Folate transport in intestinal brush border membrane: involvement of essential histidine residue(s)

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We examined the possible existence of histidine residue(s) in the folate transporter of rabbit intestine. This was done with use of the histidine-specific reagent diethyl pyrocarbonate (DEPC) and purified intestinal brush-border-membrane vesicles. DEPC caused significant concentration- and time-dependent inhibition of folic acid transport. The inhibition was only seen when transport was examined in vesicles incubated in buffer at pH 5.2 and not in those incubated in buffer at pH 7.4. The addition of unlabelled folic acid to vesicle suspension before treatment with DEPC (2.5 mM) led to a significant ($P < 0.01$) protection (84 %) against the inhibition of folic acid transport. Treating vesicles pretreated with DEPC (2.5 mM) with reducing reagents (dithiothreitol, 2-mercaptoethanol and 2,3-dimercaptopropanol, all at a final concentration of 10 mM) did not reverse the in-

hibitory effect of DEPC on folic acid transport. On the other hand, treating the DEPC-pretreated vesicles with hydroxylamine (140 mM) led to a significant reversal ($P < 0.01$) (54 %) of the inhibition of folic acid transport. The inhibitory effect of DEPC on carrier-mediated folic acid transport was found to be mediated through a decrease in the $V_{\max}$ (i.e. a decrease in the number and/or activity) of the carriers and an increase in the apparent K_m (i.e. a decrease in their affinity), classifying the effect as a mixed-type inhibition. These results demonstrate the existence of critical histidine residue(s) in the intestinal brush-border-membrane folate transporter which is essential for its interaction with, and transport of, the vitamin. These findings also suggest that the histidine residue(s) is located at (or near) the substrate-binding site.

INTRODUCTION

The transport mechanism of folate monoglutamate across mammalian small intestine has been the subject of intense investigation over the past two decades [1–10]. The transport process across the brush border membrane has been shown to occur via a carrier-mediated system which recognizes both reduced and oxidized forms of folate and the folate antagonist methotrexate, and is mainly localized in the proximal part of the small intestine [1–10]. This system was found to be driven by a transmembrane pH gradient (acidic outside with maximum uptake at pH 5.0–5.5), to be electroneutral in nature and severely inhibited by the stilbene derivatives 4,4'-di-isothiocyano-2,2'-disulphonic acid stilbene (DIDS) and 4-acetamido-4-isothiocyanostilbene-2,2'-disulphonic acid (SITS) [4,8–10]. Based on these findings it was suggested that folate transport occurred via a folate[−]:OH[−] exchange and/or a folate[−]:H⁺ co-transport mechanism [8–10]. So far, however, little is known about the molecular mechanism of action of this transporter with regard to important amino acid moieties and reactive groups that are important for its interaction with, and transport of, folate. The use of amino-acid-specific reagents have proven to be of great value in identifying such moieties in a number of membrane transporters [11–15]; in some cases this has assisted in the isolation and further characterization of the carrier involved [13]. In this study we report the existence of critical histidine residue(s) in the folate carrier of rabbit intestinal brush border membrane that is essential for its normal function of interacting with and transporting folate.

MATERIALS AND METHODS

Materials

[3,5,7,9-³H]Folic acid (specific radioactivity 35 Ci/mmol; radiochemical purity > 98 %) was purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO, U.S.A.). All other chemicals and reagents used in this study were of analytical quality and were obtained from commercial sources. Filters (cellulose nitrate, pore size 0.4 mm) were purchased from Millipore Corp. (Bedford, MA, U.S.A.).

Preparation of jejunal membrane vesicles and transport investigation

Brush-border-membrane vesicles (BBMV) were isolated from the jejunum of New Zealand White rabbits using a Mg²⁺-precipitation method [16] as described in detail by us previously [10,17]. Isolated BBMV were used fresh on the day of isolation. Transport investigations were performed at 37 °C by a rapid-filtration technique [18] described by us previously [10,17]. Incubation was done in incubation buffer containing 100 mM NaCl/80 mM mannitol/10 mM Hepes/10 mM Mes (at pH 5.2 or 7.4), with labelled and unlabelled folic acid and other constituents. Reaction was stopped by the addition of ice-cold stop solution (100 mM NaCl/100 mM mannitol/10 mM KH₂PO₄, pH 7.4). Protein concentrations of vesicle preparations were determined using the method of Lowry et al. [19], with BSA

Abbreviations used: DEPC, diethyl pyrocarbonate; BBMV, brush-border-membrane vesicles; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

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as the standard. Each transport study was done on multiple BBMVs preparations from different rabbits. Transport results are expressed as mean $\pm$ S.E.M. in terms of pmol of folic acid transported/mg of protein per unit time.

Pretreatment of rabbit jejunal BBMVs with diethyl pyrocarbonate (DEPC)

Pretreatment of intestinal BBMVs with DEPC was done as described previously [11,12,20]. Briefly, the final pellet of jejunal BBMVs was suspended in a treatment buffer containing 280 mM mannitol and 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6.4. Freshly prepared stock solution of DEPC was added to membrane suspensions (1–2 mg of protein) to give the desired final concentration of the reagent in a total suspension volume of 2.5 ml. The suspension was shaken (10 s) to ensure complete mixing and the reaction was allowed to proceed for the desired period of time at room temperature. During the pretreatment stage, the concentration of ethanol did not exceed 0.3%, and an equal amount of ethanol was added to control BBMVs suspensions, which were then taken through the same steps as those of DEPC-treated BBMVs. At the end of the pretreatment period, the reaction was terminated by the addition of 30 ml of ice-cold treatment buffer and the suspension was centrifuged for 20 min at 47800 g. The pellet was suspended in 30 ml of transport (intravesicular) buffer (280 mM mannitol and 20 mM Hepes/Tris, pH 7.4) and centrifuged for 20 min at 47800 g. This washing step was then repeated once more. Finally, BBMVs were suspended in suspension buffer and used for transport investigations.

Substrate-protection study and effect of reducing agents and hydroxylamine on DEPC inhibition of folic acid transport

In the substrate-protection study, unlabelled folic acid (100 μM) was added to BBMVs suspensions (pH 6.4) before addition of DEPC. Control vesicles were treated in the same manner but without DEPC.

In the experiments where the reversal of the inhibitory effect of DEPC on folic acid transport by reducing agents or by hydroxylamine was to be examined, BBMVs were first pretreated with DEPC for a specified period of time, then 30 ml of ice-cold treatment buffer was added and the membrane suspension was centrifuged at 47800 g for 20 min. The BBMVs pellet was resuspended in 30 ml of the treatment buffer containing freshly prepared reducing agent (10 mM) or 140 mM hydroxylamine (the solution of the latter was adjusted for osmolarity by reducing the mannitol concentration accordingly), allowed to stand for 45 min (90 min in the case of hydroxylamine) at room temperature, centrifuged, and the pellet was then washed twice with transport buffer as described above. The final pellet was then suspended in the desired volume of transport buffer and used for transport assays.

RESULTS

Effect of pretreatment of BBMVs with different concentrations of DEPC on folic acid transport

The effect of pretreatment of jejunal BBMVs with different concentrations of DEPC (0.5–5 mM) on the initial rate of transport (5 s; see [8,10]) of [^3H]folic acid (0.14 μM) was examined in this study. Transport was examined in incubation buffer at pH 5.2 and pH 7.4 (in both cases intravesicular pH was 7.4). The results (Table 1) showed concentration-dependent inhibition of [^3H]folic acid transport in vesicles incubated in buffer at pH 5.2, which became significant at 1 mM DEPC. No inhibition, on the

other hand, was seen of the transport of [^3H]folic acid in vesicles pretreated with the same concentrations of DEPC but incubated in buffer at pH 7.4. As expected, transport of folic acid in control (untreated) vesicles showed its usual pH-dependence by being significantly higher in incubation buffer at pH 5.2 than at pH 7.4.

In another study we examined the effect of the pretreatment time (5, 10, 15 and 20 min) of BBMVs with DEPC (2.5 mM) on the inhibitory effect of DEPC on [^3H]folic acid (0.14 μM) transport. Transport was measured after 5 s in an incubation buffer at pH 5.2. The results (Figure 1) showed a linear increase ($r = 0.99$) in inhibition of folic acid transport with increasing

Table 1 Effect of pretreatment of jejunal BBMVs with different concentrations of DEPC on transport of folic acid

Jejunal BBMVs were pretreated for 10 min with different concentrations of DEPC. Transport of folic acid (0.14 μM) was examined after 5 s in incubation buffers at pH 5.2 and pH 7.4. In both cases the vesicles' suspension pH was 7.4. Results are expressed in the form of mean $\pm$ S.E.M. Values in parentheses represent numbers of separate transport determinations from different rabbits. Statistical significance values were calculated using Student's *t*-test. Comparison was made relative to the simultaneously performed controls. Abbreviation: NS, not significant.

DEPC concn. (mM)	pH of incubation buffer...	Transport of folic acid (pmol/mg of protein per 5 s)	
		pH 5.2	pH 7.4
0 (Control)		1.09 ± 0.15 (8)	0.12 ± 0.01 (5)
0.5		0.75 ± 0.12 (6) NS	0.11 ± 0.02 (5)
1.0		0.63 ± 0.07 (8)†	0.09 ± 0.01 (5)
2.5		0.46 ± 0.07 (10)†	0.10 ± 0.006 (5)
5.0		0.22 ± 0.02 (6)†	0.09 ± 0.004 (6)

† $P < 0.01$.

Figure 1 Effect of pretreatment time of BBMVs with DEPC on folic acid transport

BBMVs were pretreated with 2.5 mM DEPC for different periods of time, followed by examination of the transport of 0.14 μM folic acid. Incubation was performed in buffer at pH 5.2 for 5 s at 37 °C. Each data point is the mean $\pm$ S.E.M. of four to eight separate transport determinations from two different rabbits. $y = -0.035x + 1.03$, $r = -0.99$.

exposure time of the BBMV to DEPC. A pretreatment time of 10 min was chosen as the standard time in these studies.

Substrate protection against the inhibitory effect of DEPC

In this study we examined the effect on subsequent transport of [3 H]folic acid ($0.14 \mu\text{M}$) of adding $100 \mu\text{M}$ of unlabelled folic acid to jejunal BBMV suspensions (pH 6.4) before addition of DEPC (2.5 mM). Transport was examined after 5 s in incubation buffer at pH 5.2. The results showed that such treatment is capable of providing significant ($P < 0.01$) protection (84%) against the inhibitory effect of DEPC on [3 H]folic acid transport compared with vesicles pretreated with DEPC but without prior addition of unlabelled folic acid. In fact, transport of folic acid under these conditions became insignificantly different from that of untreated control vesicles [transport levels of 0.88 ± 0.05 ($n = 5$), 0.32 ± 0.04 ($n = 6$) and 0.79 ± 0.12 ($n = 5$) pmol/mg of protein per 5 s were found in control vesicles, vesicles pretreated with DEPC, and vesicles to which unlabelled folic acid was first added followed by treatment with DEPC respectively].

Nature of inhibitory effect of DEPC on folic acid transport

The possibility that DEPC inhibition of folic acid transport is mediated through interaction of DEPC with thiol rather than histidyl groups was investigated by examining the ability of dithiothreitol, 2-mercaptoethanol and 2,3-dimercaptopropanol (all at 10 mM) to reverse the inhibition caused by 2.5 mM DEPC on [3 H]folic acid ($0.14 \mu\text{M}$) transport. These reducing agents are known to be able to reverse the inhibition in thiol groups in proteins. Uptake was performed after 5 s in a buffer at pH 5.2. The results showed that none of the reducing agents tested could reverse the inhibitory effect of DEPC on folic acid transport [transport levels of 1.13 ± 0.06 ($n = 7$), 0.52 ± 0.05 ($n = 7$), 0.55 ± 0.07 ($n = 7$), 0.53 ± 0.06 ($n = 7$) and 0.53 ± 0.09 ($n = 7$) pmol/mg of protein per 5 s were found in control vesicles, vesicles pretreated with DEPC, and vesicles pretreated with DEPC and then treated with dithiothreitol, 2-mercaptoethanol and 2,3-dimercaptopropanol respectively].

In another study we examined whether treatment with 140 mM hydroxylamine (the hydrochloride) of BBMV pretreated with DEPC (2.5 mM) could reverse the inhibitory effect of the reagent on [3 H]folic acid ($0.14 \mu\text{M}$) transport [11,21]. Transport was examined after 5 s in an incubation buffer at pH 5.2. The results showed that hydroxylamine is indeed capable of significantly reversing ($P < 0.01$) (54%) the inhibitory effect caused by DEPC on folic acid transport [transport levels of 0.88 ± 0.08 ($n = 5$), 0.32 ± 0.04 ($n = 5$) and 0.62 ± 0.06 ($n = 5$) pmol/mg of protein per 5 s were found in control vesicles, vesicles pretreated with DEPC, and those pretreated with DEPC and then treated with 140 mM hydroxylamine respectively].

Effect of DEPC on folic-acid-transport kinetic parameters

The effect of pretreatment of BBMV with 2.5 mM DEPC on the kinetic parameters (i.e. the V_{max} and the apparent K_m) of the carrier-mediated DIDS-sensitive folic acid transport system was examined in this study. The initial rate of folic acid transport (5 s) was simultaneously examined as a function of concentration (0.14 – $6 \mu\text{M}$) in control and DEPC-pretreated vesicles incubated in buffer at pH 5.2. Non-mediated DIDS-insensitive folic acid transport was determined as described before [8] by examining the transport of folic acid in control and DEPC-pretreated vesicles in the presence of 1 mM DIDS. Uptake by this

Figure 2 Effect of pretreatment of BBMV with DEPC on kinetic parameters of carrier-mediated DIDS-sensitive folic acid transport

Folic acid transport was simultaneously examined as a function of concentration (0.014 – $6 \mu\text{M}$) in BBMV pretreated with 2.5 mM DEPC and in control vesicles. Incubation was done for 5 s in buffer at pH 5.2. Closed circles represent total transport. Open circles represent transport by the carrier-mediated DIDS-sensitive component determined as described in the text. Each data point is the mean $\pm$ S.E.M. of four to six separate transport determinations from two different rabbits.

component was then subtracted from total uptake to determine carrier-mediated DIDS-sensitive transport. The results are presented in Figure 2. In both control and DEPC-pretreated vesicles, transport of folic acid by the carrier-mediated process was found to be saturable and to obey Michaelis–Menten kinetics. Pretreatment of vesicles with DEPC was found to cause a marked inhibition of the V_{max} of the folic acid transport process (2.76 and 4.56 pmol/mg of protein per 5 s for DEPC-pretreated and control vesicles respectively) and a marked increase in the apparent K_m of the transport system ($0.42 \mu\text{M}$ and $0.23 \mu\text{M}$ for DEPC-pretreated and control vesicles respectively).

DISCUSSION

In both mammalian and bacterial cells a number of H^+ (pH)-driven transport systems have been shown to possess histidine residues which are essential for their function [12,22,23]. Our aim in the present study was to investigate the possible existence of such a residue(s) in the folate membrane transporter of rabbit small intestine, a pH-driven transport system. To do so we used the histidyl-specific reagent DEPC [21]. DEPC pretreatment of jejunal BBMV was found to cause concentration-dependent inhibition of the transport of folic acid in vesicles incubated in buffer at pH 5.2, but not in vesicles incubated in buffer at pH 7.4. Because folate transport is carrier-mediated only at acidic pH, and is non-mediated in incubation buffer of pH 7.4 and above [2,24], these findings indicate that the inhibitory effect of DEPC is directed exclusively toward the carrier-mediated system, with no effect on the diffusion (non-mediated) component. The inhibitory effect of DEPC on folic acid transport was also found to be time-dependent in nature, and increased in a linear manner with the increase of the exposure time of BBMV to the reagent.

Under certain conditions, DEPC has been reported to react with groups other than the histidine residues (e.g. thiol groups). This possibility, however, can reasonably be excluded in the present study. This is because during treatment of a protein with

DEPC in buffer at pH values of 5.5–7.5, the reagent interacts specifically with histidine residues [12]. In our case treatment of BBMV with DEPC was done at pH 6.4. Furthermore, if the interaction was indeed happening with thiol groups, then the reducing agents dithiothreitol, 2-mercaptoethanol and 2,3-dimercaptopropanol (compounds which are known to reverse inhibition of thiol groups in proteins) would be expected to reverse the inhibition of folate transport. Such treatment did not reverse DEPC inhibition of folate transport (see the Results section). Finally, the ability of hydroxylamine (which is known to reactivate DEPC-inhibited histidyl groups in proteins; [11,21]) to significantly reverse the inhibitory effect of DEPC on folic acid transport further supports the conclusion that DEPC was specifically interacting with histidyl groups in the folic acid transporter.

An important observation was the ability of unlabelled folic acid, when added to the vesicle suspension prior to treatment with DEPC, to protect the folate transporter. This finding suggests that the histidine residue(s) with which DEPC is interacting is located at (or near) the substrate-binding site. The possibility that the interaction of unlabelled folic acid with the membrane transporter might have caused conformational changes in the carrier protein that subsequently led to alteration in reactivity of histidine residues that are located outside the folic-acid-binding site could not, however, be excluded at present.

The inhibitory effect of DEPC on folic acid transport was found to be mediated through a marked inhibition of the $V_{\max}$ of the transport process, with an increase in apparent K_m of the transporter. These findings suggest that DEPC inhibition is mediated through a decrease in the number (and/or activity) of the functional folic acid transporters and a decrease in their affinity respectively. The above findings also indicate that DEPC inhibition of folic acid transport is a mixed-type inhibition.

In summary, the present study demonstrates the existence of histidine residue(s) in the folic acid transporter of rabbit small intestine which is essential for its normal function. Furthermore,

our findings suggest that the histidine residue(s) is located at (or near) the substrate-binding site.

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