Identification and Conformational Changes of the Intestinal Proline Carrier*

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Fluorescein isothiocyanate (FITC) was used to selectively label the rabbit intestinal brush-border imino carrier, identify the binding protein on SDS-polyacrylamide gel electrophoresis, and monitor the effect of ions on fluorescein quenching. FITC inhibits Na+-dependent L-proline transport irreversibly, but transport is protected by physiological concentrations of Na* and L-proline. About 1 nmd of FITC/mg of protein binds specifically to the transporter, which was identified by SDS-polyacrylamide gel electrophoresis as a 100 ± 5-kDa peptide. Na+ produced a specific, saturable quench in the fluorescence of FITC bound to the proline carrier. Both transport and FITC quenching are inhibited by n-acetylimidazole, and membranes are protected from acetylation by Na*. We conclude that Na* binds to the proline carrier (100-kDa peptide) to produce a change in conformation that results in an increase in the affinity of the carrier for proline.

Na+-induced changes in substrate affinity have been observed in a number of Na+/organic solute co-transporters. These include the glucose and proline carrier from small intestine brush-border membranes (1-5) and the succinate, lactate, and glucose carriers from renal brush-border membranes (4-6). Although the mechanism for this change in carrier affinity is poorly understood, recent work with the fluorescent group specific reagent FITC has suggested that Na* induces a change in carrier conformation (7, 8) in the intestinal Na+-glucose carrier.

In this study we have extended the use of FITC to intestinal brush-border amino acid transport. Proline was chosen as the test amino acid since it is largely handled by a fairly specific, Na+-dependent carrier, the imino carrier (9-11). This imino carrier is also found in renal and choroid plexus brush borders (12, 13). Prolinuria is probably a genetic defect of this carrier (9, 11).

MATERIALS AND METHODS

Rabbit intestinal brush-border vesicles were isolated by a Ca**+-differential centrifugation procedure and purified further by KSCN treatment (7, 8). These vesicles were enriched 70- ±5-fold in alkaline phosphate, 125- ± 5-fold in γ-glutamyl transpeptidase, and 129- ± 10-fold in sucrase, relative to the initial mucosal homogenate. Initial rates of Na+-dependent L-proline uptake were measured using a rapid quench and filtration method (9). Na+-dependent proline uptake (mol mg protein-1 s-1) is defined as the uptake in the presence of 100 mM cis-NaCl minus the uptake in the presence of 100 mM cis-ECl under zero-trans conditions. The kinetics of L-proline uptake as a function of cis-L-proline concentration gave a maximum rate of transport (Vmax) of 900 ± 27 for KSCN-treated membranes versus 60 ± 18 pmol mg protein-1 s-1 for the Ca**+-precipitated membranes. There was no difference in the apparent affinity (Km) between the two preparations. As in the case of D-glucose (7, 8), KSCN produced 10-fold enrichment in the L-proline carrier. Kinetics measured in the presence and absence of excess L-alanine (50 mM) indicate that about 75% of the total Na+-dependent L-proline uptake is insensitive to alanine, i.e. the imino carrier accounts for the greater fraction of the total Na+-dependent L-proline transport (10).

Vesicles were treated with FITC or FITC in 50 mM Tris-Cl, pH 9.2, or 50 mM phosphate buffer, pH 9.2, plus 2 mM EDTA as described previously (7, 8). Substrate protection during exposure to FITC was routinely performed at 22 °C for 30 min with 0.5 or 10 mM L-proline and 100 mM NaCl, followed by a 15-min exposure to 60 μM FITC with and without substrates.

Fluorescence measurements were performed on an SLM-Aminco SPF 500 spectrophotometer set in the ratio mode with substrate-protected FITC-labeled membranes in the reference cuvette. The emission was monitored at 522 nm with excitation at 495 nm and slit widths of 2 nm. All experiments were performed at 22 °C and results are expressed as uncorrected emission spectra.

SDS-gel electrophoresis was performed according to the method of Laemmli (14) on 10-15% linear gradient slab gels. Following electrophoresis, one track was stained with Coomassie Blue and parallel tracks were sliced into 3-mm fractions and mashed, and the FITC fluorescence of the supernatant solutions was determined as described above. All enzyme assays were performed as described previously (7, 8) and protein was determined with the Bio-Rad protein assay with γ-globulin as protein standard. Transport and binding data are expressed as the mean ± S.E. of 3-6 estimates.

RESULTS

Na+-dependent L-proline uptake was sensitive to FITC and FITC in a substrate-protectable manner. Fig. 1a shows the effect of FITC on proline uptake. The concentration of FITC required for 50% inhibition (Ki) was 40 μM, and for FITC, 50 μM (not shown). Maximum inhibition (80%) occurred at 2 mM FITC and was irreversible. There was no inhibition of Na+-independent proline uptake. Figs. 1b and 2 demonstrate that L-proline transport can be protected against isothiocyanate inhibition by the presence of L-proline and Na+ during exposure of the membranes to FITC or FITC. There was no protection by glucose and Na+. The proline concentration required for 50% protection in the presence of 100 mM NaCl is 300 ± 30 μM. In the absence of Na+, proline offered no protection against FITC or FITC.

Binding of FITC to the brush-border proline transporter was determined after reacting nonspecific binding sites with FITC in the presence of substrates (Fig. 2). FITC binding was reduced from 18 ± 2 to 2.9 ± 0.2 nmol mg-1 after pretreatment with FITC in the presence of 10 mM L-proline and 100 mM NaCl. This binding was further reduced to 1.8 ± 0.2 nmol of FITC mg-1 when proline and Na+ were included during FITC treatment. Note that FITC inhibited proline transport from 0.89 ± 0.17 to 0.31 ± 0.3 nmol mg-1 s-1 after FITC pretreatment and that this was reversed completely by the presence
of substrates. These results indicate that about 1 nmol of FITC mg⁻¹ is bound specifically to the L-proline carrier.

Vesicles pretreated with PITC in the presence of 0.5 mM proline and 100 mM NaCl and then reacted with FITC were examined for the presence of Na⁺-dependent FITC fluorescence quenching. The addition of 75 mM NaCl, but not KCl, resulted in a 12.5% quench of the FITC fluorescence (Fig. 3a). A Hill analysis of the Na⁺ quench yields a Hill coefficient of 1.7 ± 0.1 indicating that a minimum of two Na⁺ ions bind to the proline carrier to produce the fluorescence quench. Li⁺, Cs⁺, Rb⁺, and choline⁺ all failed to quench the fluorescence.

Both Na⁺-dependent proline transport and the Na⁺ quench of FITC fluorescence were sensitive to the tyrosine group specific reagent n-acetylimidazole. Fig. 4a shows that n-acetylimidazole inhibited proline uptake 98% with 50% inhibition at 15 μM, and that transport was protected in the presence of 100 mM NaCl. Fig. 4b shows that n-acetylimidazole completely inhibited the Na⁺-induced fluorescence quench with 50% inhibition occurring at 20 μM, and that this action of n-acetylimidazole was also blocked by the inclusion of 100 mM NaCl during exposure of the membrane to the acetylating reagent.

The covalent binding of FITC to the proline carrier was used to identify the transport peptide in a SDS-polyacrylamide gel. Fig. 5 shows a gel with Coomassie Blue staining for protein at the top and FITC fluorescence. The molecular weight standards are indicated by the arrows. Only a single polypeptide band bound FITC after pretreatment of the membranes with PITC in the presence of 0.5 mM L-proline and 100 mM NaCl. The inclusion of substrates during FITC treatment reduces FITC binding at the 100-kDa peptide band by...
Under appropriate experimental conditions, FITC covalently labels the glucose binding site on the carrier. SDS-polyacrylamide gel electrophoresis has revealed that the glucose binding site is on a 75-kDa peptide.

We have now extended this approach to Na+-dependent L-proline transport across intestinal brush borders. Proline transport is inhibited irreversibly by PITC and FITC, and transport is protected by the simultaneous presence of Na+ and L-proline during exposure of membranes to the isothiocyanates. This latter observation enabled us to determine specific FITC binding to the proton transporter. Nonspecific FITC binding to the membranes was blocked by first incubating membranes with PITC in the presence of proline and Na+. Then specific FITC binding was obtained from the difference in FITC binding in the presence and absence of the substrates. This binding amounted to ~1 nmol/mg of protein, which is comparable to the amount of specific FITC binding to the glucose carrier (8). Judging from the amount of binding and the $J_{\text{max}}$ of Na+-dependent L-proline transport in these preparations (0.5 nmol mg$^{-1}$ s$^{-1}$), the turnover numbers for the proline and glucose carriers are similar (~3-5 s$^{-1}$). The molecular weight of the proton transporter (100-kDa) is significantly higher than that for glucose (75-kDa). These experiments clearly establish that the proline and glucose Na+-dependent carriers are at least separate subunits, if not separate proteins.

Na+ causes a specific, saturable fluorescence quench of FITC bound to the proline and glucose carriers. In the case of proline, the maximal quench was smaller than with glucose (15 versus 20%) and the $K_{\text{d}}$ values for Na+ were similar (22-25 mM). However, Hill analysis of the quench curve shows that the Hill coefficient was higher for proline than glucose (1.7 ± 0.1 versus 1.4 ± 0.3). This suggests that a minimum of two Na+ ions bind to the proline transporter to produce the conformation change.

There is good, albeit circumstantial, evidence that the Na+-induced fluorescence quench is related to Na+ binding to the Na+ sites on the transporter. First, the fluorescence quench, i.e. conformation change, is consistent with the Na+ effect on the $K_{\text{d}}$ for proline transport (3); second, both transport and the fluorescence quench are inhibited by n-acetylimidazole with similar $K_{\text{d}}$ values (15 and 20 mM), and these effects are completely blocked by the presence of Na+ during acetylation; and third, the Na+ Hill coefficients for quenching and transport were 1.7 suggesting that there are at least two ligand sites for Na+ on the proline carrier (15). Our interpretation of these results is that Na+ binds to the proline carrier to produce a change in conformation that results in an increase in the affinity of the carrier for proline.

Although the L-proline and D-glucose carriers of intestinal brush-borders are different peptides, they have much in common. Isothiocyanate inhibition of transport indicates that a lysine residue is close to both the glucose and proline sites, and n-acetylimidazole inhibition indicates that tyrosyl residues are close to the Na+ sites on both carriers. However, it remains to be established whether or not the Na+ sites are on the same peptides as the proline and glucose binding sites, and whether or not there is any topological relationship between the two carriers in the membrane.

Finally, in view of the fact that proline transport occurs via two pathways in brush-border membranes (10), we must address the question of which proline carrier we have identified. Since the imino carrier, operationally defined as the Na+-dependent, L-alanine insensitive uptake (11), accounts for 60-75% of the total, it is probable that FITC reacts chiefly with the imino carrier. This conclusion is supported by the

**DISCUSSION**

In previous reports we have shown that FITC can be used to identify and study conformational changes of the brush-border Na+/D-glucose cotransporter (8). Under appropriate experimental conditions, FITC covalently labels the glucose site on the carrier, and Na+ is able to produce a specific, saturable quench in the fluorescently labeled transporter. Detailed studies of transport, phlorizin binding, FITC binding, and the action of tyrosyl and sulphydryl reagents (7, 8) have led to the conclusion that the Na+ quench of fluorescence

![Figure 4. Effect of N-acetylimidazole on Na+-dependent proline uptake and Na+-induced FITC fluorescence quenching.](image-url)
**FIG. 5.** SDS gel of FITC-labeled vesicles. 250 µg of vesicles were treated with 2 mM PITC in the presence of 100 mM NaCl and 500 µM proline in 50 mM Tris-Cl, pH 9.2, and 2 mM EDTA for 30 min at 22°C. Substrates and unreacted PITC were removed by centrifugation. Vesicles were then treated with 50 µM FITC in the presence (broken line) or absence (solid line) of 100 mM NaCl and 500 µM proline in 50 mM Tris-Cl, pH 9.2, and 2 mM EDTA for 30 min at 22°C. Substrates and unreacted FITC were removed by centrifugation. Vesicles were then suspended in 10 mM Tris-Cl, pH 6.8, 1% SDS, and 0.4% β-mercaptoethanol for 15 min at 22°C and centrifuged for 120 min at 130,000 × g. Electrophoresis was performed on 10–15% linear gradient slab gels. Following electrophoresis, one track was stained with Coomassie Blue (100 µg of protein), and a parallel track was processed for FITC binding. The arrows indicate the position of the standards used to generate the molecular weight scale.

Observation that the \( K_{0.5} \) for proline protection of isothiocyanate inhibition (300 µM) is identical to the \( K_{0.5} \) for the imino transporter (234 µM). Furthermore, varying the proline concentration from 0.5 to 10 mM during PITC treatment did not change the pattern of FITC binding to polypeptides on SDS-polyacrylamide gel electrophoresis. This implies either that FITC only reacts with the imino carrier, that the alanine-sensitive proline transporter has the same molecular weight as the imino carrier, or that the density of alanine-sensitive carriers are low relative to the imino carriers.

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