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Involvement of histidine residues and sulfhydryl groups in the function of the biotin transport carrier of rabbit intestinal brush-border membrane

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Possible involvement of histidine residues and sulfhydryl groups in the function of the intestinal brush-border membrane (BBM) transporter of biotin was investigated. This was done by examining the effects of pretreatment of BBM vesicle (BBMV) isolated from rabbit intestine with the histidine-specific reagent diethyl pyrocarbonate (DEPC) and the sulfhydryl group-specific reagents *p*-chloromercuribenzenesulfonic acid (*p*-CMBS) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) on carrier-mediated biotin transport. Pretreatment of BBMV with DEPC caused significant inhibition in the initial rate of biotin transport without affecting the substrate uptake at equilibrium. Addition of biotin plus Na^+ to vesicle suspensions prior to treatment with DEPC provided significant protection to biotin transport. Treatment of DEPC-pretreated vesicles with the reducing agents dithiothreitol and 2,3-dimercaptopropanol failed to reverse the inhibitory effect of DEPC on biotin transport. The inhibitory effect of DEPC was found to be mediated through a marked decrease in the number of the functional biotin transport carriers with no change in their affinity, as indicated by the severe inhibition in the V_{max} but not the apparent K_m of the biotin transport process, respectively. Pretreatment of BBMV with *p*-CMBS and NBD-Cl also caused significant inhibition in the initial rate of biotin transport without affecting the substrate uptake at equilibrium. Addition of biotin plus Na^+ to vesicle suspensions prior to treatment with *p*-CMBS (or NBD-Cl) failed to protect biotin transport from inhibition. On the other hand, treatment of vesicles pretreated with *p*-CMBS (or NBD-Cl) with the reducing agents dithiothreitol and mercaptoethanol caused significant reversal in the inhibition of biotin transport. The inhibitory effects of *p*-CMBS (and NBD-Cl) on biotin transport was also found to be mediated through inhibition in the V_{max}, but not the apparent K_m, of biotin transport process. These results indicate the involvement of histidine residues and sulfhydryl groups in the normal function of the biotin transport system of rabbit intestinal BBM. Furthermore, the results also suggest that the histidine residues are probably located at (or near) the substrate-binding site while the sulfhydryl groups are located at a site other than the substrate binding region.

Introduction

Previous studies from our laboratory have shown that transport of the water-soluble vitamin biotin across the brush-border membrane domain of rabbit, rat and human intestine is via a specialized, carrier-mediated system [1–3]. This system was found to be Na^+ gradient-dependent and is capable of accumulating the substrate against a concentration gradient in the intravesicular space [1–3]. Furthermore, this system was found to be regulated by the level of the vitamin in the diet [4] and by ontogeny [5]. Nothing, however, is known about the functional residues and groups in this transporter that are important for its normal function. In recent years, the use of amino acid residue- and group-specific-reagents have proven to be a valuable tool in identifying moieties of membrane transporters that are important for their function [6–16]. These, in certain cases, have subsequently assisted in the effort toward isolation of these carriers [9]. In this study, we investigated the possible involvement of histidine residues and sulfhydryl groups in the normal function of the intestinal biotin transport carrier using residue- and group-specific reagents.

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Materials and Methods

Materials

[8,9-^{3}H]Biotin (spec. act. > 40 Ci/mmol; radiochemical purity > 95%) and ^{22}Na (spec. act. 110 Ci/g) were obtained from New England Nuclear. Cellulose nitrate filters (0.45 mm pore size) were obtained from Millipore Corp. All other chemicals and reagents were obtained from commercial sources and were of analytical quality.

Methods

Isolation of intestinal brush border membrane vesicles (BBMV) and transport studies

BBMV were isolated from the jejunum of New Zealand white rabbits using a Mg^{2+} precipitation method [17] as described by us previously [1,3]. BBMV were used fresh on the day of isolation and were suspended in a buffer (transport buffer) of 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4). Transport studies were performed at 37°C by a rapid-filtration technique [18] as described by us previously [1,3]. The incubation buffer was 100 mM NaCl or KCl, 80 mM mannitol and 20 mM Hepes, pH 6.5 (pH was adjusted with NaOH or KOH, respectively) (we used this pH because it represents the physiological pH at the luminal surface of the small intestine [19]), containing (unless otherwise specified) 0.08 μM [^{3}H]biotin. The stop solution was 100 mM NaCl, 100 mM mannitol and 10 mM KH_2PO_4 (pH 7.4). Protein concentrations of vesicles suspensions were determined using the method of Lowry et al. [20] with bovine serum albumin as the standard. Each transport study was done on multiple BBMV preparations from different rabbits. Quantitative, but not qualitative, variations were observed in the absolute amount of biotin transported in some BBMV preparations. For this reason, appropriate controls were run simultaneously with each study to allow proper comparison of the effect of different reagents and conditions on carrier-mediated biotin transport. Results of biotin transport by the carrier-mediated system (determined as described previously [3] are presented as mean ± S.E. in pmol/mg protein per unit time.

Pretreatment of BBMV with amino acid- and group-specific-reagents

Pretreatment with DEPC. Intestinal BBMV were pretreated with DEPC using the method and conditions described by Bindslev and Wright [14] and Miyamoto et al. [12] with renal BBMV. Briefly, BBMV were suspended in a treatment buffer of 280 mM mannitol and 20 mM KH_2PO_4/K_2HPO_4 (pH 6.4). Concentrated DEPC solution (60 mM) was prepared fresh immediately prior to use and 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 then shaken for 10 s to insure complete mixing and the reaction was allowed to proceed for 10 min at room temperature. During the pretreatment with DEPC, ethanol concentration did not exceed 0.3% and an equal amount of ethanol was added to control membrane suspensions which were then taken through the identical steps as that of DEPC-treated vesicles. At the end of 10 min, 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 then suspended in 30 ml of transport buffer (pH 7.4) and centrifuged for 20 min at 47800 × g. This step was then repeated one more time. Finally, BBMV were suspended in transport buffer to achieve a protein concentration of 2–4 mg/ml.

Pretreatment with p-CMBS, NBD-Cl and N-acetylimidazole. Pretreatment of intestinal BBMV with *p*-CMBS, NBD-Cl and *N*-acetylimidazole was done as described previously by Miyamoto et al. [12] with renal BBMV. In principal pretreatment and subsequent washing were similar to those described above with DEPC, except that the treatment buffer pH was 7.5. Stock solutions of *p*-CMBS and *N*-acetylimidazole were prepared fresh, in treatment buffer and that of NBD-Cl were prepared fresh in ethanol. BBMV were pretreated with *p*-CMBS for 30 min and with NBD-Cl and *N*-acetylimidazole for 60 min at room temperature as described before [12]. In BBMV pretreated with *p*-CMBS, an equal concentration of EDTA was included in the *p*-CMBS solution in order to chelate any free mercury.

Substrate protection and reversal of inhibition of biotin transport by reducing agents

In the substrate protection studies, biotin (70 μM) plus Na^+ (100 mM) were added to BBMV suspensions prior to the addition of the inhibitor. Control vesicles were treated in the same manner but without the inhibitor.

In the experiments when the reversal of the inhibitory effects of DEPC, *p*-CMBS and NBD-Cl on biotin transport by reducing agents were to be examined, vesicles were first pretreated with the inhibitor as described above, then 30 ml of treatment buffer was added and the vesicles suspension was centrifuged at 47800 × *g* for 20 min. The BBMV pellet was resuspended in 30 ml of the treatment buffer containing 10 mM of the reducing agent which was prepared fresh immediately before use. The suspension was then allowed to stand for 45 min at room temperature, centrifuged and the pellet was then washed twice with transport buffer as described earlier. Treatment of

BBMV with the reducing agents alone did not affect biotin transport compared to untreated controls, data not shown.

Results

Effect of pretreatment of BBMV with DEPC on biotin carrier-mediated transport

The effect of pretreatment of rabbit jejunal BBMV with 1,2.5 and 20 mM of the histidine-specific reagent DEPC [12,14,21] on the initial rate of transport (10 s) and equilibrium uptake (60 min) (1 and 3) of 0.08 μM [^{3}H]biotin was examined. It was found that DEPC causes significant ($P < 0.01$) inhibition in the initial rate of biotin transport (Table IA) (the inhibition was significantly higher when pretreatment of BBMV with DEPC was done at 37°C for longer times and became complete when the pretreatment was done for 40 min; data not shown). Equilibrium uptake of biotin, on the other hand, was not affected by DEPC (0.251 ± 0.041 ($n = 9$) and 0.255 ± 0.030 ($n = 6$) pmol/mg protein per 60 min for control and 20 mM DEPC, respectively).

We also examined the effect of pretreatment of BBMV with DEPC (2.5 mM) on the uptake of 100 mM ^{22}Na. The results showed slight, but significant ($P < 0.05$), inhibition in ^{22}Na uptake in DEPC-pretreated vesicles compared to control vesicles (61.8 ± 2.8 ($n = 11$) and 79.8 ± 6.1 ($n = 10$) nmol/mg protein per 10 s, respectively).

Substrate protection against the inhibitory effect of DEPC. In this study, we examined the effect of adding biotin (70 μM) plus Na^+ (100 mM) to BBMV suspensions prior to treatment of these vesicles with DEPC (2.5 mM), on subsequent transport of 0.08 μM [^{3}H]biotin. The result (Table II) showed that the addition of biotin plus Na^+ prior to the addition of the inhibitor provided significant ($P < 0.01$) (53%) protection to biotin transport against the inhibitory effect of DEPC. (Higher protection (73%) was seen when the concentration of unlabeled biotin was increased to 0.5 mM, the concentration of Na^+ was increased to 140 mM and the pretreatment was done at 37°C; data not shown.)

TABLE I

Effect of pretreatment of rabbit jejunal BBMV with different concentrations of DEPC, p-CMBS and NBD-Cl on the initial rate of biotin carrier-mediated transport

	Concn. (mM)	Transport (pmol/mg protein per 10 s)	Inhibition (%)
A. DEPC	0 (Control)	0.089 ± 0.005 (16) [a]	
	1.0	0.059 ± 0.005 (7)	34
	2.5	0.050 ± 0.005 (9)	44
	20	0.036 ± 0.004 (5)	60
B. *p*-CMBS	0 (Control)	0.086 ± 0.006 (17)	
	0.5	0.054 ± 0.005 (5)	37
	1.0	0.036 ± 0.005 (15)	58
C. NBD-Cl	0 (Control)	0.077 ± 0.006 (13) [a]	
	0.1	0.044 ± 0.008 (9)	43
	0.3	0.034 ± 0.003 (16)	56

[a] Number of transport measurements from three to five different BBMV preparations.

TABLE II

Substrate protection against the inhibitory effect of DEPC

Condition	Transport (pmol/mg protein per 10 s)
Control	0.098 ± 0.003 (12) [a]
DEPC (2.5 mM)	0.051 ± 0.003 (14)
Biotin (70 μM) + Na^+ (100 mM) then DEPC (2.5 mM)	0.076 ± 0.004 (11)

[a] Number of transport measurements from at least four different BBMV preparations.

Nature of the inhibitory effect of DEPC. The possibility that the inhibitory effect of DEPC on biotin transport is mediated through interaction of the inhibitor with sulfhydryl groups rather than histidine groups in the carrier site was addressed in these experiments. This was done by testing the ability of the reducing agents dithiothreitol and 2,3-dimercaptopropanol (10 mM), which are known to reverse the inhibition in sulfhydryl groups in proteins, to reverse the inhibition caused by 2.5 mM DEPC on the transport of 0.08 μM [^{3}H]biotin. The results showed that neither of the reducing agents was able to significantly reverse the inhibitory effect of DEPC on biotin transport (transport of 0.083 ± 0.006 ($n = 9$), 0.048 ± 0.003 ($n = 8$), 0.052 ± 0.004 ($n = 6$) and 0.050 ± 0.004 ($n = 8$) pmol/mg protein per 10 s were found in control vesicles, vesicles pretreated with DEPC and vesicles pretreated with DEPC then with dithiothreitol and 2,3-dimercaptopropanol, respectively).

Hydroxylamine has been shown to be able to reverse the inhibitory effect of DEPC on histidine residues in certain proteins [21]. Thus, we also examined whether treatment of DEPC-pretreated vesicles with 100 mM hydroxylamine(-hydrochloride) could reverse the inhibitory effect of DEPC (2.5 mM) on [^{3}H]biotin (0.08 μM) transport. The results, however, showed that hydroxylamine could not reverse the inhibitory effect of DEPC on biotin transport to any significant extend (transport of 0.104 ± 0.003 ($n = 14$), 0.055 ± 0.003 ($n = 12$) and 0.061 ± 0.002 ($n = 5$) pmol/mg protein per 10 s were reported in control vesicles, those pretreated with DEPC, and those pretreated with DEPC then with 100 and 140 mM hydroxylamine, respectively). (We also examined the effect of 250 and 500 mM hydroxylamine and again did not see significant reversal in biotin transport inhibition; data not shown.)

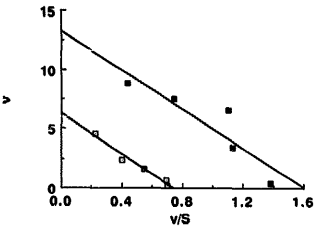

Fig. 1. Effect of pretreatment of rabbit jejunal BBMV with DEPC on the kinetic parameters of biotin carrier-mediated transport. Carrier-mediated biotin transport was examined in BBMV pretreated with DEPC (2.5 mM) and in control vesicles as a function of increasing the substrate concentration (0.3–20 μM). Incubation was performed for 10 s at 37°C. Transport results are from 3–8 separate transport determinations from four different BBMV preparations and are presented as the Woolf-Augustinsson-Hofstee plot, i.e., v/S against v. v is expressed in pmol/mg protein per 10 s; S is expressed in μM. Kinetic parameters (i.e., the apparent K_m and V_{max}) of the biotin carrier-mediated transport were calculated using linear regression analysis. For DEPC-pretreated vesicles (opened squares) $Y = -8.48X + 6.28$, $r = 0.99$. For control vesicles (closed squares) $Y = -8.29X + 13.28$, $r = 0.90$.

Effect of DEPC on the kinetic parameters of biotin carrier-mediated transport. In this study, we examined 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 biotin carrier-mediated transport system. Initial rate of biotin transport (10 s) was simultaneously examined in control and DEPC-pretreated vesicles as a function of concentration (0.3–20 μM). The results are presented in Fig. 1 in the form of the Woolf-Augustinsson-Hofstee plot. In both cases, the data produced straight lines with a correlation coefficient of 0.90 and 0.99 for control and DEPC-pretreated vesicles, respectively, indicating that biotin transport is saturable and obeys Michaelis-Menten kinetics. Pretreatment of vesicles with DEPC was found to cause a marked inhibition in the V_{max} of the biotin transport process (13.28 and 6.28 pmol/mg protein per 10 s for control and DEPC-pretreated vesicles, respectively) with no effect on the apparent K_m of the transport system (8.29 and 8.48 μM for control and DEPC-pretreated vesicles, respectively).

Effect of pretreatment of BBMV with sulfhydryl group reagents on carrier-mediated biotin transport

The effect of pretreatment of BBMV with the sulfhydryl group reagent *p*-CMBS on the initial rate of biotin transport (10 s) and on equilibrium uptake (60 min) of 0.08 μM [^{3}H]biotin was examined in these studies. The results showed that *p*-CMBS inhibits, in a significant manner ($P < 0.01$), the initial rate of biotin transport (Table IB) (the degree of inhibition was significantly higher when pretreatment was done at 37°C for 45 and 60 min; data not shown). No inhibition in biotin uptake at equilibrium, on the other hand, was seen (0.283 ± 0.038 ($n = 5$), 0.251 ± 0.075 ($n = 5$) and 0.255 ± 0.095 ($n = 5$) pmol/mg protein per 60 min for control and 0.5 and 1 mM *p*-CMBS, respectively). Similarly, pretreatment of BBMV with the sulfhydryl group reagent NBD-Cl (Ref. 10) caused significant ($P < 0.01$) inhibition in the initial rate of biotin transport (Table IC) but not equilibrium uptake of the substrate (equilibrium uptake of 0.199 ± 0.034 ($n = 6$), 0.196 ± 0.050 ($n = 6$) and 0.217 ± 0.018 ($n = 6$) pmol/mg protein per 60 min for control and 0.1 and 0.3 mM NBD-Cl, respectively). Neither of these reagents caused rapid dissipation of the Na^+ gradient as no increase in ^{22}Na influx was seen in vesicles pretreated with these reagents, data no shown.

Substrate protection against the inhibitory effect of sulfhydryl group reagents. The effect of adding biotin (70 μM) plus Na^+ (100 mM) to vesicle suspensions prior to pretreatment with *p*-CMBS (1 mM) on transport of 0.08 μM [^{3}H]biotin was examined in this study. The results showed that such a treatment does not protect the biotin transport system against the inhibitory effect of *p*-CMBS (biotin transport of 0.077 ± 0.005 ($n = 11$), 0.031 ± 0.001 ($n = 8$) and 0.032 ± 0.005 ($n = 9$) pmol/mg protein per 10 s were found in control vesicles, vesicles pretreated with *p*-CMBS and those pretreated with *p*-CMBS after the addition of biotin plus Na^+, respectively). Similarly, the addition of biotin and Na^+ prior to pretreatment with NBC-Cl failed to protect the biotin transport carrier against inhibition (biotin transport of 0.077 ± 0.006 ($n = 11$), $0.042 + 0.004$ ($n = 10$) and 0.046 ± 0.003 ($n = 10$) pmol/mg protein/10 s were found in control vesicles, vesicles pretreated with NBD-Cl and those pretreated with NBD-Cl after the addition of biotin plus Na^+, respectively).

Nature of the inhibitory effect of sulfhydryl group reagents on biotin transport. To confirm that the inhibition of biotin transport by *p*-CMBS and NBD-Cl is mediated through their interaction with sulfhydryl groups in the biotin transport system, we examined the ability of the reducing agents dithiothreitol and mercaptoethanol (10 mM) to reverse the inhibition caused by *p*-CMBS (1 mM) and NBD-Cl (0.3 mM) on [^{3}H]biotin (0.08 μM) transport. The results (Table III) showed that these reducing agents are indeed capable of significantly ($P < 0.01$) reversing the inhibition caused by *p*-CMBS on biotin transport; dithiothreitol and mercaptoethanol caused 71% and 35% reversal, respectively. Similarly, reducing agents caused significant ($P < 0.01$) reversal in the inhibition caused by NBD-Cl on biotin transport; dithiothreitol and mercaptoethanol caused 56% and 67% reversal, respectively.

Effect of sulfhydryl group reagents on the kinetic parameters of biotin transport. The effect of pretreatment of BBMV with the sulfhydryl group inhibitors *p*-CMBS (1 mM) and NBD-Cl (0.3 mM) on the kinetic parameters of biotin carrier-mediated transport was examined in this study. Biotin initial rate of transport (10 s) was examined simultaneously in control and *p*-CMBS- (or NBD-Cl-) pretreated vesicles as a function of increasing the substrate concentration (0.3–20 μM) in the incubation medium. The results presented as v/S against v (Fig. 2) showed that pretreatment of BBMV with *p*-CMBS leads to marked inhibition in the V_{max} of biotin transport (16.62 and 8.09 pmol/mg protein per 10 s in control and *p*-CMBS-pretreated vesicles, respectively) without affecting the apparent K_m (7.53 and 8.10 μM in control and *p*-CMBS-pretreated vesicles, respectively). Similarly, pretreatment of BBMV with NBD-Cl caused sever inhibition in the V_{max} of biotin transport (14.17 and 4.34 pmol/mg protein/10 s in control and NBD-Cl-pretreated vesicles, respectively) but not the apparent K_m (6.73 and 7.65 μM in control and NBD-Cl-pretreated vesicles, respectively).

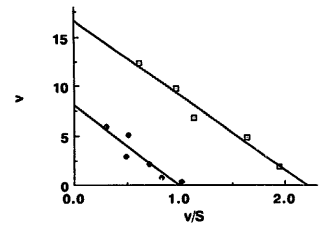

Fig. 2. Effect of pretreatment of rabbit jejunal BBMV with *p*-CMBS on the kinetic parameters of biotin carrier-mediated transport. Carrier-mediated biotin transport was examined in BBMV pretreated with *p*-CMBS (1 mM) and in control vesicles as a function of increasing the substrate concentration (0.3–20 μM). Incubation was performed for 10 s at 37°C. Transport results are from 3–6 separate transport determinations from three different BBMV preparations and are presented as the Woolf-Augustinsson-Hofstee plot, i.e., v/S against v. v is expressed in pmol/mg protein per 10 s; S is expressed in μM. Kinetic parameters (i.e., the apparent K_m and V_{max}) of the biotin carrier-mediated transport were calculated using linear regression analysis. For *p*-CMBS pretreated vesicles (closed squares) $Y = -8.10X + 8.09$, $r = 0.93$. For control vesicles (opened squares) $Y = -7.53X + 16.62$, $r = 0.98$.

Effect of pretreatment of BBMV with N-acetylimidazole on carrier-mediated biotin transport

Among a number of tyrosine-specific reagents that have been used in previous studies to produce chemical modifications in tyrosine residues in proteins, *N*-acetylimidazole appears to be the most specific [22]. Thus, to investigate the possible involvement of tyrosine residues in the function of the biotin transport carrier, we examined the effect of pretreatment of BBMV with different concentrations of this reagent (1–10 mM) on the transport of [^{3}H]biotin (0.08 μM).

TABLE III

Ability of reducing agents to reverse the inhibitory effect of p-CMBS and NBD-Cl on biotin transport

	Transport (pmol/mg protein per 10 s)
A. *p*-CMBS	
Control	0.787 ± 0.005 (11) [a]
p-CMBS (1 mM)	0.035 ± 0.006 (10)
p-CMBS (1 mM) then dithiothreitol (10 mM)	0.072 ± 0.008 (8)
p-CMBS (1 mM) then mercaptoethanol (10 mM)	0.053 ± 0.003 (10)
B. NBD-Cl	
Control	0.070 ± 0.006 (7)
NBD-Cl (0.3 mM)	0.031 ± 0.003 (10)
NBD-Cl (0.3 mM) then dithiothreitol (10 mM)	0.053 ± 0.005 (10)
NBD-Cl (0.3 mM) then mercaptoethanol (10 mM)	0.057 ± 0.005 (10)

[a] Number of separate transport measurements from three to four different BBMV preparations.

The results showed no inhibition in biotin transport occur by any of the *N*-acetylimidazole concentrations tested (transport of 0.073 ± 0.04 (n = 8), 0.078 ± 0.004 (n = 8), 0.075 ± 0.003 (n = 8) and 0.08 ± 0.005 (n = 7) pmol/mg protein per 10 s were found in control vesicles and vesicles pretreated with 1, 5 and 10 mM *N*-acetylimidazole, respectively). These findings indicate that tyrosine groups does not appear to be involved in the normal functioning of the biotin transport carrier.

Discussion

The aim of the present study was to examine the possible involvement of histidine residues and sulfhydryl groups in the normal function of the biotin transport carrier of intestinal BBM with the use of residue- and group-specific reagents. Recent studies have shown that these groups are involved in the normal function of a number of membrane transporters in renal and intestinal cells [6,7,12–14]. The results of our studies with the histidine-specific reagent DEPC [12,14] showed that this reagent causes a significant inhibition in the initial rate of biotin transport without affecting the substrate uptake at equilibrium. The latter finding also indicate that DEPC does not affect vesicles size and physical integrity.

As we stated earlier, biotin transport by the carrier-mediated system is dependent on Na^+ gradient [1–3].

Thus, it is possible that DEPC inhibition of biotin transport is mediated through rapid dissipation of the Na^+ gradient imposed across the BBM. To test this possibility, we examined the effect of pretreatment of BBMV with DEPC on the transport of ^{22}Na. The results showed that the uptake of ^{22}Na in DEPC-pretreated vesicles is not increased. In fact a slight (though significant) decrease in ^{22}Na uptake was observed. This finding excludes the possibility that the inhibitory effect of DEPC on biotin transport is mediated through dissipation of the Na^+ gradient.

DEPC is considered to be a specific histidine reagent when used at a pH range of 5.5 to 7.5 [21]. At higher pH values, DEPC specificity, however, decreases and the compound begins to interact with other groups such as sulfhydryl groups and tyrosine residues. Because pretreatment of our BBMV with DEPC was performed at pH 6.4, it is reasonable, therefore, to assume that DEPC is interacting with histidine residue(s) in the biotin transporter. This conclusion was further supported by the finding that the reducing agents dithiothreitol and 2,3-dimercaptopropanol could not reverse the inhibitory effect caused by DEPC on biotin transport. In addition, the inability of *N*-acetylimidazole, a highly specific tyrosine modifying reagent [11,12,22] to inhibit biotin transport argue against the possibility that DEPC is interacting with tyrosine residues.

Hydroxylamine has been reported to be able to reveres DEPC inhibition of proteins [21]. In our study, however, hydroxylamine (100–500 mM) failed to reverse the inhibitory effect of DEPC on biotin transport, a finding which was also seen with other enzymes and membrane transport proteins [12,14,23,24]. The reason(s) for that is not clear, but could be because at these concentrations of DEPC, carbethoxylation reaction between DEPC and the second nitrogen atom in the imidazole ring of the histidine residue might occur [14,25]. This second reaction is known to be irreversible in nature and does not respond to hydroxylamine treatment [14,25].

The inhibitory effect of DEPC on biotin transport was significantly prevented by the addition of biotin plus Na^+ to vesicle suspensions prior to the addition of DEPC. This finding suggests that DEPC is interacting with histidine residues that are located at (or near) the substrate-binding site. The possibility that binding of biotin and Na^+ to the biotin transport carrier might have initiated a conformational changes in the carrier that led to changes in the reactivity of histidine groups located outside the substrate binding site with DEPC, however, could not be excluded at this stage.

The inhibitory effect of DEPC on biotin transport was found to be mediated through a decrease in the number of the functional biotin transport carriers without changes in the affinity of these carriers. This conclusion is based on the observation that pretreatment of BBMV with DEPC led to a severe inhibition in the V_{max} of the biotin transport process without a marked change in the apparent K_m, respectively.

The results of the studies with the sulfhydryl group reagents *p*-CMBS and NBD-Cl showed that these reagents cause significant inhibition in the initial rate of carrier-mediated biotin transport, indicating the involvement of sulfhydryl group(s) in the function of the biotin transport system. Uptake of biotin at equilibrium, however, was not affected by either inhibitor indicating lack of effect of these inhibitors on vesicles size and integrity. Also, neither of these reagents was found to affect the Na^+ gradient imposed across the membrane as no stimulation in ^{22}Na transport was seen in vesicles treated with these reagents. Previous studies have reported that NBD-Cl could, under certain conditions, reacts with not only sulfhydryl groups but also with amino and tyrosine residues in enzymes and transport proteins [26–28]. However, since the reaction of NBD-Cl with amino groups occur at a significant rate only at pH greater than 8 and is almost negligible at pH 7–7.5 [26], it is unlikely that NBD-Cl inhibition of biotin transport is due to interaction with amino groups since treatment of our BBMV with the reagent was done at pH 7.5. Similarly, the possibility that NBD-Cl inhibition of biotin transport is mediated through its interaction with tyrosine residues is unlikely because *N*-acetylimidazole, a more specific inhibitor of tyrosine residues [22], failed to inhibit biotin transport to any significant extent. Finally, the ability of the reducing agents dithiothreitol and mercaptoethanol to significantly reverse the inhibitory effect caused by *p*-CMBS and NBD-Cl further indicate that these inhibitors are indeed interacting with sulfhydryl groups. These sulfhydryl groups appear to be located at a site other than the substrate-binding site because the addition of biotin plus Na^+ to BBMV suspensions prior to the addition of *p*-CMBS or NBD-Cl failed to protect the biotin transport system from inhibition.

The inhibitory effect caused by *p*-CMBS and NBD-Cl was found to be mediated through a marked decrease in the number of the functional biotin transport carriers with no changes in the affinity of these carriers. This conclusion is again drawn from the observation that these inhibitors caused severe inhibition in the V_{max} of the biotin transport process without markediy affecting the apparent K_m, respectively.

In summary, the present study demonstrate the involvement of histidine residues and sulfhydryl groups in the biotin transport system of the intestinal BBM which are important for its function. Furthermore, the results suggest that the histidine residues are probably located at (or near) the substrate binding-site while the sulfhydryl groups are located at a site other than the substrate binding region.

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