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Oxalate binding to rat intestinal brush-border membrane in pyridoxine deficiency: a kinetic study

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Oxalate bound specifically to the intestinal brush-border membrane (BBM) of pyridoxine-deficient rats, but not to BBM of control rats. The binding of oxalate to intestinal BBM of pyridoxine-deficient rats was rapid, reversible, dependent on concentration of oxalate, temperature sensitive and competitively inhibited by oxalate analogues. Kinetic analysis of the oxalate binding data revealed induction of two distinct classes of receptor site for oxalate. The high-affinity oxalate binding sites, reached saturation at 60–70 nM oxalate, had a K_d of 24.29 nM and the number of binding sites were 30 pmoles (i.e., $1.8 \cdot 10^{13}$ molecules). The low-affinity oxalate binding sites, could not be saturated under experimental conditions upto 1 μM oxalate. It had a K_d of 487.5 nM and the number of binding sites were 156 pmoles (i.e., $9.4 \cdot 10^{13}$ molecules). The apparent energy of activation was 19 kcal/mol. The half-saturation concentration of inhibitor (IC_{50}) of oxalate was $0.4 \cdot 10^{-5}$ M, while all other structural analogues of oxalate had higher IC_{50} values. Among the competitive inhibitors tested IC_{50} was in the following order, pyruvate > maleate > oxaloacetate > glyoxylate > parabonate > oxalate. These kinetic characteristics indicate involvement of a membrane protein in oxalate binding and transport in rat intestinal brush-border membrane in pyridoxine deficiency.

Introduction

Idiopathic calcium oxalate nephrolithiasis is now recognized as a metabolic disorder characterized by a generalized defect in cellular transport of oxalate [1]. Several reports suggest that the primary cause of hyperoxaluria and nephrolithiasis is hyperabsorption of dietary oxalate [2,3] mainly due to marginal pyridoxine deficiency [4]. Oxalate absorption from the gut takes place by a simple passive diffusion in man and experimental animals [5–7]. However, in pyridoxine deficiency, oxalate absorption follows biphasic carrier-mediated characteristics in which a carrier-mediated saturable component facilitates oxalate uptake from lumen into enterocytes at low mucosal oxalate concentrations [8–10]. Further treatment of animals with inhibitors of protein synthesis i.e., cyclohexamide and actinomycin-D, inhibited carrier-mediated uptake of oxalate in pyridoxine-deficient animals [11]. Thus, these studies suggest that hyperabsorption of dietary oxalate in pyridoxine deficiency arises as a result of induction of a biphasic carrier-mediated transport process for oxalate in intestinal brush-border membrane. These findings, although contributing to an understanding of overall oxalate transport system in intestinal brush-border membrane in pyridoxine deficiency, do not provide direct information on the basic mechanisms of intestinal oxalate transport in pyridoxine deficiency. It has been generally assumed that trans-membrane transport involves and, in fact, may begin with interaction or binding of oxalate to the receptor site in the brush-border membrane.

In this paper, the initial interaction of oxalate with intestinal brush-border membrane in pyridoxine deficiency is characterized and essential parameters as well as kinetics of the system are described.

Methods

Male albino rats (Wistar strain) initially weighing about 45 g were divided into two groups of ten each. The first group was fed ad libitum on pyridoxine-deficient diet (as described previously) [4,8–10] for 6 weeks.

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TABLE I

Effect of pyridoxine deficiency on erythrocyte alanine transaminase (ALT) activities in male weanling rats

All values are mean ± S.E. of ten rats.

Group	Alanine transaminase (ALT)	
	U [a]/mg protein ($\times 10^6$)	Stimulation index (%) [b]
Pair-fed control	58.62 ± 4.31	6.09 ± 1.46
Pyridoxine deficient	14.85 ± 2.21 ***	55.68 ± 3.62 ***

[a] One International Unit of ALT is defined as one mole of pyruvate formed per min at 37°C.

[b] Percent PALP stimulation index = $100(A(\text{Sat}) - A(\text{Test}))/A(\text{Test})$. $A(\text{Sat})$ = difference in A in the presence of 0.1 nM pyridoxal phosphate; $A(\text{Test})$ = difference in A in the absence of pyridoxal phosphate.

*** $P < 0.001$ as compared to pair-fed controls.

The second group was pair-fed along with the first group with the same diet supplemented with 24.0 mg pyridoxine HCl/kg diet.

At the end of experimental period (6 weeks), the clinical symptoms of pyridoxine deficiency, i.e., acrodynia, alopecia and peripheral neuritis, were prominent. The overnight fasted animals were killed and their pyridoxine status was assessed biochemically by assaying the erythrocyte alanine transaminase activity together with the percent pyridoxal phosphate stimulation index of the erythrocyte alanine transaminase activity as described previously [4,8–10].

The animals fed on pyridoxine-deficient diet showed a significant ($P < 0.001$) decrease in erythrocyte alanine transaminase activity and an about 9-fold increase in percent pyridoxal phosphate stimulation index of erythrocyte alanine transaminase activity as compared to pair-fed control animals (Table I), thus confirming the pyridoxine-deficient status of these animals.

Intestinal brush-border membrane was prepared as described [12] and modified [13] except that $CaCl_2$ was replaced by $MgCl_2$. The final brush-border membrane pellet was reconstituted in 5 mM Tris-HCl buffer (pH 7.0), containing 50 mM mannitol. The specific activities of marker enzymes, sucrase and alkaline phosphatase in brush-border membrane preparations were approximately 17–21-fold, those in mucosal homogenate, while the specific activity of Na^+/K^+-ATPase, a basolateral marker enzyme, showed 83–93% decrease in brush-border membrane preparations as compared to mucosal homogenate (Table II).

The membrane sites for oxalate binding were determined in a total volume of 1.0 ml at 30°C in 5 mM Tris-HCl buffer (pH 7.0) containing 400 mM mannitol. [^{14}C]Oxalate was isotopically diluted with unlabelled oxalate to accommodate the wide concentration range (10 nM–1.0 μM). To differentiate specific and non-specific binding, parallel incubations were performed in presence of 1 mM unlabelled oxalate. The final concentration of membrane protein was 0.05–0.10 mg/ml. Membrane bound oxalate was separated from unbound oxalate by rapid filtration assay on 0.45 μm Millipore filters. The filters were washed four times with ice-cold 5 mM Tris-HCl buffer containing 1.0 M mannitol and dried. The filtrate was routinely checked and after the 3rd washing no radioactivity could be seen in the filtrate. The filters were dried, mixed with Bray's scintillation fluid and the radioactivity counted in Packard's Tricarb liquid scintillation spectrometer. The radioactivity retained on the filters was taken as oxalate binding.

Protein was determined according to a modified Lowry method [14] using bovine serum albumin as standard.

Results and Discussion

In previous studies [8–10] we have reported the development of a biphasic carrier-mediated phenomenon of oxalate uptake in rat intestine in pyridoxine deficiency, in which the carrier-mediated saturable component has been shown to facilitate oxalate uptake at

TABLE II

Biochemical determination of the purity of intestinal brush-border membrane

All values are mean ± S.E. of 8–10 observations.

	Activity (mol/min per mg protein)					
	pair-fed control			pyridoxine deficient		
	sucrase	alkaline phosphatase	Na^+/K^+-ATPase	sucrase	alkaline phosphatase	Na^+/K^+-ATPase
Homogenate	0.038 ± 0.001	0.197 ± 0.002	0.191 ± 0.02	0.058 ± 0.004	0.164 ± 0.01	0.260 ± 0.013
Brush-border membrane	0.840 ± 0.02	3.011 ± 0.023	0.012 ± 0.001	1.528 ± 0.051	2.952 ± 0.071	0.022 ± 0.007
Enrichment factor	21.1	14.28	0.06	25.34	17.0	0.08

TABLE III

Oxalate binding to intestinal brush border of normal and pyridoxine deficient rats

All values are means ± S.E. of 8–10 independent observations. n.d., not detected.

Condition	Oxalate bound (pmol/mg protein per 10 min)	
	total	specific
Pyridoxine deficient	22.35 ± 0.002	21.15 ± 0.04
Pair-fed control	10.47 ± 0.004	n.d.

lower oxalate concentration in vitro. When oxalate binding to the intestinal BBM was determined by incubating 0.1 mg BBM protein from pyridoxine-deficient and control rats with 100 nM [^{14}C]oxalate for 10 min at 30°C at zero osmotic space, the results indicated that oxalate bound specifically to the intestinal BBM of pyridoxine-deficient rats, but not to the intestinal BBM of control rats (Table III). Therefore, it has been speculated that differences in mechanism of oxalate uptake in pyridoxine deficiency as compared to normals may be due to the alterations in membrane binding to oxalate.

To ascertain this, the kinetic properties of oxalate binding to intestinal BBM of pyridoxine-deficient rats, the effect of time, oxalate concentration and temperature were determined. Binding of oxalate to BBM with respect to time showed 80% of maximal binding in the first 4 minutes of incubation. Equilibrium was reached

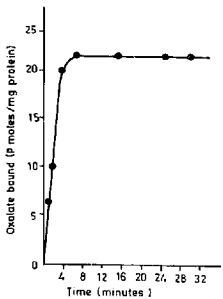

Fig. 1. Effect of time on oxalate binding. Pyridoxine-deficient rat intestinal brush-border membrane aliquots (100 μg protein) were incubated with 100 nM [^{14}C]oxalate in a total volume of 1.0 ml at 30°C. Binding was measured as detailed in Methods. The reaction was terminated by addition of 5.0 ml of ice cold saline at the indicated time intervals, filtered rapidly and radioactivity counted. Each point is the mean ± S.E. of six observations.

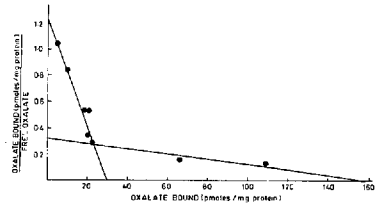

FIG.15/SCATCHARD PLOT OF OXALATE BINDING

Fig. 2. Scatchard plot characterizing relationship between the concentration of oxalate and its binding. Pyridoxine-deficient rat intestinal brush-border membrane aliquots (100 μg membrane protein) were incubated at 30°C for 10 min with varying concentrations of oxalate (10 nM–1.0 μM) and oxalate binding was measured as detailed in Methods. Each point is the mean of 8–10 independent observations.

after 6 min of incubation, the level remained constant upto 30 min (Fig. 1).

The binding of oxalate to the BBM (expressed as oxalate bound pmol/mg of membrane protein) studied over concentration range of 10 nM–1.0 μM revealed two distinct binding sites for oxalate. One site reached saturation at 60–70 nM of oxalate with a half-saturation concentration of 25 nM. There was no further increase in binding until the oxalate concentration reached 100 nM. The increased binding was again observed with higher oxalate concentrations of 500 and 800 nM. Complete saturation of the second system was not obtained experimentally even at oxalate concentration of 1.0 μM. These two binding systems for oxalate were quantitated by Scatchard plot analysis [15] by plotting the ratio of bound oxalate to free oxalate against the amount of oxalate bound/mg protein. As shown in Fig. 2, the curve describing this relationship comprised of two components, each with a different slope indicating the presence of at least two affinity sites for oxalate in the brush-border membrane. The high-affinity site that was saturated at low levels of oxalate, had a K_d of 24.29 nM while 30 pmol of oxalate could interact with 1 mg membrane protein. At the other non-saturable low-affinity site, K_d was 487.5 nM, while 156 pmol interacted with 1 mg of protein. The binding system, which was saturated at low oxalate concentration, had a low K_d value but had a lower number of binding sites, is considered to have high-affinity classes of binding sites. The binding system, which had the high K_d value but had the greater number of binding sites per unit of membrane, is designated as having low-affinity classes of binding sites. These observed differential systems for oxalate binding to intestinal BBM from pyridoxine-deficient rats are comparable to the biphasic transport systems of oxalate in intestinal brush-border membrane vesicles from B_6 deficient rats

[10]. It might be tempting to speculate on the presence of two distinct oxalate binding proteins in the brush-border membrane, however, evidence to this date is not sufficient for such a proposal. It is entirely feasible to point out that, two binding systems were mediated by a single membrane-carrier protein (unpublished observation), whose affinity for oxalate, as shown here, was dependent on the oxalate concentration. A precedent to this kind of alterations, i.e., conformational changes with substrate concentration is an isolated galactose binding protein from *Escherichia coli* [16]. Similar types of receptor sites have been previously reported for a number of compounds viz. glycosides [17–19] and phloridzin [20] in kidney luminal membrane. Thus the development of both components of binding, i.e., the low- and high-affinity systems in pyridoxine deficiency might be attributed to the induction of an oxalate binding protein in intestinal brush border.

One of the characteristics of the carrier-mediated uptake processes is the competitive type of inhibition observed with structurally related compounds. In view of this phenomenon and also to find out the specificity of oxalate to the carrier, kinetic studies of oxalate binding to BBM in the presence of dicarboxylate ions were carried out. The half-saturation concentration of the inhibitor (IC_{50}) is the concentration of the competitor that displaces 50% of the oxalate binding. IC_{50} of oxalate was $0.40 \cdot 10^{-5}$ M. Among the inhibitors tested IC_{50} was in the following order, pyruvate > maleate > oxaloacetate > glyoxylate > parabonate > oxalate (Fig. 3).

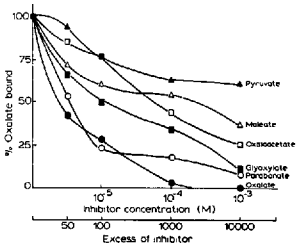

Fig. 3. Specificity of [^{14}C]oxalate binding to brush-border membrane. Aliquots of brush-border membrane (100 μg protein) were incubated at 30°C for 10 min in total volume of 1.0 ml (400 mM D-mannitol, 5 mM Tris-HCl (pH 7.0)) containing 100 nM [^{14}C]oxalate, with or without 5–10000-fold excess of unlabelled competing substrate as indicated. Binding was studied as detailed in Methods. IC_{50} is the concentration of inhibitor needed to displace 50% of [^{14}C]oxalate binding and was calculated to be, oxalate $0.4 \cdot 10^{-5}$ M, parabonate $0.6 \cdot 10^{-5}$ M, glyoxylate $1.0 \cdot 10^{-5}$ M, oxaloacetate $7.0 \cdot 10^{-5}$ M, maleate $30.0 \cdot 10^{-5}$ M and pyruvate $100 \cdot 10^{-5}$ M. All values are means of 8–10 independent observations.

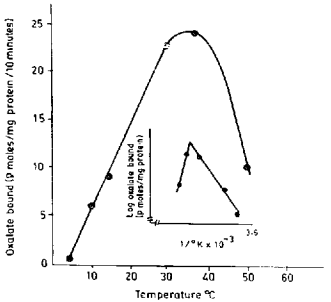

Fig. 4. Effect of temperature on oxalate binding. Pyridoxine-deficient rat intestinal brush-border membrane aliquots (100 μg membrane protein) were incubated with 100 nM [^{14}C]oxalate for 10 min. Incubations were carried out at 4–50°C. All values are means of 8–10 independent observations.

These results indicate that the compounds structurally related to oxalate viz. parabonate and glyoxylate had lower IC_{50} values as compared to other dicarboxylates tested. Moreover, 10000-fold excess concentration of the rest of the dicarboxylates tested was ineffective in competing with oxalate, demonstrating a high specificity of oxalate binding to intestinal brush-border membrane in pyridoxine deficiency. Additionally, the high-affinity binding of oxalate was highly sensitive to temperature (Fig. 4). Increase in temperature from 0°C to 40°C increased oxalate binding. The break point was found to be at 34°C for oxalate binding in intestinal BBM from pyridoxine-deficient rats. The apparent energy of activation was 19 kcal/mol for the slope below the break transition point as calculated from maximal binding at 30°C and 20°C.

The transition point in the Arrhenius plot (Fig. 4 inset) for oxalate binding in intestinal BBM derived from pyridoxine-deficient rats, is compatible with the hypothesis that 'Carrier' proteins of the transport system are part of an ordered lipoprotein structure. This system undergoes a phase transition at a temperature which is a function of the fatty acid composition of the lipid components. There is a considerable evidence that membrane transport systems in human erythrocytes membrane [21], endoplasmic reticulum [22], microsomal membrane [23] and intestinal BBM [24] are mediated by proteins which are intimately associated with the membrane lipids spanning the hydrophobic region of the membrane and generally required for their transport function. Previously we have reported that the prior administration of protein synthesis inhibitors like cyclohexamide and actinomycin-D, reversed the

carrier-mediated uptake of oxalate in pyridoxine-deficient rats [4].

Thus, several of the kinetic properties of high-affinity oxalate binding sites, i.e., saturability, substrate specificity, reversibility, ligand specificity and temperature dependence indicate involvement of a binding protein for oxalate transport. On the basis of all above findings, it is attractive to speculate that the binding of oxalate to intestinal BBM of pyridoxine-deficient rats represents the initial interactions in the transport of oxalate by pyridoxine-deficient rat intestine. The specific binding of oxalate, as demonstrated in this study and the excellent correlation between binding of analogues of oxalic acid, including parabonate and glyoxylate and uptake of oxalate by intestinal sacs, everted gut rings and BBM vesicles, as observed previously [8–10] provide further support to such a hypothesis.

The question whether the initial interaction of oxalate with isolated brush-border membranes (as described in this paper) represented specific binding of oxalate to receptor sites on the membrane or reflected the specific binding of oxalate followed by its uptake into membrane-bound vesicles is complex, but has been answered unequivocally from our studies, which will be described in detail elsewhere (Koul, H.K., Thind, S.K. and Nath, R., in preparation). Firstly, we performed experiments at hyptonic, isotonic and hypertonic media, by altering D-mannitol concentrations (0.0 mM–1.0 M), but the specific binding of oxalate was essentially the same in all experiments. Secondly, oxalate was found to be actively bound to membranous particles obtained by total disruption of isolated brush-border membranes by deoxycholate. Thus, in both of the above experiments the binding of oxalate with brush-border membranes was essentially unchanged by procedures which would have caused major alterations, if oxalate accumulated into vesicles constituted a significant share of the interaction.

The experiments described in this paper in addition to describing initial interactions of oxalate with BBM of pyridoxine-deficient rats, provide an insight into the component part of the transport system. Based on the above evidence it can be concluded that transport protein of BBM is involved as an oxalate carrier in pyridoxine deficiency, thus providing a new evidence to our current knowledge of oxalate transport in pyridoxine deficiency. Attempts to identify and characterize the protein and its transport capability by reconstitution are currently in progress and the results will be communicated in the near future.

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