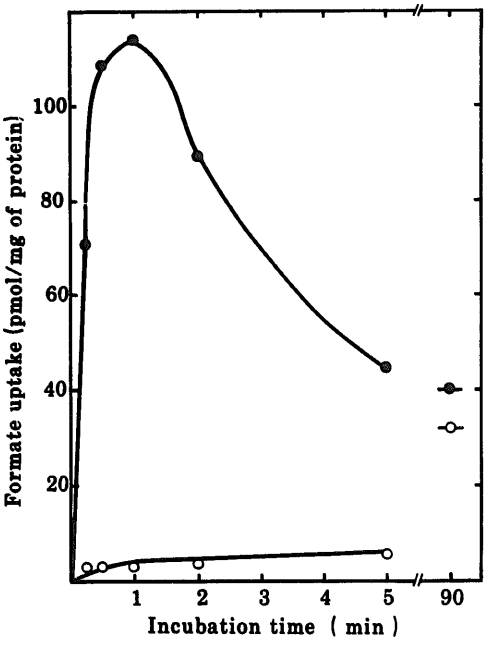

Fig. 4. Influence of an inward-directed H^+ gradient on formate uptake. Membrane vesicles were preloaded with either 50 mM Hepes/75 mM Tris buffer (pH 8.4) containing 100 mM K_2SO_4 or 40 mM Mes/40 mM Hepes/45 mM Tris buffer (pH 6.7)/100 mM K_2SO_4. Uptake was measured in either 50 mM Mes/50 mM Hepes/25 mM Tris buffer (pH 5.8)/300 mM mannitol or 40 mM Mes/40 mM Hepes/45 mM Tris buffer (pH 6.7)/300 mM mannitol. Uptake was initiated by mixing 40 μl of membrane suspension with 160 μl of uptake buffer containing radiolabelled formate. Final concentration of formate was 30 μM. ●, $pH_i = 8.4$; $pH_o = 6.7$; and ○, $pH_i = pH_o = 6.7$.

served in these experiments might be due to nonspecific alterations induced by these reagents on the properties and integrity of the membrane vesicles, rather than due to direct interaction with the dipeptide transport protein. For example, treatment of the membrane vesicles with these reagents might alter permeability of the vesicles to H^+ or induce changes in intravesicular volume, and these changes may lead to the observed effects on Gly-Sar uptake. In order to probe these possibilities, we investigated the uptake characteristics of another organic solute, formate, in control vesicles and in vesicles which have been treated with dimercaptopropanol. Formate uptake in renal cortical brush-border membrane vesicles occurs via nonionic diffusion [23], and therefore accumulation of formate within the vesicles against a concentration gradient can be demonstrated in the presence of an inward-directed H^+ gradient (Fig. 4). Initial uptake (15 s incubation) as well as equilibrium uptake (90 min incubation) of formate were measured in the presence of an H^+ gradient ($pH_i = 8.4$; $pH_o = 6.7$) in control and treated membrane vesicles. Any nonspecific change which occurs in the H^+ permeability of the membrane vesicles is expected to affect the H^+ gradient and hence alter the initial uptake of formate. Similarly, changes which occur in the intravesicular volume will be reflected in the equilibrium uptake. The data given in Fig. 5b show that neither the initial uptake nor the equilibrium uptake of formate was

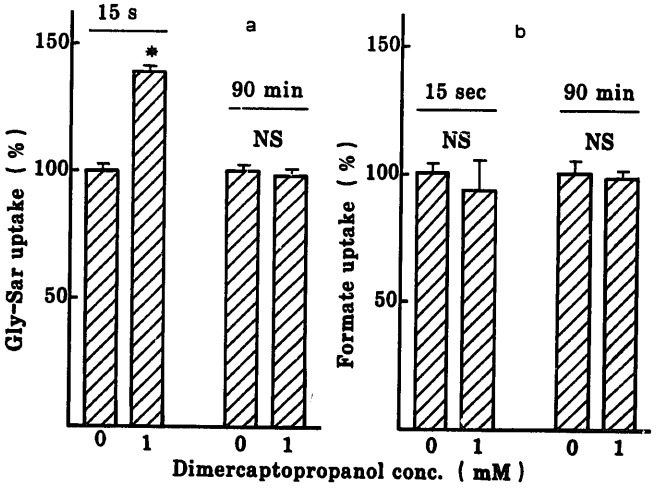

Fig. 5. Effects of dimercaptopropanol on initial uptake and equilibrium uptake of Gly-Sar (a) and formate (b). Membrane vesicles were treated with or without 1 mM dimercaptopropanol for 30 min at 37°C in 50 mM Hepes/75 mM Tris buffer (pH 8.4)/100 mM K_2SO_4. The treated membrane vesicles were directly used in uptake measurements. Uptake was measured in the presence of an inward-directed H^+ gradient ($pH_i = 8.4$; $pH_o = 6.7$). Initial uptake was measured with a 15 s incubation and equilibrium uptake was measured with a 90 min incubation. Final concentration of Gly-Sar and of formate was 20 μM. The data represent means ± S.D. for three determinations. *, $P < 0.001$; N.S., not significant.

affected by dimercaptopropanol. These results indicate that treatment of membrane with dimercaptopropanol does not alter the H^+ permeability of the membrane vesicles, nor does it change the intravesicular volume. However, when Gly-Sar uptake was measured in these vesicles, initial uptake was significantly greater in dimercaptopropanol-treated vesicles than in control vesicles (Fig. 5a). There was, however, no change in the equilibrium uptake, again indicating that the reagent does not affect the integrity of the vesicles.

Therefore, it appears that these reagents elicit their effects on Gly-Sar uptake by directly interacting with thiol groups of the transport protein. The stimulation by dimercaptopropanol and inhibition by DTNB suggest that these essential thiol groups must exist in the reduced from for the renal dipeptide transporter to remain active. Treatment of the membrane vesicles with dimercaptopropanol would convert oxidized thiol groups to the active reduced form, thereby increasing the activity of the transport protein. Similarly, treatment with DTNB would result in covalent modification of the essential thiol groups and would thus decrease the transport activity.

To provide additional evidence for the obligatory role of the reduced thiol groups in Gly-Sar uptake, the effects of two lipophilic oxidants, plumbagin and phenazine methosulfate, were studied. Treatment of the membrane vesicles with these reagents would result in oxidation of thiol groups in the transport protein. Fig. 6

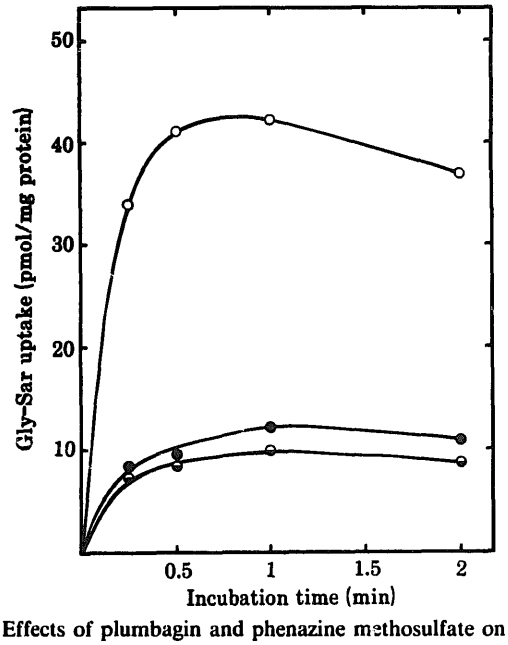

Fig. 6. Effects of plumbagin and phenazine methosulfate on Gly-Sar uptake. Membrane vesicles were treated with 1 mM plumbagin or 1 mM phenazine methosulfate at 37 °C for 30 min in 50 mM Hepes/75 mM Tris buffer (pH 8.4) containing 100 mM K_2SO_4. The treated vesicles were used directly in uptake measurements. Uptake of 15 μM Gly-Sar was measured in the presence of an inward-directed H^+ gradient. ○, control; ●, plumbagin-treated; ⊖, phenazine-methosulfate-treated.

TABLE I

Irreversibility of the effects of dimercaptopropanol, DTNB, plumbagin and phenazine methosulfate on Gly-Sar uptake

Membrane vesicles were treated with dimercaptopropanol (1 mM), DTNB (0.3 mM), plumbagin (1 mM) or phenazine methosulfate (1 mM) at 37 °C for 30 min in 50 mM Hepes/75 mM Tris buffer (pH 8.4)/100 mM K_2SO_4. Following the treatment, a portion of the membrane suspension was directly used (no washing) in Gly-Sar uptake measurements. The rest of the membrane suspension was washed twice with the treatment buffer before it was used in uptake measurements. Gly-Sar uptake was measured in membrane vesicles in the presence of an inward-directed H^+ gradient. The concentration of the dipeptide was 15 μM and the incubation time was 30 s. The results are given as the mean ± S.D. for three determinations.

Reagent	Gly-Sar uptake			
	without washing		with washing	
	pmol/mg protein per 30 s	%	pmol/mg protein per 30 s	%
None	32.5 ± 6.9	100	35.6 ± 1.6	100
Dimercapto-propanol	74.3 ± 0.4	229	67.4 ± 1.5	189
DTNB	13.8 ± 0.8	42	15.4 ± 4.0	43
Plumbagin	8.2 ± 3.5	25	10.0 ± 0.6	28
Phenazine methosulfate	5.9 ± 0.4	18	8.0 ± 1.1	22

shows that such a maneuver almost totally inactivated the Gly-Sar transport system, providing strong support for the concept that the transport protein is active if the thiol groups exist in the reduced state and inactive if they exist in the oxidized state.

If the effects of these reagents on Gly-Sar uptake were due to the changes in the redox state of the essential thiol groups, the alterations in the transport function induced by these reagents should be irreversible. This was investigated by measuring Gly-Sar uptake in membrane vesicles which had been washed free of the reagents following the treatment. The stimulatory effect of dimercaptopropanol as well as the inhibitory effects of DTNB, plumbagin and phenazine methosulfate were observed even in these washed vesicles (Table 1). Furthermore, the effects were quantitatively similar to those seen in membrane vesicles which had not been washed subsequent to the treatment with the reagents.

The results presented thus far clearly suggest that a redox-sensitive step involving thiol groups plays a crucial role in the function of the renal dipeptide transport system. A similar role for thiol groups in the activities of many H^+-gradient-dependent solute transport systems in *E. coli* has been demonstrated [3–6]. In these cases, it has been shown that the essential thiol groups are present as vicinal dithiols because these transport systems are sensitive to treatment with phenylarsine oxide [6]. This reagent specifically reacts with dithiols [24,25]. We have, however, shown in a previous publication [21] that treatment of rabbit renal brush-border

membrane vesicles with this reagent failed to inhibit dipeptide transport, thus apparently ruling out the possibility that the essential thiol groups in the transport protein exist as vicinal dithiols. In view of the strong evidence gathered in the present investigation in support of the participation of a redox-sensitive step involving thiol groups in dipeptide transport, we reinvestigated the effect of phenylsarsine oxide on Gly-Sar transport in rabbit renal brush-border membrane vesicles. Careful examination of various parameters, such as ionic composition of the treatment buffer, temperature and pH of the reaction mixture and the ratio between the concentrations of the reagent and the membrane protein during the treatment, revealed that, among these different parameters, the reaction was highly sensitive to the temperature. When the membrane vesicles were treated with phenylarsine oxide at room temperature (21–22°C), there was no effect on Gly-Sar uptake. These results are similar to those reported earlier [21]. However, if the treatment was done at 37°C, Gly-Sar uptake was significantly reduced by phenylarsine oxide (data not shown). Subsequent experiments to characterize the phenylarsine-oxide-induced inhibition of the renal dipeptide transporter were therefore done by incubating the membrane vesicles with the reagent at 37°C for 20 min. Fig. 7 describes the dose–response of phenylarsine-oxide-induced inhibition on the uptake of Gly-Sar in renal brush-border membrane vesicles. The uptake of the dipeptide was significantly inhibited at a concentration as low as 0.05 mM of the reagent and a 50% inhibition was observed at about 0.25 mM.

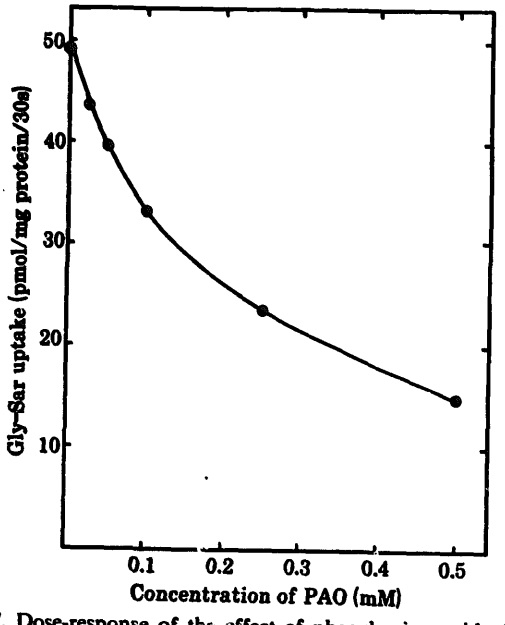

Fig. 7. Dose-response of the effect of phenylarsine oxide (PAO) on Gly-Sar uptake. Membrane vesicles were treated with varying concentrations of phenylarsine oxide at 37°C for 20 min at pH 8.4. Uptake of 15 μM Gly-Sar was measured with a 30 s incubation in the presence of an inward-directed H^+ gradient.

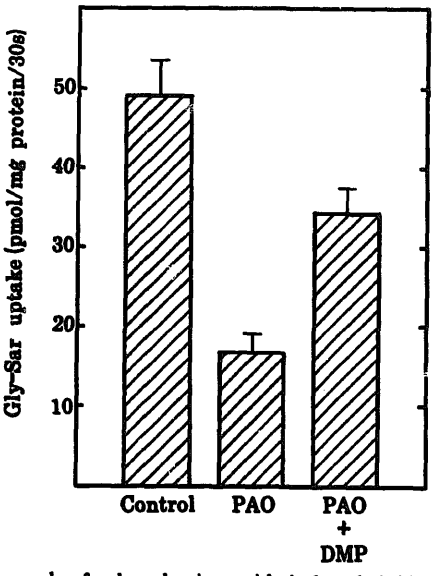

Fig. 8. Reversal of phenylarsine-oxide-induced inhibition by dimercaptopropanol. The experimental procedure for treatment with the reagents is given in the Methods and Materials section. Gly-Sar uptake in control and treated vesicles was measured in the presence of an inward-directed H^+ gradient with a 30 s incubation. Concentration of Gly-Sar was 15 μM. The data represents means ± S.D. for three determinations. PAO, phenylarsine oxide; DMP, 2,3-dimercaptopropanol.

Kinetic analysis of Gly-Sar uptake in control vesicles and in phenylarsine-oxide-treated vesicles revealed that the inhibition caused by the reagent was noncompetitive in nature because the treatment reduced velocity of the transport system, without having any significant effect on the apparent K_t value (data not shown).

The reversibility of the oxidant-induced inhibition by subsequent treatment with thiol-reducing agents such as dimerceptopropanol was then investigated. As shown in Fig. 8, dimercaptopropanol was able to reverse the phenylarsine-oxide-induced inhibition. Similarly, the inhibition caused by treatment with plumbagin, another thiol oxidant, was also found to be significantly reversed by dimercaptopropanol (data not shown).

Taken collectively, the data presented here demonstrate that one or more vicinal dithiol groups are essential for the function of the renal dipeptide transport system. They also show that the transport system is active if these thiol groups exist in the reduced form and inactive if they exist in the oxidized form.

The mode of involvement of thiol groups in the function of the $\Delta\mu H^+$-driven dipeptide transport system in renal brush-border membrane vesicles is similar to the participation of thiol groups in the function of a number of $\Delta\mu H^+$-driven solute transport systems in microorganisms [2–7]. One major difference, however, is that while oxidation of thiol groups in bacterial transport systems reduces their affinities for substrates without affecting maximal velocities, such a maneuver in the case of the renal dipeptide transport system

reduces the maximal velocity without affecting the substrate affinity. It has been hypothesized that the essential thiol groups undergo a dithiol–disulfide interconversion during the catalytic cycle of the bacterial transport systems [2,5], though a recent study has raised doubts on the validity of this hypothesis [7]. The essential role of the thiol groups in the renal dipeptide transport system, the sensitivity of the transport system to treatment with phenylarsine oxide and the effects of thiol-group-reducing and thiol-group-oxidizing agents on the function of the transport system indirectly suggest that a dithiol–disulfide interchange may play a catalytic and/or regulatory role in the function of the renal dipeptide transport system.

Acknowledgements

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