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Characterization of essential arginine residues implicated in the renal transport of phosphate and glucose

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We have characterized the reaction of arginine-specific reagents with the phosphate and glucose carriers of the kidney brush-border membrane. The inhibition of phosphate and glucose transport by phenylglyoxal follows pseudo-first-order kinetics. The rate of inactivation of phosphate transport by 50 mM phenylglyoxal was about 3-fold higher than that for glucose transport (k_{app} was 0.052 s^{-1} for the uptake of phosphate and 0.019 s^{-1} for the uptake of glucose). The order of the reaction, n, with respect to phenylglyoxal was 1.25 and 1.31 for the inactivation of phosphate and glucose transport, respectively. The inactivation of phosphate flux by *p*-hydroxyphenylglyoxal also follows pseudo-first-order kinetics, but the inhibition rate (k_{app} = 0.0012 s^{-1}) was slower than with phenylglyoxal. The inactivation increased with the alkalinity of the preincubation medium for both phosphate and glucose fluxes and was maximal at pH 9.0. The inactivation of phosphate flux by phenylglyoxal depends upon the presence of an alkaline intravesicular pH. Extravesicular pH does not affect the reaction. Phenylglyoxal does not interfere with the recycling of the protonated carrier since phosphate uptake is inhibited independently of the pH used for transport measurements. Moreover, phenylglyoxal completely abolished trans stimulation by phosphate. Trans sodium inhibited phosphate uptake and abolished the pH profile of phosphate uptake.

Introduction

One of the most important functions of the epithelial cells of the proximal tubule is the reabsorption of solutes from the tubular fluid. Reabsorption is an active process that depends upon the presence of sodium in the tubular fluid [1–3] and is carried out by carrier proteins which are located in the luminal membrane of tubular cells [4–8]. Several studies have analyzed the kinetics of these transport systems in brush-border membrane vesicles [9–12], but little is known about the structure-function relationship of these carrier proteins.

The identification of specific residues within the active sites of carrier molecules is of importance for understanding their molecular mechanisms. Amino acid-specific reagents have been used to identify some functionally active amino acid residues in carrier molecules. HS- and -NH_2 groups have been implicated in glucose transport by intestinal brush-border membrane vesicles [13–15]. A tyrosine residue has been identified at the Na^+-binding site of the renal Na^+-glucose cotransporter [16]. It has been shown that arginine residues are important in the translocation of glucose, phosphate and alanine in renal brush-border membrane vesicles [17]. An arginine residue has also been implicated in the conformational changes of the phosphate carrier induced by the sodium gradient [18]. More recently, the use of a fluorescent reagent, specific for arginine residues, has led to the identification of a putative intestinal phosphate carrier [19]. In this paper, we characterize the reaction of arginine-specific reagents with the phosphate and glucose carriers of the kidney brush-border membrane.

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Materials and Methods

Preparation of brush-border membrane vesicles

Brush-border membrane vesicles were isolated from rat kidney cortex with the $MgCl_2$ precipitation technique [20]. The purified vesicle preparations were resuspended in 300 or 450 mM mannitol and 20 mM

Hepes-Tris (pH 7.5) to a final protein concentration of 15 to 25 mg/ml and stored in liquid nitrogen for up to two weeks. The purity of the membrane preparations was evaluated by measuring the enrichment factors for alkaline phosphatase and Na^+/K^+-dependent ATPase (12.9 ± 1.4 and 0.42 ± 0.07, respectively).

Preloading of the vesicles

In some experiments, it was necessary to preload the vesicles with media containing different salts and buffers. This was accomplished by diluting the vesicle suspensions (100 μl) in 10 ml of the desired medium, followed by an homogenization with a glass-teflon homogenizer. The suspensions were centrifuged at 33 000 × *g* for 30 min, resuspended in the same medium, passed ten times through a fine needle (27½ gauge), and allowed to equilibrate for 1 h at 25°C.

Transport measurements

The uptake of phosphate and D-glucose was measured at 25°C with a rapid filtration technique [21]. The reaction was initiated by the addition of 80–100 μg of protein. Transport was stopped by adding an ice-cold stop solution containing 200 mM KCl, 50 mM mannitol, and 20 mM Hepes-Tris (pH 7.5). Membrane suspensions were filtered through a 0.45 μm pore-size Millipore filter under vacuum. Filters were washed with 8 ml of the cold stop solution and processed for liquid-scintillation counting. Nonspecific binding by vesicles and filters, measured by adding the incubation medium and the membrane preparation directly to the stop solution, was subtracted from the transport measurements. Sodium-independent uptake was measured by replacing $NaNO_3$ by KNO_3 in the incubation media. Protein concentration was evaluated with the method of Bradford [22].

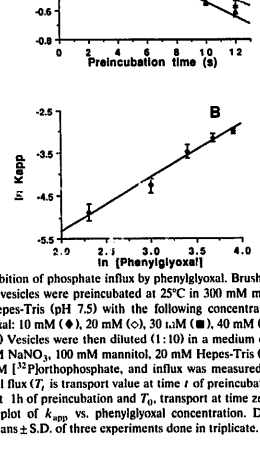

Fig. 1. Inhibition of phosphate influx by phenylglyoxal. Brush-border membrane vesicles were preincubated at 25°C in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5) with the following concentrations of phenylglyoxal: 10 mM (♦), 20 mM (○), 30 mM (■), 40 mM (□) and 50 mM (▲) Vesicles were then diluted (1:10) in a medium containing 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.2 mM [^{32}P]orthophosphate, and influx was measured at 5 s. (A) residual flux (T_i is transport value at time t of preincubation; T_∞, transport at 1h of preincubation and T_0, transport at time zero). (B) Double-ln plot of k_{app} vs. phenylglyoxal concentration. Data are means ± S.D. of three experiments done in triplicate.

Chemical modification of brush-border membrane vesicles with phenylglyoxal

Membrane vesicles were modified as described previously [17,18]. Membrane vesicles were preincubated with phenylglyoxal or hydroxyphenylglyoxal in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5) for 2 min at 25°C and washed by centrifugation in 300 mannitol, 20 mM Hepes-Tris (pH 7.5) (33 000 × *g*, 30 min, 4°C) before the transport measurements.

Chemicals

Phenylglyoxal and *p*-hydroxyphenylglyoxal were purchased from Sigma Chemical Company. [^{32}P]Orthophosphate was obtained from Du Pont-New England Nuclear. All other chemicals were reagent grade.

Results

Preincubation of brush-border membrane vesicles with phenylglyoxal, at pH 7.5, resulted in a time- and concentration-dependent inhibition of phosphate (Fig. 1A) and glucose influx (Fig. 2A). The inhibition of phosphate and glucose influx followed pseudo-first-order kinetics. A linear relationship was observed between the pseudo-first-order rate coefficient k_{app} (s^{-1}) and the concentration of phenylglyoxal. The order of the reaction, n, with respect to phenylglyoxal was determined by the application of a simplified form of the Hill equation described by Levy et al. [23]. The dependence of k_{app} on phenylglyoxal concentration is described by the following equation:

$$\ln k_{app} = \ln k' + n \ln[PGO]$$

where [PGO] is the concentration of phenylglyoxal, k', a constant, and n, the Hill coefficient. It corresponds to the slope of the plot of ln k_{app} versus ln[PGO]. The calculated Hill coefficient, n, was 1.25 for the inactivation of phosphate transport and 1.31 for that of glucose transport (Figs. 1B and 2B). This suggests that one molecule of phenylglyoxal per molecule of phosphate or glucose carrier is involved in the rate-limiting step of the inactivation of these transport processes.

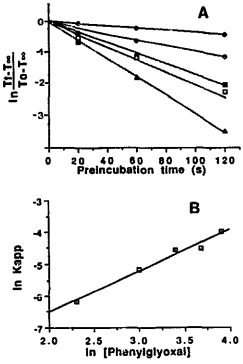

Fig. 2. Inhibition of glucose influx by phenylglyoxal. Brush-border membrane vesicles were preincubated as described in Fig. 1. Vesicles were then diluted in a medium containing 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.1 mM D-[^{3}H]glucose and influx was measured at 5 s. (A) Residual flux (T_t is transport value at time t; T_∞, transport at infinite time and T_0, transport at time zero). (B) Double-ln plot of k_{app} vs. phenylglyoxal concentration.

We have also studied the time course of inactivation of phosphate influx with another arginine-specific reagent, *p*-hydroxyphenylglyoxal. As shown in Fig. 3, the preincubation of brush-border membrane vesicles with 50 mM *p*-hydroxyphenylglyoxal resulted in a time-dependent inhibition of phosphate transport: as

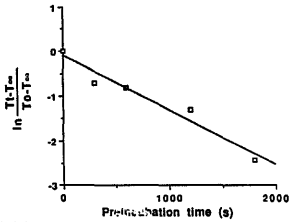

Fig. 3. Inhibition of phosphate influx by preincubation with *p*-hydroxyphenylglyoxal. Brush-border membrane vesicles were preincubated at 25°C in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5) with 50 mM *p*-hydroxyphenylglyoxal. Vesicles were then diluted (1:10) in a medium containing 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.2 mM [^{32}P]orthophosphate and influx was measured at 5 s. T_t is transport at time t; T_∞, transport at infinite time and T_0 transport at time zero. Data are means ± S.D. of three experiments done in triplicate.

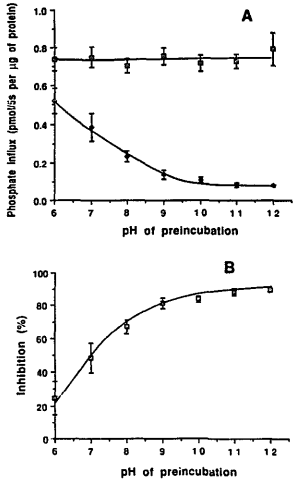

Fig. 4. Effect of preincubation pH on the inhibition of phosphate influx by phenylglyoxal. Brush border membrane vesicles were equilibrated as described in Materials and Methods with 300 mM mannitol and 20 mM of either one of the following buffers: Mes-Tris (pH 6.0), Hepes-Tris (pH 7.0), Tris-Hepes (pH 8.0), Ches-Tris (pH 9.0) or Caps-KOH (pH 10.0, 11.0 and 12.0). Vesicles were then preincubated for 2 min at 25°C in the same media with (◆) or without (□) 50 mM phenylglyoxal. They were then diluted 50-fold in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and centrifuged at 33000 × *g* for 20 min, at 4°C. The vesicles were resuspended in the same medium and allowed to equilibrate for 1 h at 25°C. Phosphate influx was measured in 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.2 mM [^{32}P]orthophosphate. Data are means ± S.D. of six experiments done in triplicate. Panel B represents the percentage of inhibition obtained for each pH of preincubation.

observed for the inhibition by phenylglyloxal, the reaction followed pseudo-first-order kinetics. The reaction of *p*-hydroxyphenylglyoxal with the phosphate carrier was about 40-times slower than that of phenylglyoxal, the k_{app} being 0.0012 s^{-1} compared to 0.052 s^{-1} with the same concentration of phenylglyoxal. A slower inactivation with *p*-hydroxyphenylglyoxal which is more hydrophilic than phenylglyoxal suggests that the arginine residue that is modified may not be exposed directly to the extravesicular medium.

Figs. 4A and 5A show the effect of pH on the inactivation of phosphate and glucose transport activities by phenylglyoxal. The inhibition increased with the alkalinity of the preincubation medium; phosphate uptake was inhibited by 25% at pH 6.0 and 81% at pH

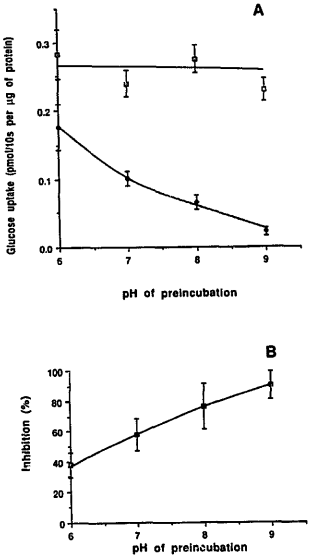

Fig. 5. Effect of preincubation pH on the inhibition of glucose influx by phenylglyoxal. Brush-border membrane vesicles were equilibrated and modified as described in the legend of Fig. 4. Glucose flux was measured in 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.1 mM D-[^{3}H]glucose. Data are means ± S.D. of three experiments done in triplicate. Panel B represents the percentage of inhibition obtained for each pH of preincubation.

9.0 (Fig. 4B), and glucose uptake was inhibited by 38 and 93% at pH 6.0 and 9.0, respectively (Fig. 5B).

The pH dependency of phenylglyoxylation was used to study the localization of the functional arginine residues within the phosphate and glucose carrier molecules. When preincubation was carried out at an intravesicular pH of 9.0, the inhibition of phosphate flux was 67% compared to 23% when the intravesicular medium was at pH 6.0, independently of the extravesicular pH (Fig. 6). This strongly suggests that the phosphate carrier has a functional arginine residue located within the portion of the carrier that is accessible from the cytosolic side of the membrane. In contrast, when glucose flux was measured under the same conditions (Fig. 7), inhibition was maximal when both intra and extravesicular compartments were at pH 9.0 and minimal at pH 6.0.

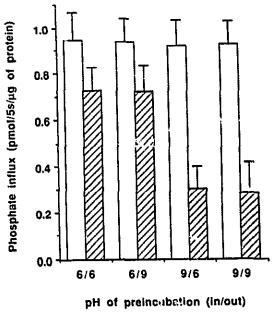

Fig. 6. Effect of different intravesicular and extravesicular pH of preincubation on the inhibition of phosphate influx by phenylglyoxal. Brush-border membrane vesicles were equilibrated as described in Materials and Methods with 300 mM mannitol and 20 mM Mes-Tris (pH 6.0), or 20 mM Ches-Tris (pH 9.0). Vesicles were diluted 6-fold in the appropriate buffers with (hatched bars) and without (empty bars) 50 mM phenylglyoxal and were incubated for 2 min at 25°C. They were then diluted 50-fold in an ice-cold solution containing 300 mM mannitol and Hepes-Tris (pH 7.5) and centrifuged at 33000× g for 20 min, at 4°C. Vesicles were resuspended in the same medium and allowed to equilibrate for 1 h at 25°C. Phosphate flux was measured in 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.2 mM [^{32}P]orthophosphate. Data are means ± S.D. of nine experiments done in triplicate.

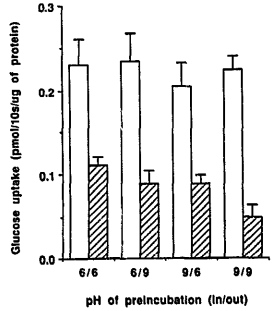

Fig. 7. Effect of different intravesicular and extravesicular pH of preincubation on the inhibition of glucose uptake by phenylglyoxal. Brush-border membrane vesicles were equilibrated and modified as described in the legend of Fig. 6. Glucose flux was measured in 100 mM $NaNO_3$, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 0.1 mM D-[^{3}H]glucose. Data are means ± S.D. of nine experiments done in triplicate.

In the presence of an external pH of 7.5, an internal pH of 6.5 stimulated phosphate uptake by 43%, and an internal pH of 8.5 inhibited phosphate uptake by 31% when compared with an internal pH of 7.5 (Fig. 8A). These results are in agreement with a model where the phosphate carrier is protonated at the inner face of the membrane [24]. Fig. 8A also shows the stimulation of phosphate uptake by trans phosphate at different internal pH values with a constant external pH (7.5); the trans stimulation was 0, 74 and 48% at pH 6.5, 7.5 and 8.5, respectively. Phenylglyoxal treatment reduced

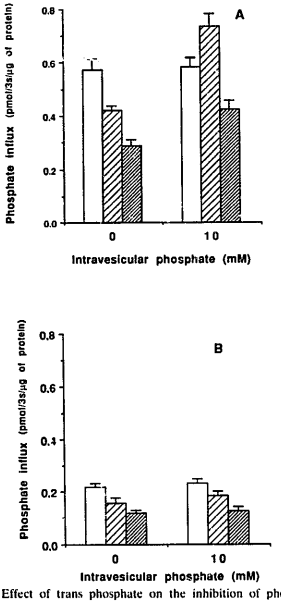

Fig. 8. Effect of trans phosphate on the inhibition of phosphate uptake by phenylglyoxal. Membrane vesicles in 450 mM mannitol and 20 mM Hepes-Tris (pH 7.5) were treated with 50 mM phenylglyoxal as described in Materials and Methods. Membrane vesicles were then loaded in a medium containing 200 mM KNO_3, 50 mM mannitol, 20 mM of either one of the following buffers: MES-Tris (pH 6.5, empty bars), Hepes-Tris (pH 7.5, hatched bars) and Tris-HCl (pH 8.5, double hatched bars), and either 10 mM phosphate or 10 mM K_2SO_4. Preparations were incubated 2 h to allow equilibration before transport measurements. 5 µl of brush-border membrane vesicles were diluted with 245 µl of the incubation medium (200 mM $NaNO_3$, 50 mM mannitol and 20 mM Hepes-Tris (pH 7.5) and 0.2 mM [^{32}P]orthophosphate) and transport was measured. (A) Control vesicles; (B) phenylglyoxal-treated vesicles.

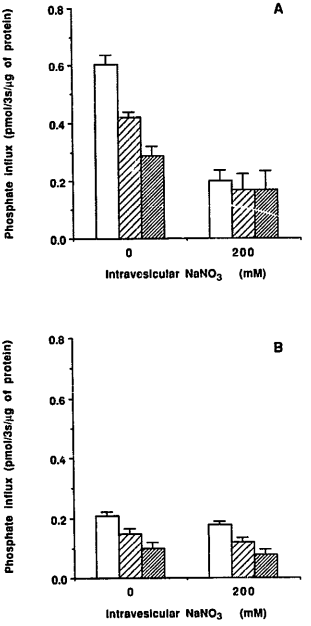

Fig. 9. Effect of trans sodium on the inhibition of phosphate uptake by phenylglyoxal. Membrane vesicles were modified with 50 mM phenylglyoxal as described in Materials and Methods. Membrane vesicles were then loaded with 50 mM mannitol, 200 mM $NaNO_3$ or 200 mM KNO_3 and 20 mM of either one of the following buffers: Mes-Tris (pH 6.5, empty bars), Hepes-Tris (pH 7.5, hatched bars), and Tris-HCl (pH 8.5, double hatched bars). Transport measurements were performed as described in the legend of Fig. 8. (A) Control vesicles; (B) phenylglyoxal-treated vesicles.

phosphate uptake into membrane vesicles with an internal pH of 6.5, 7.5, and 8.5 by 62, 63 and 58%, respectively (Fig. 8B). The effect of trans pH remained present with phenylglyoxal-modified vesicles, indicating that phenylglyoxal does not affect the proton binding site of the transporter. Trans stimulation by phosphate, however, was completely abolished in phenylglyoxal-modified vesicles.

Under sodium equilibrium conditions, phosphate uptake into control membrane vesicles was reduced by 65, 57 and 33% at pH 6.5, 7.5 and 8.5, respectively (Fig. 9A). Phenylglyoxal treatment reduced phosphate uptake by 60% at pH 6.5, 7.5 and 8.5, (Figs. 9A and

9B, left). The inhibition by trans sodium was stronger with control than with phenylglyoxal-treated vesicles (Fig. 9A and 9B). Phosphate uptake was inhibited by phenylglyoxal in a pH-dependent manner: 52, 45 and 37% at pH 6.5, 7.5 and 8.5, respectively.

Discussion

α-Dicarbonyls offer a major advantage over many other amino acid modifiers, since they cause no change in the charge of the protein [25] and no gross alteration in the structure of the protein after modification of their functional arginine residues [26]. Phenylglyoxal, which has been used previously to study transport activities in kidney brush-border membrane vesicles, does not alter the physical integrity of these membranes [17,18].

The aim of the study reported in this paper was to characterize the action of phenylglyoxal on the phosphate and glucose carriers of kidney brush-border membrane vesicles. Phenylglyoxal inhibited both phosphate and glucose influx, but the rate of inactivation of phosphate transport was about three times greater than that of glucose. With 50 mM phenylglyoxal, the k_{app} for the inhibition of phosphate and glucose fluxes were 0.052 s^{-1} and 0.019 s^{-1}, respectively. This corresponds to a halftime of inactivation ($T_{1/2}$) of 19 s for phosphate transport and 53 s for glucose transport. These are among the fastest reactions ever reported for the modification of an arginine residue. Only the anion exchanger of red blood cells was reported to have a shorter half-time of inactivation [27].

The inhibition of both phosphate and glucose fluxes increased with alkalinisation of the medium (Figs. 4 and 5). This pH-dependency is characteristic of the modification of arginine residues [28] and reflects the level of ionization of the guanidinium group. Inhibition of phosphate flux increased as the preincubation pH increased from 6.0 to 9.0 and remained at a stable level of about 82% as the pH was raised to 12.0. The pK_a of the guanidinium group of free arginine is, however, above 12. Arginine residues located within the catalytic site of active proteins have been shown to react faster than free arginine with α-dicarbonyls [29,31]. To explain the discrepancy between the reactivity of free arginine and the apparent pK_a of functional arginine residues it has been suggested that the catalytic site is surrounded by a special microenvironment which contributes to a lowering of the pK_a of the guanidinium group of the arginine residue [32].

The order of the inactivation reaction, n, with respect to phenylglyoxal, obtained from the kinetics of inactivation (Figs. 1B and 2B) was close to 1 for both phosphate and glucose fluxes. This indicates that the covalent binding of one molecule of phenylglyoxal to the glucose and the phosphate carriers is the rate-limiting step in the inhibition process. This selectivity of phenylglyoxal for specific arginine residues of a polypeptide chain has been reported before [32] for other proteins, where even prolonged incubation periods yielded a modification of only 1 residue on a total of up to 18 arginine residues in the polypeptide chain. Here again, the involvement of adjacent amino acid residues in creating an adequate microenvironment for modification has been proposed to explain this phenomenon.

The pattern of trans stimulation by phosphate at various internal pH values from 6.5 to 8.5 (Fig. 8A) suggests that the protonation site of the carrier involves a residue with a pK_a within the physiological range of pH. Internal pH affected phosphate uptake into control and phenylglyoxal-modified membranes in a similar fashion (Figs. 8A and 8B, controls, without trans phosphate) suggesting that the phenylglyoxal-sensitive arginine residue is not located at the protonation site of the carrier.

Trans sodium was strongly inhibitory, confirming that the binary complex C-N does not translocate from one side of the membrane to the other. In the presence of trans sodium, phosphate uptake was independent of internal pH (Fig. 9A), indicating that the complex C-N is not protonated at the inner face of the membrane. However, it seems possible that the protonated complex C-N-H translocates much slower than the unprotonated form (C-N) since the inhibition by trans sodium is higher at pH 6.5 (65%) and 7.5 (57%) than at pH 8.5 (33%). Phosphate uptake measured under sodium equilibrium conditions was not significantly inhibited by phenylglyoxal. The effect of trans Na^+ was somewhat smaller in PGO-treated vesicles. We have previously shown that PGO causes no collapse of the sodium gradient, since initial rates of sodium influx remained unaffected by this reagent [17].

Our results indicate that the arginine residue that is functionally active in the translocation process of the phosphate carrier is located on the cytosolic side of the membrane. The alkaline dependence of modification by phenylglyoxal combined with the fact that the inhibition of phosphate was increased from 25% to 81% when intravesicular pH was raised from 6.0 to 9.0, clearly indicates that phenylglyoxal modified a component located at the cytosolic side of the membrane. Also, the rate coefficient for the inactivation of phosphate transport by *p*-hydroxyphenylglyoxal, a less lipophilic reagent, is about 40-times smaller than that obtained with phenylglyoxal (0.0012 s^{-1} vs. 0.052 s^{-1}), indicating that the arginine residue functionally implicated in the translocation process is less accessible to a reagent which is presumably less permeant through the lipid bilayer. The preferential localization of positively charged amino acid residues of proteins on the cytoplasmic side of the membrane has been emphasized

before [33]; lysine and arginine residues of some membrane proteins are 4-times more prevalent in the cytoplasmic than in the extracellular loops [34]. These positive residues are thought to play a major role in the correct orientation of membrane proteins and would thus be crucial factors for the transmembrane topology. In the case of the glucose transport system, however, the arginine residue implicated in the translocation process appears to be accessible from either side of the membrane since both intra- and extravesicular pH affect the inhibition.

Acknowledgments

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