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CHARACTERIZATION OF AN ESSENTIAL DISULFIDE BOND ASSOCIATED WITH THE ACTIVE SITE OF THE RENAL BRUSH-BORDER MEMBRANE D-GLUCOSE TRANSPORTER

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In a previous report (J. Biol. Chem. 258 (1983) 3565–3570) we have demonstrated that the disulfide-reducing agent dithiothreitol has two effects on the sodium-dependent outer cortical brush border membrane D-glucose transporter; the first results in a reversible increase in the affinity of the transporter for the non-transported competitive inhibitor phlorizin, while the second results in a partially reversible loss of phlorizin binding and glucose-transport activity. Evidence was presented that both of these effects are the result of the reduction of disulfide bonds on the transport molecule. In the present paper we extend our observations on the inactivation of the transporter by dithiothreitol. We provide evidence here (i) that the inactivation of the transporter by dithiothreitol is independent of the effect of the reducing agent on the affinity of the transporter, (ii) that this inactivation process is first-order in dithiothreitol and thus presumably due to the reduction of a single disulfide bond essential to the functioning of the transporter. (iii) that it is the reduction of this disulfide bond and not some subsequent conformational or other change in the transporter which results in its inactivation, (iv) that phlorizin and substrates of the transporter provide protection against inactivation by dithiothreitol and that the degree of protection provided correlates well with the known specificity and phlorizin-binding properties of the transporter, and (iv) that the reactivity of the transporter with dithiothreitol is pH-dependent, decreasing with increasing pH over the pH range 6.5–8.5. We conclude that this site of action of dithiothreitol is a single essential disulfide bond intimately associated with the glucose-binding site on the transport molecule.

Introduction

D-Glucose is actively reabsorbed from the urine in the renal proximal tubule via secondary active cotransport with sodium across the brush border membrane [1]. Previous reports from this [2–4] and other [5] laboratories indicate that the bulk of the glucose load is reabsorbed by a low-affinity, high-capacity system identified in outer cortical brush border membrane vesicles (early proximal tubule). This carrier cotransports sodium and glucose in a 1 : 1 tightly coupled manner [3] and is competitively inhibited by phlorizin [4], a well-known non-transported inhibitor of renal D-glucose reabsorption [1,6]. Evidence from a number of studies indicates that glucose and phlorizin compete for the active site on this transporter in a 1 : 1 fashion and that the high-affinity sodium-dependent component of phlorizin binding typically observed in renal brush border membrane preparations represents the binding of phlorizin to this site [1,3,4,6–14].

In an earlier paper [15] we have demonstrated

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

that the disulfide-reducing agent dithiothreitol has two effects on the outer cortical brush-border membrane D-glucose transporter; the first results in an increase in phlorizin-binding affinity and the second in a loss of phlorizin-binding sites and glucose-transport activity. The former phenomenon is completely reversed and the latter partially reversed by washing the membranes free of dithiothreitol. Reversal of both effects could be prevented by including an excess of the sulfhydryl-alkylating agent *N*-ethylmaleimide in the washing solution. We have also shown that the magnitudes of both effects of dithiothreitol on phlorizin binding are influenced by the presence of substrates of the carrier during dithiothreitol treatment. More specifically, the increase in the affinity of phlorizin binding is further enhanced in the presence of substrate, while the rate of loss of binding sites is reduced. These and other results presented in Ref. 15 provide strong evidence that both the increase in phlorizin-binding affinity and the loss of phlorizin-binding sites observed with dithiothreitol treatment are the results of the reduction of disulfide bonds located on the D-glucose transporter.

In the present paper we extend our observations on the second of the two effects described above; namely, the inactivation of the transporter by dithiothreitol.

Methods and Materials

Vesicle preparation and characterization

Outer cortical brush border membrane vesicles were prepared from the kidneys of white New Zealand rabbits as previously described [2]. Relative to the starting tissue homogenate the activity of the brush border membrane marker maltase is enriched 10–12-times in this membrane vesicle preparation, while activities of $(Na^+ + K^+)$-ATPase (an antiluminal membrane marker), succinic dehydrogenase (a mitochondrial marker) and glucose-6-phosphatase (an endoplasmic reticulum marker) are reduced by factors of 5, 5 and 2.5, respectively [2]. Freshly prepared vesicles were suspended in buffer A (10 mM Tris-Hepes (10 mM Hepes buffered with Tris to pH 7.45) containing 100 mM mannitol) at a protein concentration of 10 mg/ml, fast-frozen in an ethanol/solid CO_2 slush in 300-μl aliquots and stored above liquid nitrogen. On the day of the experiment aliquots were thawed for 30 min at 37°C, diluted into appropriate media for the experiment, reincubated at 37°C for 30 min and stored on ice until use.

Phlorizin-binding measurements

(a) Rate of inactivation of phlorizin binding. Unless otherwise noted the rate of inactivation of phlorizin binding by dithiothreitol was measured as follows. Vesicles (1–2 mg protein/ml) were preincubated in an appropriate buffer (see figure captions) at 37°C. At time zero, 50 μl of a dithiothreitol stock solution were added to 950 μl of vesicles. At the times indicated on the figures 50-μl aliquots of this mixture were removed and added to 100 μl of prewarmed (37°C) buffer N (buffer A plus 60 mM NaCl) containing [^{3}H]phlorizin (typically 0.1–0.25 μM). 3 min later these samples were diluted 10-fold with ice-cold stop solution (10 mM Tris-Hepes plus 300 mM NaCl, 200 mM mannitol and 1 mM phlorizin) and immediately applied to a Millipore filter (HAWP 0.45 μ) under light suction. The filter, which retained the membrane vesicles, was then washed by a further 4.2 ml of stop solution, dissolved in scintillation fluid and counted along with samples of the incubation medium and appropriate standards.

(b) Equilibrium phlorizin binding. The procedure for equilibrium phlorizin binding was as previously described [3,14]. Briefly, aliquots of vesicles (50–100 μl, 1–2 mg protein/ml) were combined with 100 μl of incubation medium containing [^{3}H]phlorizin and other additions as required. After suitable incubation at 37°C ($\geq$ 3 min) samples were diluted in stop solution and filtered as described above.

Corrections for nonspecific (sodium-independent) phlorizin binding were carried out as previously described [15]. The sodium-dependent component of binding is illustrated in all cases. Results of representative experiments are shown.

Protein was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin as the standard.

Criteria of purity

The purity of [^{3}H]phlorizin (> 97%) was regularly verified by thin-layer chromatography using

the solvent system chloroform/methanol/water (65/25/4, v/v).

Calculations

Linear fits to the experimental data were obtained by the method of least squares. Non-linear least-squares fits were carried out using the program P3R (BMD, Biomedical Computer Programs, University of California, Los Angeles). The errors quoted and illustrated on experimental and least-squares parameters are standard deviations. Student's *t*-test was used in the statistical evaluation of data and *P* values below 0.05 were taken to indicate statistically significant differences.

Materials

Radiolabeled compounds were from New England Nuclear Corp (Boston, MA). Dithiothreitol was from Bethesda Research Laboratories (Gaithersburg, MD). Choline chloride was obtained from Eastman Kodak Co. (Rochester, NY) and was recrystalized from ethanol before use. All monosaccharides were from Sigma Chemical Co. (St. Louis, MO). Phloretin was from K&K Laboratories (Plainview, NY). Other chemicals were from standard commercial sources.

Results

Time course of the two effects of dithiothreitol on the outer cortical brush border membrane D-glucose transporter

In the experiment shown in Fig. 1 membrane vesicles were incubated in the presence of 3.33 mM dithiothreitol for 0, 5, 10, 15, 20 or 25 min, then diluted and washed in the presence of excess *N*-ethylmaleimide. Scatchard plots of the sodium-dependent component of phlorizin binding after the various periods of incubation in dithiothreitol are illustrated in Fig. 1a. The time course of the two effects of dithiothreitol on phlorizin binding to the glucose transporter can be seen directly from these data. The increase in affinity (decrease in K_d) is essentially complete within 10 min ($T_{1/2} \approx 5$ min). A progressive decrease in the number of phlorizin-binding sites (N_0) with time is also readily apparent from Fig. 1a. A semilogarithmic plot of N_0 vs. time is shown in Fig. 1b. This figure demonstrates that the number of binding sites falls

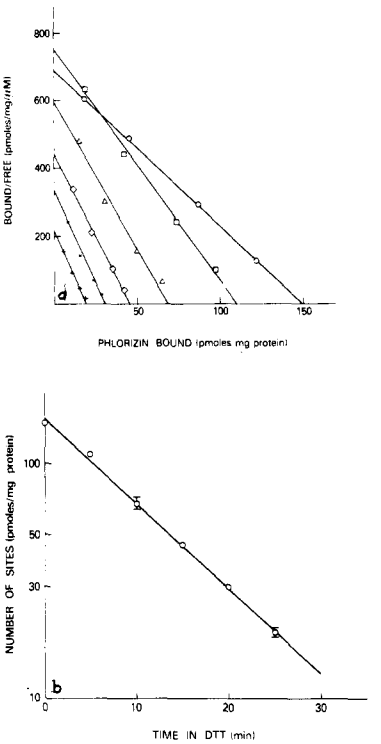

Fig. 1. (a) Scatchard analysis of sodium-dependent phlorizin binding to membrane vesicle incubated for various times in the presence of 3.33 mM dithiothreitol and washed in excess *N*-ethylmaleimide. Vesicles were incubated at 37°C in buffer N containing 3.33 mM dithiothreitol for the times indicated, then diluted 21-fold into cold buffer N containing 0.5 mM *N*-ethylmaleimide. The vesicles were then pelleted (20 min, 43000 × *g*), resuspended in buffer A, repelleted and taken up in buffer A for the binding studies. Phlorizin binding was measured in buffer A containing 60 mM NaCl or 60 mM choline chloride. The sodium-dependent component of binding was calculated by subtraction. Least-squares fits to the data yield: zero time, $K_d = 0.217 \pm 0.004$ μM, $N_0 = 150.0 \pm 1.5$ pmol/mg protein, $r = 0.999$ (○); 5 min, $K_d = 0.147 \pm 0.007$ μM, $N_0 = 110.0 \pm 2.8$ pmol/mg protein, $r = 0.998$ (□); 10 min $K_d = 0.115 \pm 0.012$ μM, $N_0 = 68.0 \pm 3.8$ pmol/mg protein, $r = 0.990$ (△); 15 min, $K_d = 0.103 \pm 0.005$ μM, $N_0 = 45.2 \pm 1.2$ pmol/mg protein, $r = 0.997$ (◇); 20 min, $K_d = 0.090 \pm 0.006$ μM, $N_0 = 29.8 \pm 1.1$ pmol/mg protein, $r = 0.996$ (×); 25 min, $K_d = 0.088 \pm 0.007$ μM, $N_0 = 19.1 \pm 0.8$ pmoles/mg protein, $r = 0.993$ (+). (b) A semilogarithmic plot of number of binding sites (N_0) from (a) vs. time of incubation in dithiothreitol (DTT). The least-squares fit to the data yields $T_{1/2} = 8.31 \pm 0.15$ min, $r = 0.999$.

off exponentially with time over the entire incubation period as expected for a single independent inactivation process beginning at time zero. The $T_{1/2}$ for this process is 8.31 ± 0.15 min (see figure

caption). ($T_{1/2}$ values for inactivation of phlorizin-binding sites by 3.33 mM dithiothreitol typically range from 8–10 min from experiment to experiment (see below and Ref. 15). Values from duplicate runs in the same experiment are reproducible to within 5%.)

Owing to the simultaneous occurrence of the two effects illustrated above, the action of dithiothreitol on the glucose transporter over short time periods ($\ll 10$ min) is relatively complex. However, for times of 10 min or more the change in K_d produced by dithiothreitol is essentially complete and the effect of dithiothreitol on phlorizin binding is due only to a loss of binding sites. Over these longer time intervals a semilogarithmic plot of binding at a single phlorizin concentration vs. time is expected to be linear and thus to give a reliable estimate of the rate of inactivation of binding sites. That this is indeed the case is illustrated in Fig. 2. Here the rate of inactivation of phlorizin binding by dithiothreitol is measured by two methods. In the first (open symbols) aliquots of vesicles incubating in dithiothreitol were diluted into excess *N*-ethylmaleimide to terminate the reaction with the reducing agent, then equilibrium phlorizin binding was measured by the usual procedure (see figure caption and Methods). This method is analogous to the one used to obtain the data in Fig. 1b. In the second method (solid symbols) phlorizin binding was measured as described in Methods (Rate of inactivation of phlorizin binding) without the intermediate treatment with *N*-ethylmaleimide. Two dithiothreitol concentrations were studied (2.0 and 5.0 mM). Both methods yield linear semilogarithmic plots with identical $T_{1/2}$ values at each phlorizin concentration (see figure caption). Owing to its simplicity the latter method has been employed in the experiments which follow.

As discussed later in the paper, the fact that the two methods used in Fig. 2 yield identical results also has important implications regarding the mechanism of inactivation of the transporter by dithiothreitol.

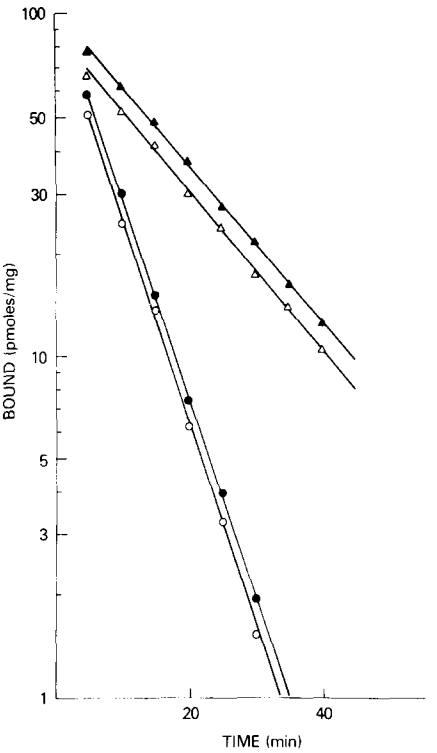

Fig. 2. Inactivation curves for phlorizin binding in the presence of dithiothreitol. The experiment was carried out in buffer N. The points represented by the open symbols were obtained by diluting 50-μl aliquots of vesicles incubating (37°C) in 5.0 mM or 2.0 mM dithiothreitol into 50 μl-aliquots of buffer N containing 5.5 mM *N*-ethylmaleimide (5.0 mM dithiothretiol) (circles) or 2.5 mM *N*-ethylmaleimide (2.0 mM dithiothreitol) (triangles) at the times indicated, leaving the samples on ice for 50 min then measuring equilibrium phlorizin biniding at 37°C ([phlorizin] = 0.15 μM). The points represented by the closed symbols were obtained simultaneously, without the intermediate treatment with *N*-ethylmaleimide, using the procedure for measuring the 'rate of inactivation of phlorizin binding' described in Methods ([phlorizin] = 0.2 μM). Least-squares fits to the data yield $T_{1/2}$ values of 13.1 ± 0.2 (▲), 13.0 ± 0.2 (△), 5.09 ± 0.04 (●) and 4.97 ± 0.08 (○) min with $r \geqq 0.999$ in each case.

Protection of the transporter by phlorizin

The experiment shown in Fig. 3 illustrates the effect of the presence of phlorizin on inactivation of the glucose transporter by dithiothreitol. Here we have preequilibrated membrane vesicles with various concentrations of [^{3}H]phlorizin then monitored phlorizin binding as a function of time after the addition of 3.33 mM dithiothreitol. As illustrated in Fig. 3a, the rate of inactivation of phlorizin binding decreases with increasing phlorizin concentration (note that only three of the five phlorizin concentrations studied in the experiment are illustrated in Fig. 3a).

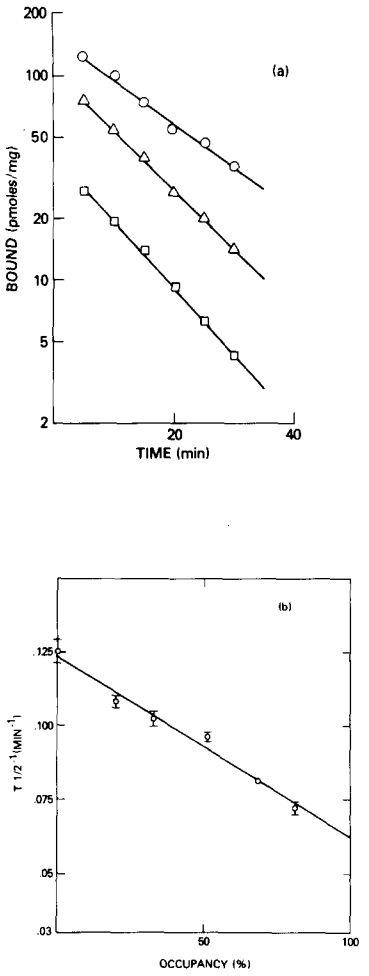

Fig. 3. Effect of phlorizin on the rate of inactivation of the transporter by dithiothreitol. (a) Vesicles were preincubated at 37°C in buffer A containing various concentrations of [^{3}H]phlorizin and either 60 mM NaCl or 60 mM chloline chloride. At time zero 50 μl of a dithiothreitol stock solution in the same buffer were added to 950 μl of vesicles to give a final dithiothreitol concentration of 3.33 mM. At the times indicated 50 μl of this mixture were removed, added to 1.4 ml cold stop solution and assayed for phlorizin binding by Millipore filtration (see Methods). The sodium-dependent component of binding was determined by subtraction. The plots shown are for phlorizin concentrations of 0.7 μM (○), 0.17 μM (△), and 0.04 μM (□). Runs were also carried out at 0.35 and 0.08 μM (see (b)). (b) $T_{1/2}$ values for all of the phlorizin concentrations studied in the experiment shown in (a) were calculated by the method of least squares and replotted as shown. Occupancy of the transporter was calculated using a K_{prot} value of 0.15 μM (see text). The $T_{1/2}$ value for zero occupancy ([phlorizin] = 0) was determined as described in Methods (Rate of inactivation of phlorizin binding). A linear least-squares fit to Fig. 3b yields $T_{1/2} = 16.0 \pm 1.5$ min at 100% occupancy.

It can be shown that, if the protection of the transporter provided by phlorizin arises as a result of phlorizin binding at a site with half-saturation constant K_{prot}, then $T_{1/2}$ will be related to the occupancy of this site (the ratio of occupied to total binding sites) by the equation

$$\frac{1}{T_{1/2}} = \text{occupancy}\left(\frac{1}{T^{o}_{1/2}} - \frac{1}{T^{u}_{1/2}}\right) + \frac{1}{T^{u}_{1/2}} \quad (1)$$

where

$$\text{occupancy} = [P]/(K_{prot} + [P]),$$

and $T^{o}_{1/2}$ and $T^{u}_{1/2}$ are the half-times for inactivation of the occupied and unoccupied transporters, respectively. A non-linear least-squares fit of the $T_{1/2}$ values from the experiment shown in Fig. 3 to the above equations yields $T^{o}_{1/2} = 18 \pm 3$ min, $T^{u}_{1/2} = 8.3 \pm 0.3$ min and $K_{prot} = 0.23 \pm 0.09$ μM. In previous experiments we have demonstrated that the K_d for sodium-dependent phlorizin binding measured under the same experimental conditions as employed in Fig. 3 (viz., 60 mM NaCl, 3.33 mM dithiothreitol and 37°C; see Ref. 15, Fig. 2) is 0.15 μM. Thus there is good agreement between the K_{prot} of the hypothetical protective site and the K_d of phlorizin binding to the active site of the glucose transporter.

Eqn. 1 above predicts that $1/T_{1/2}$ should be a linear function of the occupancy of the protective site. This prediction is tested and confirmed in Fig. 3b using $K_{prot} = 0.15$ μM.

Protection of the transporter by substrates

The ability of various glucose analoques to protect the transporter against inactivation by dithiothreitol is illustrated in Table I. The protection by monosaccharides closely parallels the known specificity properties of the transporter [2]; i.e., in order of decreasing affinity, methyl β-glucoside, D-glucose, methyl α-glucoside and galactose share the transporter, while mannose and fructose do not. Table I also demonstrates that the protection provided by substrates is concentration-dependent (see data for D-glucose).

In addition, Table I illustrates that sodium and phloretin, the aglycone of phlorizin, increase the rate of inactivation of the transporter by dithioth-

TABLE I

EFFECT OF MONOSACCHARIDES, PHLORETIN AND SODIUM ON THE RATE OF INACTIVATION OF THE OUTER CORTICAL BRUSH BORDER MEMBRANE VESICLE D-GLUCOSE TRANSPORTER BY DITHIOTHREITOL

The experiment was carried out in buffer N containing 3.33 mM dithiothreitol as described in Methods ([phlorizin] = 0.1 μM). In A an additional 25 mM mannitol was present and the various compounds tested replaced mannitol isoosmotically. In B NaCl was replaced isoosmotically by choline chloride during dithiothreitol treatment.

A. Substrate tested	$T_{1/2}$ (min)
25 mM mannitol (control)	8.01 ± 0.05
5 mM D-glucose	9.59 ± 0.21
10 mM D-glucose	11.42 ± 0.16
25 mM D-glucose	17.03 ± 0.25
25 mM α-methyl-D-glucoside	15.46 ± 0.16
25 mM β-methyl-D-glucoside	21.97 ± 0.55
25 mM D-galactose	8.72 ± 0.13
25 mM mannose	8.19 ± 0.11
25 mM fructose	7.99 ± 0.21
100 μM phloretin	7.03 ± 0.09
B. [NaCl]	**$T_{1/2}$ (min)**
60 mM	8.31 ± 0.10
30 mM	8.44 ± 0.14
15 mM	8.66 ± 0.15
0 mM	9.82 ± 0.11

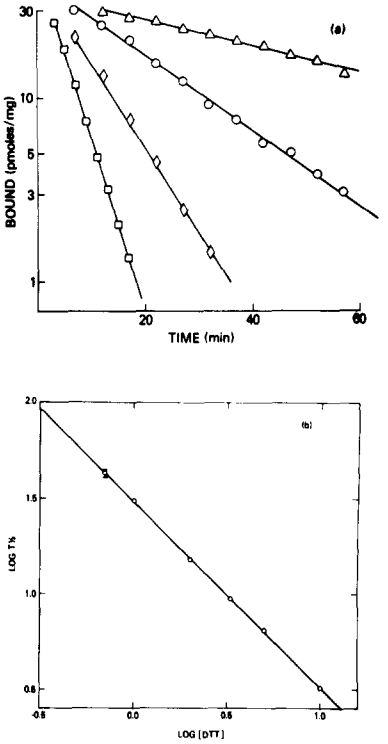

Fig. 4 (a) Rate of inactivation of the transporter at various dithiothreitol (DTT) concentrations. The experiment was carried out in buffer N as described in Methods. The dithiothreitol concentrations illustrated are 10 mM (□), 5 min (◇), 2 mM (○) and 0.7 mM (△). Runs were also carried out at 3.33 mM and 1.0 mM dithiothreitol ([phlorizin] = 0.1 μM). (b) Order of reaction of dithiothreitol with the transporter. $T_{1/2}$ values for all dithiothreitol concentrations studied in the experiment illustrated in (a) were calculated by the method of least squares and replotted as shown. A least-squares fit to Fig. 4b yields $n = 0.97 \pm 0.01$ for the order of the reaction with respect to dithiothreitol.

reitol. The effect of increasing the concentration of sodium is, however, not large.

The order of the reaction of dithiothreitol with the transporter

In the experiment presented in Fig. 4 we have measured the rate of inactivation of phlorizin binding at various dithiothreitol concentrations over the range 0.7 to 10 mM. A selection of the inactivation curves is illustrated in Fig. 4a. The $T_{1/2}$ values obtained from this experiment have been replotted in Fig. 4b to determine the order of the reaction of the transporter with dithiothreitol. This plot of log $T_{1/2}$ vs. log[dithiothreitol] is linear with a slope very close to one (see figure caption), demonstrating that the inactivation reaction is first-order with respect to dithiothreitol.

pH dependence of the effect of dithiothreitol

Fig. 5 illustrates the pH dependence of the effect of dithiothreitol on inactivation of phlorizin-binding sites. The results have been plotted in two ways; the raw data (open symbols) illustrate the actual $T_{1/2}$ values obtained at the various pH values tested, while the corrected data (solid symbols) take into account the fact that only the anionic form of dithiothreitol (the thiolate ion) is active in the reduction of disulfide bonds. This correction is made using the following equation [16]

$$T_{1/2}^{corr} = T_{1/2}\left(1 + 10^{pK_a^{DTT} - pH}\right)^{-1}$$

where $T_{1/2}$ is the observed half-time, $T_{1/2}^{corr}$ is the

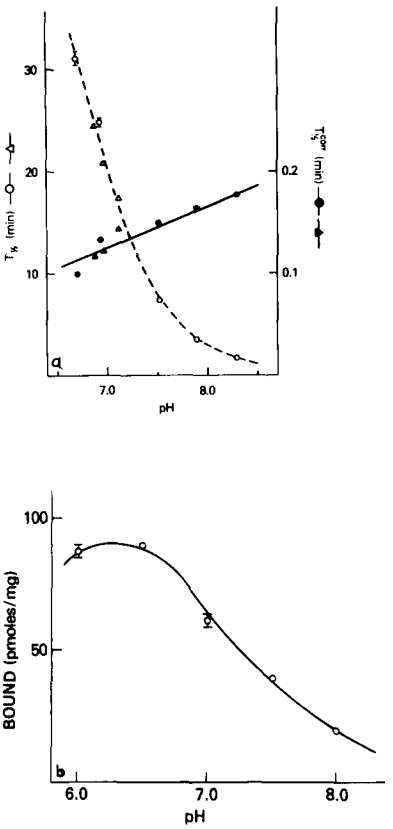

Fig. 5 (a) Effect of pH on the rate of inactivation of the transporter by dithiothreitol. The experiment was carried out as described in Methods except that 2 mM Tris-Hepes buffered to the pH values indicated was used in place of 10 mM Tris-Hepes. $T_{1/2}$ values from the inactivation curves (not shown) were calculated by the method of least squares (open symbols). Corrected $T_{1/2}$ values (closed symbols) were calculated as described in the text. Results of two experiments (○ and △, respectively) have been combined ([phlorizin] = 0.1 μM). (b) Effect of pH on equilibrium phlorizin binding to membrane vesicles. Equilibrium phlorizin binding was measured at 60 mM NaCl and 37°C as described in Methods except that 10 mM Tris-Hepes was replaced by 30 mM Mes titrated to the pH values indicated with Tris ([phlorizin] = 0.1 μM).

corrected half-time and the pK_a for dithiothreitol (pK_a^{DTT}) is 9.2 [16]. The corrected data show that over the pH range 6.5–8.5 the reactivity of the glucose transporter with dithiothreitol decreases, with increasing pH. Fig. 5b demonstrates that the ability of the transporter to bind phlorizin also decreases with increasing pH over this same pH range.

Discussion

Previous observations from our laboratory [15] demonstrated that the disulfide-reducing agent dithiothreitol had two effects on the renal outer cortical brush border membrane D-glucose transporter, the first resulting in a reversible increase in affinity for phlorizin and the second in a partially reversible inactivation of glucose transport and phlorizin-binding activity. Evidence was presented that both of these effects are the result of the reduction of disulfide bonds on the transport molecule. In this paper we present a detailed study of the inactivation of the transporter by dithiothreitol. The data presented here in Figs. 1 and 4 provide strong evidence that this inactivation is an independent process unrelated to the change in affinity also produced by dithiothreitol: Fig. 1 shows that the number of phlorizin-binding sites begins to decrease exponentially with time immediately upon exposure to dithiothreitol. This result indicates that the inactivation process does not require the prior completion of the effect of dithiothreitol on the affinity of the transporter which takes place with $T_{1/2} \approx 5$ min (see Fig. 1a). Fig. 4 demonstrates that the inactivation of the transporter is first-order with respect to dithiothreitol, presumably due to the reduction of a single disulfide bond, again indicating that this process is independent of other effects of dithiothreitol.

In our earlier paper [15] we demonstrated that substrates of the carrier can provide some protection against inactivation by dithiothreitol. This phenomenon is studied in more detail in Table I of the present paper. Here we demonstrate that the degree of protection closely follows the relative specificity of various monosaccharides for the transporter. No protection is provided by non-interacting sugars. The fact that sodium alone gives no protection against dithiothreitol (Table I) indicates that the protection provided by substrates is associated with the occupancy of the glucose-binding site rather than the (simultaneously occupied) sodium site on the transporter.

We demonstrate in Fig. 3 that phlorizin, a non-transported competitive inhibitor of the D-glucose carrier, also provides protection against inactivation by dithiothreitol. Complete protection does not occur, however, even for saturating

phlorizin concentrations (Fig. 3b). A simple model of this process (Eqn. 1) illustrates that the protection provided by phlorizin is consistent with the binding of phlorizin to a protective site with half saturation constant, K_{prot}, in good agreement with the K_d of phlorizin binding to the transport site. Thus the data presented in both Table I and Fig. 3 show that there is a strong correlation between occupancy of the transport site and protection against inactivation by dithiothreitol.

It should also be noted that the fact that substrates of the transporter provide protection against inactivation by dithiothreitol does not necessarily imply that phlorizin will also protect. This is because phlorizin interacts with the transporter in a much more limited way than substrates. Since phlorizin is not transported and, since the membrane vesicle preparation is oriented right-side-out [4], phlorizin binding occurs only to the externally oriented carrier. The substrate-carrier complex, on the other hand, can exist in both externally and internally oriented forms as well as in any possible intermediate configurations. Thus the protective effect of phlorizin in our system necessarily occurs when it occupies the substrate site of the externally oriented carrier.

It is interesting to note that phloretin, the aglycone of phlorizin, increases the rate of inactivation by dithiothreitol (Table I). Phlorizin is thought to interact with the carrier by binding its glucose moiety in the active transport site and its hydrophobic phloretin moiety elsewhere on the carrier protein [6,17]. Thus the site of action of dithiothreitol appears to be protected when the glucose-binding site is occupied but more susceptible when the phloretin site is occupied. This conjecture is further supported by the observation that substrates of the transporter apparently are capable of providing more protection against dithiothreitol than phlorizin (the $T_{1/2}$ measured in the presence of 25 mM methyl β-glucoside from Table I is (2.74 ± 0.07)-times that of the control, whereas from Fig. 3b the $T_{1/2}$ for [phlorizin] $\rightarrow \infty$ is (2.2 ± 0.2)-times control).

The protection provided when the glucose-binding site is occupied may either be due to direct steric hindrance by the substrate molecule or to a conformational change in the protein induced by the binding event and leading to protection at some other site. Measurements of the rate of inactivation of the transporter by dithiothreitol as a function of pH (Fig. 5a) show that the susceptibility of the transporter to attack by dithiothreitol decreases with increasing pH. Fig. 5b illustrates that the ability of the transporter to bind phlorizin also decreases over the same pH interval. These results may indicate that the site of action of dithiothreitol is actually located at the active site of the transporter and that this site becomes physically less accessible with increasing pH.

On the basis of our previous data [15] we have proposed that the inactivation of the outer cortical brush-border membrane D-glucose carrier in the presence of dithiothreitol proceeds according to the following scheme (for simplicity we neglect the increase in affinity produced by dithiothreitol, since this apparently occurs via an independent process):

$$\text{Form 1} \overset{\text{DTT}}{\underset{k_1}{\rightarrow}} \text{Form 2} \underset{k_2}{\rightarrow} \text{Form 3} \qquad (2)$$

Form 1 is the usual state of the carrier with the essential disulfide bond in its oxidized state. Reduction of this bond by dithiothreitol (DTT) results in Form 2 of the carrier, which is unstable and undergoes a change in configuration to the inactive Form 3. This latter step is irreversible or at least not easily reversed, whereas the reoxidation of Form 2 to Form 1 occurs readily upon removal of dithiothreitol. Although more complex models are certainly possible this scheme can account for all of our previous data. In particular Eqn. 2 accounts for our observations (Ref. 15, see also below) that, when membrane vesicles are incubated in dithiothreitol then washed in the presence or absence of excess *N*-ethylmaleimide, a reduced recovery of binding sites is observed in membranes washed with the alkylating agent. Thus exposing the membranes to *N*-ethylmaleimide during the washing procedure leads to alkylation of transporters present in Form 2 and to their subsequent loss owing to the (now inevitable) change in configuration to Form 3.

We have suggested that Form 2 of the carrier is unable to bind phlorizin [15]. This conjecture was based on the observation that Scatchard plots of sodium-dependent phlorizin binding in the pres-

ence of dithiothreitol are linear [15]. Since both Form 1 and Form 2 of the carrier would be present under these conditions this result implies either that Form 2 does not bind phlorizin or that both Form 1 and Form 2 bind phlorizin with the same affinity. The results presented here provide further evidence that Form 2 cannot bind phlorizin and thus presumably is inactive. This can be seen most easily by considering the form of the inactivation curves predicted by Eqn. 2. If N_1 and N_2 are the number of transporters in Forms 1 and 2, respectively, then

$$N_1 = N_0 e^{-k_1 t} \tag{3}$$

and

$$N_2 = k_1 N_0 [e^{-k_1 t} - e^{-k_2 t}]/(k_2 - k_1) \tag{4}$$

Denoting B_t and B_0 as the phlorizin binding at times t and 0, respectively, and neglecting the change in affinity produced by dithiothreitol we have

$$B_t = B_0 e^{-k_1 t} \tag{5}$$

if only Form 1 binds phlorizin, and

$$B_t = B_0 \left[k_2 e^{-k_1 t} - k_1 e^{-k_2 t} \right]/(k_2 - k_1) \tag{6}$$

if both Forms 1 and 2 bind phlorizin.

The behaviour of Eqn. 6 at short times ($\ll T^1_{1/2}$ and $T^2_{1/2}$, where $T^1_{1/2} = 0.693/k_1$ and $T^2_{1/2} = 0.693/k_2$) is relatively complex; however, for longer times B_t approaches a single exponential function of t. There are two distinct cases:

(i) if $k_1 \geqq k_2$ then B_t has time-dependence $e^{-k_2 t}$ for $t \gg T^1_{1/2}$, i.e., all inactivation curves approach the same slope (k_2) at large time values. This case can be excluded here, since Fig. 4 shows that the inactivation curves at different dithiothreitol concentrations do not appraoch the same slope.

(ii) If $k_1 \leqq k_2$ then B_t has time-dependence $e^{-k_2 t}$ for $t \gg T^2_{1/2}$. Numerical calculations (not shown) using Eqn. 6 indicate that for $t > 4T^2_{1/2}$ the slope of a log B_t vs. t plot is within 5% of k_1.

In fact the magnitudes of both $T^1_{1/2}$ and $T^2_{1/2}$ can be estimated simultaneously from our earlier experiment illustrated in Fig. 3 of Ref. 15. In this experiment we incubated vesicles for 15 min in the presence of 3.33 mM dithiothreitol then washed them in the presence or absence of excess *N*-ethylmaleimide. Relative to appropriate controls $31 \pm 1\%$ and $52 \pm 2\%$ of the phlorizin-binding sites remained in the membranes washed in the presence and absence of *N*-ethylmaleimide, respectively. Interpreting these results in terms of Eqn. 2 we find that, after incubation in dithiothreitol, 31% of the transporters are present in Form 1, while $\geqq 21\%$ are in Form 2 (the $\geqq$ sign allows for the possibility that some transporters present in Form 2 at the end of the incubation period may be lost during the washing procedure in the absence of *N*-ethylmaleimide). Substituting these numbers into Eqns. 3 and 4 we find that $T^1_{1/2} = 8.9$ min and $T^2_{1/2} \geqq 4.4$ min.

In the experiment illustrated in Fig. 2 the rate of inactivation of phlorizin binding by dithiothreitol was measured in two ways. In the first method (open symbols) membranes were exposed to excess *N*-ethylmaleimide before phlorizin binding was measured. In this case any transporters present in Form 2 will be alkylated and lost and only Form 1 will be available for phlorizin binding. Thus the inactivation curve will be given by Eqn. 5. In the second method (solid symbols), however, the *N*-ethylmaleimide step was omitted so that carriers present in Form 2 would remain potentially active. In this case the inactivation curve will be given by Eqn. 6 if both Forms 1 and 2 bind phlorizin, but by Eqn. 5, if only Form 1 binds phlorizin. It is clear from Fig. 2 that the slopes of the inactivation curves are the same for both methods over the entire time interval studied. If both Forms 1 and 2 bind phlorizin this result can only be reconciled with Eqn. 6 if $T^2_{1/2} \ll 5$ min. However, the calculation of the preceding paragraph shows that $T^2_{1/2} \geqq 4.4$ min. Thus we conclude that the reduced transporter (Form 2) is inactive.

It is interesting to speculate that the essential disulfide bond studied in this paper may play some role in the regulation of renal D-glucose reabsorption. Disulfide bonds and sulfhydryl groups have been shown to be involved in the regulation of a number of cellular processes. It is generally argued that, since renal D-glucose reabsorption is essentially complete under normal blood glucose levels and since blood glucose is

maintained at a relatively constant level, a control mechanism of this type is unnecessary and unlikely. However, such a mechanism could serve some purpose other than regulating the magnitude of the reabsorption of the filtered glucose load. For example, it has recently been shown that glucose transport is heterogeneous along the length of the proximal nephron with different sodium-coupled transporters apparently being expressed in different regions of the proximal tubule [2–5]. Such a control mechanism could be employed to redistribute the glucose-reabsorptive load among various proximal nephron segments in response to the physiological or metabolic state of the animal. In this regard it is worth mentioning that the cultured renal epithelial cell line LLC-PK_1, which exhibits a number of proximal-tubular functions [18] including an apical sodium-dependent D-glucose carrier, has recently been shown to regulate its apical D-glucose transport properties in response to various growth conditions [19].

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