Evidence for Tyrosyl Residues at the Na⁺ Site on the Intestinal Na⁺/Glucose Cotransporter*

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A tyrosine group has been identified at, or near, the Na⁺-binding site of the Na⁺/glucose and Na⁺/proline cotransporters of rabbit intestinal brush-borders. Three tyrosine group-specific reagents, n-acetylimidazole, tetranitromethane, and p-nitrobenzene sulfonfonyl fluoride, were used to evaluate the role of tyrosyl groups in Na⁺-dependent glucose transport, Na⁺-dependent phlorizin binding, and the Na⁺-induced fluorescence quenching of fluorescein isothiocyanate bound to the glucose site of the carrier. All three reagents inhibited glucose transport, phlorizin binding, and fluorescein isothiocyanate quenching by 50-85% with Kᵢ values in the range 7-50 μM. The presence of Na⁺ during the exposure of membranes to the reagents completely protected against inhibition, the Na⁺ concentration required to produce 50% protection was 14-36 mM. Fluorescent derivatives of n-acetylimidazole were synthesized to identify the tyrosyl residues on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A total of five polypeptide bands were labeled with eosin or fluorescein n-acetylimidazole in a Na⁺-sensitive manner. Two of these bands, previously identified as the glucose (75,000-dalton) and proline (100,000-dalton) binding sites of the glucose and proline carriers, account for 50% of the Na⁺-sensitive tyrosyl residues. On the basis of these studies, we believe that the Na⁺/glucose cotransporter contains both the Na⁺ and glucose active sites on the same polypeptide or that the cotransporter consists of two similar polypeptides, each containing one substrate binding site.

Recent progress has been made in the identification of the intestinal brush-border Na⁺/glucose cotransporter. Photoaffinity labeling with 4-azidothiorizin, covalent labeling with fluorescein isothiocyanate (FITC), and isolation of the transporter using a monoclonal antibody technique all point to a 75,000-dalton polypeptide as the glucose carrier (1-4). Furthermore, the action of isothiocyanates on glucose transport and phlorizin binding indicate that lysyl residues are at the active site for glucose binding to the carrier (2, 3, 5).

Identification of the Na⁺ active sites on the cotransporter is still lacking, even though there is a suggestion that tyrosyl residues may play a role in Na⁺ transport and/or binding by the renal brush-border Na⁺/glucose cotransporter (6). We have recently shown that two tyrosine group-specific reagents inhibit both Na⁺-dependent transport and the Na⁺-dependent conformational change of the intestinal brush-border carrier, and that physiological concentrations of Na⁺ completely protect against these reagents (3). In this paper we present further evidence that tyrosyl residues are at, or near, the intestinal cotransporter Na⁺ site, and we identify polypeptides containing Na⁺-protectable tyrosyl residues. Of the five polypeptide bands identified, a major one coincides with the FITC-labeled glucose site on the carrier. This suggests that the Na⁺ and glucose sites are on the same 75,000-dalton polypeptide band.

MATERIALS AND METHODS

Isolation of Brush-Border Membranes—Rabbit small intestinal brush-borders were prepared by a Ca²⁺-precipitation procedure (7) and were then treated with KSCN to remove core material (2, 8). Briefly, the brush-border membrane vesicles were resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, diluted to a final concentration of 0.6 M KSCN, homogenized, and held on ice for 10 min. The vesicles were then diluted 20-fold with ice-cold 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, and centrifuged for 10 min at 6,000 × g. The supernatants were pelleted at 38,000 × g for 30 min and the supernatant was discarded. The pellets consist of a white, fluffy outer corona around a darker inner layer. The fluffy outer layer was removed, resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, and repelleted. This was repeated until the pellet was uniformly white. About 20% of the Ca²⁺-precipitated membranes were recovered after this KSCN treatment, and they were routinely enriched 70-fold in alkaline phosphatase and 125-fold in γ-glutamyl transpeptidase and sucrase activities over the specific activities in the initial homogenate. The maximum velocity of Na⁺-dependent glucose and proline transport, and the maximum Na⁺-dependent phlorizin binding were all increased 10-fold over the Ca²⁺-precipitated brush-borders (2, 3, 9).

Following isolation the vesicles were stored in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, at liquid nitrogen temperature until use. Storage for up to 3 weeks was not found to affect the brush-border marker enzyme activities nor Na⁺-dependent glucose uptake. Glucose Transport Assay—Initial rates of Na⁺-dependent glucose uptake were assayed by a rapid mixing/rapid filtering procedure (7). Na⁺-dependent glucose uptake is defined as uptake in the presence of 100 mM cis NaCl minus uptake in the presence of 100 mM cis KCl. All uptakes were performed at 22 °C following a 3-s exposure to 50 μM [¹⁴C]glucose and 100 μCi of membrane protein.

Phlorizin Binding Assay—Phlorizin binding was measured using [¹³C]phlorizin (10). Na⁺-dependent phlorizin binding is defined as phlorizin binding in the presence of 100 mM cis NaCl minus binding in the presence of 100 mM cis KCl. Phlorizin binding reached a steady state in less than 3 s, and the maximum Na⁺-dependent binding was 285 ± 10 pmol mg⁻¹ with a Kᵢ of 1.4 ± 0.2 μM. We therefore used 3-s uptakes and a saturating concentration of [¹³C]phlorizin (15 μM) throughout.

Reaction with Tyrosine Group-Specific Reagents—Brush-border membranes were exposed to N-acetylimidazole (NAI) for 60 min at...
22°C and to tetraniromethane (TNM) and p-nitrobenzene sulfonyl fluoride (NBSF) for 30 min at 22°C. The reactions with TNM and NBSF were performed in 25 mM phosphate buffer, pH 8.6, and at pH 7.5 with NaI. Where indicated, NaCl, KCl, or LiCl was added during exposure to the reagents. All reactions were performed at a final osmolality of 400 mosm, which was maintained with mannitol. Following incubation with the reagent, the reaction was stopped by the addition of an excess of ice-cold buffer of the appropriate pH, and the reaction mixture was centrifuged at 38,000 g x min for 30 min. The pellets were then resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5.

N-Acetylfluorimide Assay—Nitrotyrosine formation was monitored at 428 nm (11) on an Aminco-SLM DW-2C dual beam spectrophotometer in the split beam mode. Prior to exposure to TNM, the vesicles were pretreated with 250 nM NBSF and 100 mM NaCl for 30 min at room temperature as described above. Pretreatment with NBSF and NaCl reduced the Na+-insensitive TNM binding. Vesicles (200 µg) were then added to the cuvettes and the absorbance at 428 nm was recorded as a function of time against the buffer and stored. TNM was then added to both cuvettes and the absorbance was recorded as a function of time.

Fluorescein N-Acetylimidazole—Fluorescein n-acetylimidazole was synthesized (12) from carbonyldimidazole and carboxyfluorescein dicetate in tetrahydrofuran. Carboxyfluorescein dicetate (100 mg), dissolved in 10 ml of tetrahydrofuran, was added to 22 mg of carboxyfluorescein dicetate in 10 ml of tetrahydrofuran. The reaction was allowed to proceed overnight under vacuum. The course of the reaction was followed by the release of CO₂ and the reaction was assumed to be complete when bubbling ceased. The solvent was then blown off with N₂, and the orange product was dissolved in 30 ml of benzene and recrystallized under vacuum. This step was repeated twice. The orange crystals were dried under vacuum overnight and stored in aliquots under vacuum. The reaction typically had an 82% yield.

The product was analyzed on a Nicolet MX-1 IR spectrophotometer. It showed a broad absorbance at 3500 cm⁻¹ characteristic of aromatics, an absorbance at 3000 cm⁻¹ and 2 of carbonyls at 1770 cm⁻¹ and at 1726 cm⁻¹. In addition a harmonic of the absorbance from 1620 cm⁻¹ to 1600 cm⁻¹. Peaks were present at 1500, 1450, 1420, 1375, 1200, and 1000 regions and below 1000 cm⁻¹. Comparison to fluorescein and n-acetimidazole standards indicated that the IR spectrum was consistent with the product being fluorescein n-acetimidazole.

Thin layer chromatography was performed on silica gel plates using propanol, 0.2 N ammonium (3:1) and propanol, 1 N acetic acid (3:1) as solvents. Migration was allowed to proceed for 3.5 h. The chromatograms were air-dried and tested for UV absorbance with a short wavelength UV mineral light. The chromatograms were also examined by the acidic diazo method (13). Fluorescein n-acetimidazole was not absorbed UV light strongly but did not react with the acidic diazo reagent. Carbonyldimidazole and n-acetimidazole were both negative for UV absorbance and acid diazo reagent. These results are consistent with Ames and Mitchell (13) and Dent (14) who found that substitution on the ring nitrogens of imidazole prevented the reaction with the acid diazo reagent. Imidazole and NH₂Cl gave positive reactions with the acidic diazo reagent staining orange, R₂ = 0.9, and yellow, R₂ = 0.4, respectively. The absence of a positive reaction with the acid diazo reagent indicates that no free imidazole was present. Fluorescein n-acetimidazole had an R₂ of 0.73 in propanol:NH₂Cl (3:1). Esin n-acetimidazole (ENAi) was synthesized and examined using the same protocols.

Fluorescence—Fluorescence quenching experiments were performed on an Aminco-SLM MPF 500 spectrofluorometer set in the ratio mode as previously described (3). Labeling with FITC was performed according to Peerce and Wright (2, 3). The fluorescence signals are expressed as uncorrected spectra.

Fluorescein n-acetimidazole was dissolved in absolute ethanol and the emission and excitation spectra were recorded. For the excitation spectrum, the emission monochromator was set at 525 nm with slit widths of 8 nm for the excitation slit and 2 nm for the emission slit. For the emission spectrum, the excitation monochromator was set at 471 nm and the slit widths were 8 nm for the emission slit and 2 nm for the excitation slit.

SDS-gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli on 10 to 15% linear gradient slab gels as previously described (3). Following electrophoresis, 0.3-cm slices of the fluorescently labeled tracks were cut and exposed to the reagents. All reactions were performed at a final osmolality of 400 mosm, which was maintained with mannitol. Following incubation with the reagent, the reaction was stopped by the addition of an excess of ice-cold buffer of the appropriate pH, and the reaction mixture was centrifuged at 38,000 g x min for 30 min. The pellets were then resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5.

RESULTS

Inhibition of Glucose Uptake and Phlorizin Binding by Tyrosine Group-specific Reagents—A number of tyrosine group-specific reagents with different proposed reaction mechanisms were tested for their ability to inhibit Na+-dependent glucose transport and Na+-dependent phlorizin binding. The results are summarized in Table I and Fig. 1. All of the tyrosine group reagents inhibited Na+-dependent glucose uptake (50–75%) with Kᵣ values in the range 7–50 µM. The inclusion of Na⁺ in the reaction mixture with the tyrosine reagents completely protected the transporter against inhibition, and the Na⁺ concentration required to produce 50% protection ranged from 14 to 37 mM (see also Figs. 8 and 9 in Ref. 3). For all these reagents, inhibition was dithiothreitol-insensitive, and the inclusion of D-glucose along with Na⁺ during the reaction of the reagents with the membranes did not influence glucose transport.

Fig. 1 shows the effect of n-acetimidazole on phlorizin binding in the presence and absence of Na⁺. N-Acetylimidazole was chosen as the inhibitor for these studies since it demonstrated high maximum inhibition under mild reaction conditions (1 h at pH 7.4), and it is reported to be the most selective tyrosine group reagent available (15). N-Acetylimidazole inhibited all of the Na⁺-dependent phlorizin binding without affecting nonspecific phlorizin binding with a Kᵣ of 30 µM (Fig. 1). The Kᵣ for inhibition was similar to the results shown in Table I for inhibition of Na⁺-dependent glucose uptake. Fig. 2 shows the effect of D-glucose against inhibition of Na⁺-dependent phlorizin binding by 250 µM n-acetimidazole. The apparent Kᵣ for protection by Na⁺ was 22 ± 3 mM, similar to the Na⁺ concentration required for half-maximal protection of Na⁺-dependent glucose uptake (Table I) and the Na⁺-dependent conformational change (2, 3). Similar results were seen with FNAi (fluorescein n-acetimidazole) and ENAi (esin n-acetimidazole).

Table I

| Inhibitor               | Glucose uptake | Na⁺ protection |
|-------------------------|----------------|----------------|
|                        | % control uptake | Kᵣ/µM | Kᵣ/mM |
| n-Acetylimidazole       | 26 ± 5 (6) | 50 ± 10 (4) | 18 ± 2 (4) |
| Fluorescein n-acetyl-   | 23 ± 4 (3) | 15 ± 3 (3) | 37 ± 2 (2) |
| imidazole               |                |               |               |
| p-Nitrobenzene sul-     | 25 ± 2.2 (4) | 42 ± 3 (3) | 22 ± 5 (2) |
|fonyl fluoride           |                |               |               |
| Tetranitromethane       | 48 ± 4 (4) | 7.5 ± 2.5 (4) | 14 ± 4 (3) |
compared to the time course of nitrotyrosine formation in protection against TNM binding to tyrosine. Since K+ does the Na+-sensitive binding of TNM and the formation of duplicate determinations, and they are representative of experiments. The results are the mean of duplicate estimates ± S.E. The results shown are representative of 4 separate experiments.

**FIG. 1.** Effect of N-acetylimidazole on Na+-dependent phlorizin binding in the presence and absence of Na+. Vesicles (500 μg) were treated with varying concentrations of n-acetylimidazole in the presence and absence of 100 mM NaCl for 60 min in 25 mM phosphate buffer, pH 7.5, at 22 °C. A final osmolarity of 400 mosm was maintained with mannitol. Following the incubation period the reaction mixture was diluted with ice-cold 50 mM phosphate buffer, pH 7.5, and the vesicles were centrifuged at 38,000 × g for 50 min. The pellets were collected and resuspended in 300 mM mannitol + 10 mM HEPES/Tris, pH 7.5, for determination of phlorizin binding (see “Materials and Methods”). The points shown are the mean ± S.E. of duplicate determinations, and they are representative of 3 separate experiments. The solid line indicates phlorizin binding after NAI treatment in the absence of Na+, while the dotted line indicates binding after NAI treatment in the presence of 100 mM Na+.

**Time Course of Inhibition of Glucose Transport and Nitration of Tyrosine Residues by TNM—TNM forms a spectrophotometrically measurable complex with tyrosine at pH values above 8 (11) which is sensitive to reduction to an amide which is colorless (16). Formation of nitrotyrosine was monitored by the increased absorbance at 428 nm as a function of time with correction for light scatter and autoreduction of TNM using a dual beam spectrophotometer. The time course of TNM inactivation of Na+-dependent glucose uptake is compared to the time course of nitrotyrosine formation in Fig. 3. Fig. 3A shows the difference between Na+ and K+ protection against TNM binding to tyrosine. Since K+ does not protect against inactivation of Na+-dependent glucose uptake or Na+-dependent phlorizin binding, this figure shows the Na+-sensitive binding of TNM and the formation of nitrotyrosine derivative. The time course of the formation of the nitrotyrosine derivative was similar to the time course of inactivation of Na+-dependent glucose uptake. The t50 for nitrotyrosine formation was approximately 5 min as compared to a t50 for inhibition of Na+-dependent glucose uptake of 5.5 min (Fig. 3B). Reduction of the nitrotyrosine derivative with sodium hyposulfite or dithiothreitol abolished the absorbance signal at 428 nm. These results indicate that nitration of a tyrosine group in Na+-sensitive manner results in inhibition of Na+-dependent glucose uptake and Na+-dependent phlorizin binding. Inhibition by TNM was not found to be sensitive to the addition of dithiothreitol, indicating that TNM was not binding to an SH group; however, as reported above, nitrotyrosine was dithiothreitol-sensitive.

**Tyrosine Group-specific Reagents Inhibit the Na+-induced Conformational Change of the Glucose Carrier—Na+-dependent fluorescence quenching of carrier labeled at or near the glucose site with FITC has been demonstrated to be consistent with a Na+-induced conformational change (3). Inhibition of the Na+-dependent conformational change in a Na+-sensitive manner by n-acetylimidazole and NBSF is shown in Table II. Both tyrosine-specific reagents had K0.5 values similar to the K0.5 values observed for Na+-dependent glucose uptake (40 and 25 μM as compared to inhibition of glucose uptake 50 and 42 μM, respectively). Protection against inhibition of the Na+-induced fluorescence quenching was maximal in the presence of Na+ alone, with K+ and Li+ unable to substitute. Na+ protection of the FITC fluorescence quenching gave similar K0.5 values to those seen in Table I for protection against inhibition of glucose uptake. The K0.5 for protection by Na+ against n-acetylimidazole was 25 mM for the fluorescence quenching and 18 mM for Na+-dependent glucose uptake, and 22 mM Na+ for protection of NBSF inhibition of transport and fluorescence quenching. These results indicate that the tyrosine-specific reagents are reacting at or near the same tyrosine which inhibits the proposed conformational change. Therefore, it appears that a fluorescent tyrosine group-specific reagent would label the carrier Na+ site.

**Identification of Membrane Proteins Containing Na+-binding Sites—**Fluorescent derivatives of n-acetylimidazole were synthesized by a variation on the method of Paul and Ander-
fluorescence spectra. The characteristic excitation at 490 nM and emission at 520 nM is consistent with n-acetylimidazole attachment at ring 4, since changes in fluorescence emission and excitation maxima are associated with additions to rings 1, 2, and 3 (e.g. eosin, erythrosin, and methylfluorescein). Consistent with the proposed structure is the absence of diazo reactivity (17).

Fig. 5 shows the binding of fluorescein n-acetylimidazole to membranes in the presence and absence of n-acetylimidazole. In the absence of n-acetylimidazole, 2.5 ± 0.3 nmol of FNAI were bound per mg of membrane protein with an apparent $K_{D}$ of 15 μM. The Na+-insensitive amount bound was 0.45 ± 0.01 nmol/mg of protein. Addition of n-acetylimidazole reduces FNAI binding to the Na+-insensitive level with a $K_{D}$
the gel slices were mashed and incubated overnight in the dark in polyacrylamide slab gel according to the method of Laemmli (18). Following electrophoresis, 0.3-cm slices of the tracks were made and was removed by centrifugation and the FITC fluorescence emission was determined at 522 nm with excitation at 492 nm. Eosin N-acetylimidazole emission was determined at 540 nm with excitation for binding to the brush-border membranes pretreated with 2 mM n-acetylimidazole in the presence of 100 mM Na+ were fluorescently labeled with FNAI or ENAI, and SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (18). Of 20 μM. The apparent K_{0.5} for binding to the brush-borders was similar to the K_{0.5} for inhibition of Na+-dependent glucose uptake shown in Table I. Addition of n-acetylimidazole or Na+ to the wash following exposure to FNAI had no effect on the amount of FNAI bound, indicating that FNAI binding is irreversible. Addition of 0.2 M hydroxylamine or exposure to pH 9.2 following FNAI labeling reversed FNAI labeling of the Na+-dependent phlorizin binding to control levels. These results confirm binding of FNAI to a tyrosine group in agreement with the results with TNM.

On the basis of their ability to inhibit Na+-dependent glucose uptake and Na+-dependent phlorizin binding in a Na+-sensitive manner, and their apparent irreversibility, these fluorescent n-acetylimidazole analogs appear to be good candidates as labels for the glucose carrier Na+ site. Brush-border membranes pretreated with 2 mM n-acetylimidazole in the presence of 100 mM Na+ were fluorescently labeled with FNAI or ENAI, and SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (18).

Following electrophoresis, 0.3-cm slices of the tracks were examined for fluorescence. Fig. 6A shows the Na+-sensitive FNAI binding pattern. In the absence of Na+ during the acetylation reaction, there were 5 peaks of FNAI bound to the gel, and all were markedly Na+-sensitive. On four gels of three different membrane preparations, the molecular weights of the five bands were: 1) 149,000 ± 6,000; 2) 97,000 ± 1,750; 3) 75,000 ± 1,000; 4) 38,500 ± 1,250; and 5) 29,000 ± 500. Two of these bands coincide with the polypeptides containing the glucose (75,000-dalton) and proline (100,000-dalton) binding sites of the Na+/glucose and Na+/proline cotransporters (2, 3, 9). Fig. 6B shows a parallel experiment where FITC binding to the glucose site of the glucose cotransporter was measured on the same membranes as those in Fig. 6A. This confirms that FITC and ENAI bind to a common polypeptide band. About 30% of the total Na+-sensitive ENAI binding was to the 75,000-dalton band, and 20% to the 97,000-dalton polypeptide band. Identical results were obtained using FNAI (data not shown). In two experiments the addition of 100 mM K+ or Li+ did affect fluorescent labeling of any bands.

The identity of the three other ENAI binding polypeptides is not known. However, none appear to be the brush-border Na+/H+ antiporter since 100 mM Li+ (another substrate) failed to protect against FNAI.

DISCUSSION

To elucidate the mechanism of Na+/glucose cotransport across membranes, it is essential to develop probes for both the Na+ and glucose-binding sites on the transporter. Such probes would allow the identification of the polypeptides interacting with each substrate and enable us to determine if both active sites are on the same, or different, polypeptide subunits of the carrier. So far, the identity of the glucose site on a 75,000-dalton polypeptide has been settled using photofluorophores (4-azidophorizin) and fluorescent (fluorescein isothiocyanate) probes (1–3). In this paper, we have presented evidence that a tyrosyl residue is at, or close to, the Na+ site on the glucose carrier, and that fluorescent acetylating agents (fluorescein or eosin n-acetylimidazole) may be used to label this site. The results suggest that the Na+ and glucose sites reside on the 75,000-dalton polypeptide band.

Three tyrosine group-specific reagents, n-acetylimidazole, tetranitromethane, and p-nitrobenzene sulfonyl fluoride, were found to inhibit Na+-dependent glucose transport, Na+-dependent phlorizin binding, and the Na+-induced fluorescence quenching of FITC bound to the glucose site of the carrier. The inhibitor constants ranged from 7 to 50 μM. The presence of Na+ during the reaction of the reagents with the membrane completely protected against inhibition of transport, binding, and fluorescence quenching. The Na+ concentration required to produce 50% protection was 14–36 mM, which is in the same range of the concentration to activate glucose transport (3). Alkaline pH and hydroxylamine reverse FNAI labeling and the NAI inhibition of phlorizin binding, which further indicates the involvement of tyrosine residues (20). These observations, together with direct measurement of the nitration of tyrosine residues with TNM (Fig. 3A), clearly demonstrate the involvement of a tyrosyl residue(s) at the Na+ site on the glucose carrier. This tyrosyl residue is essential for glucose transport, phlorizin binding, and the Na+-induced conformation change responsible for the Na+-dependent increase in the affinity of the carrier for glucose (2, 3, 19).

Acetylimidazole is considered to be most selective in acetyling phenolic hydroxyls (15), and TNM is used widely for the modification of tyrosine residues in proteins (20). The clearest advantage of TNM is the fact the nitrophenoxide ion...
exhibits strong absorption at 428 nm, and that this is rapidly abolished by reduction to the amino derivative. NBSF is another reagent that is thought to modify tyrosine residues selectively under mild reaction conditions (21). Other tyrosine reagents that inhibit intestinal glucose transport include N-ethylmaleimide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Glucose transport by calf renal brush-borders was also inhibited partially by n-acetylimidazole, TNM, and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, and some protection was noted by 500 mM Na+ (6).

Fluorescent acetylating reagents, FNAI and ENAI, were developed to identify polypeptides containing Na+-sensitive tyrosyl residues. Fluorescein n-acetylimidazole inhibited glucose uptake and phlorizin binding in a Na+-sensitive manner with a $K_i$ of 15 $\mu$M (Table 1). The Na+-sensitive binding of FNAI (2.5 nmol/mg) was blocked by NAI with a $K_i$ of 20 $\mu$M (Fig. 5). These results indicate that the fluorescent n-acetylimidazole derivates also acetylate the tyrosine residues at the Na+ site on the glucose carrier.

The irreversible acetylation of the Na+-sensitive tyrosyl residues by the fluorescent reagents then allows the identification of the Na+ sites on SDS-PAGE of the brush-border membranes. Five polypeptides were labeled with ENAI (Fig. 6) in a Na+-sensitive manner. A major band containing 30% of the total sites (750 pmol/mg) coincides with the 75,000-dalton glucose binding polypeptide band. We estimate that 285 pmol/mg of phlorizin and 800 pmol/mg of FITC bind to the glucose brush-border glucose carrier (3). Since there is evidence that there is one Na+-binding site on the glucose carrier (Hill plots of glucose transport versus Na+ concentration gave slopes of 1; see Ref. 3), the amount of ENAI binding to the 75,000-dalton band is then sufficient to account for the Na+-binding sites on the glucose carrier. At this juncture it is not clear whether both the Na+ and glucose sites are on the same polypeptide or not, as it is possible that the Na+ and glucose sites are on different 75,000-dalton subunits of the carrier.

One other major Na+-sensitive polypeptide band can be tentatively identified, i.e. the 97,000 band which coincides with the proline site on the brush-border Na+/proline cotransporter. FITC labels the proline site on this cotransporter, and both Na+-dependent proline transport and Na+-induced fluorescence quenching of FITC bound to the proline site are inhibited by n-acetylimidazole in a Na+-sensitive way (9). Although tyrosyl residues are at the Na+ sites on both the glucose and proline cotransporters, there appear to be differences in that there are at least two Na+ sites on the proline carrier but only one on the glucose carrier.

The identity of the other 3 polypeptide bands containing Na+-sensitive tyrosyl residues has yet to be established. None appear to be related to the brush-border Na+/H antiporter since Li+ fails to reduce ENAI binding (22). Possible candidates for these include cotransporters for phosphate, sulfate, phenylalanine, and other neutral amino acids.

Finally, the fluorescent n-acetylimidazole analogs will prove useful in the examination of Na+ sites when the glucose and proline carriers are isolated and purified to homogeneity. It should be possible to determine if the active sites for both substrates (Na+ and glucose or proline) are on the same polypeptide subunit, and to examine the structural relationship between the two active sites.

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