

Distance between substrate sites on the Na–glucose cotransporter by fluorescence energy transfer

(intestine/Na–glucose symporter/brush border membranes)

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ABSTRACT Covalent fluorescent probes were used to label the rabbit intestinal brush border Na⁺–glucose cotransporter at the putative glucose and Na⁺ binding sites, and a steady-state fluorescence energy transfer technique was used to measure the distance between the two binding sites. In both intact brush border membrane vesicles and partially purified soluble protein, the distance ($R_{2/3}$) between the Na⁺ and glucose sites was ≈ 35 Å. This distance was the same with four different donor/acceptor pairs with different transfer efficiencies, by donor quantum yield measurements, or sensitized acceptor fluorescence. The fact that the Na⁺ site and glucose site probes bind to a 75-kDa polypeptide, copurify with the same isoelectric point (pI 5.3) and retain function, and exhibit energy transfer indicates that the sites are on the same 75-kDa polypeptide. The large distance between the Na⁺ and glucose site probes raises questions about simple models of frictional interactions between the two substrates during the transport cycle.

Uphill glucose transport across the intestinal brush border membrane occurs by Na⁺–glucose cotransport (1). We have identified this cotransporter as a 75-kDa polypeptide using fluorescent group-specific reagents: fluorescein isothiocyanates (FITCs) react with lysine residues at or near the glucose binding site, whereas fluorescent *N*-acetylimidazoles react with tyrosine residues at or near the Na⁺ binding sites (2–4). In the present study, we have measured the distance between the sodium and glucose binding sites of the glucose cotransporter using the technique of fluorescence energy transfer (5, 6). In both native brush border membrane vesicles and partially purified, soluble proteins, our results demonstrate that the sodium and glucose sites are on the same polypeptide (75 kDa; pI 5.3) and that the Na⁺ site is 35 Å away from the glucose site.

METHODS

Rabbit intestinal brush border membrane vesicles were prepared by a Ca²⁺ precipitation procedure and treated with KSCN (3, 4). The vesicles were then resuspended in 300 mM mannitol and 10 mM Hepes/Tris·HCl, pH 7.5, and stored at –80°C until needed.

Chromatofocusing of vesicles was performed as described by Lin *et al.* (7), except we have substituted the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for octyl glucoside. Chromatofocusing was performed in a 35 cm × 1.5 cm column at a flow rate of 30 ml/hr and over the pH range of 7.2–4.0. Fractions were precipitated with (NH₄)₂SO₄ and resuspended in buffer and

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the (NH₄)₂SO₄ was removed by passage through a Sephadex G-50 column equilibrated with buffer. Following removal of (NH₄)₂SO₄, the protein was lyophilized and stored at –80°C.

FITC and other isothiocyanate derivative labeling of the cotransporter at the glucose site and *N*-acetylimidazole derivative labeling of the cotransporter at the Na⁺ site were carried out as reported (2–4). The conditions were optimal for specific, complete labeling of the sites on the transport protein.

NaDodSO₄/polyacrylamide electrophoresis was performed according to Laemmli (8) as described by Pearce and Wright (3). High-pressure liquid chromatography (HPLC) was performed on a Bio-Rad HPLC and a C₁₈ reverse-phase column using a linear gradient of 6 M formic acid, 0.13 M triethylamine, H₂O/4 M formic acid, 0.09 M triethylamine, 72% propanol, H₂O. Proteolytic digestion was performed at 37°C for 3 hr with a 10:1 protein:chymotrypsin or trypsin ratio according to the method of Allen (9). Fluorescent fragments were identified using a Gilson 121 fluorometer with appropriate excitation and emission filters.

Extinction coefficients for fluorescent isothiocyanate lysine derivatives and fluorescent *N*-acetylimidazole tyrosine derivatives were obtained from absorbance measurements on a SLM-DW2C spectrophotometer. For isothiocyanates at pH 9.2 and acetylimidazoles at pH 7.5 (50 mM potassium phosphate buffers), the extinction coefficients were 2-anthracene isothiocyanate (AITC) and 2-anthracene *N*-acetylimidazole (ANAI), 28×10^3 ; 3-(4-isothiocyanatophenyl)-7-diethyl-4-amino-4-methylcoumarin (IPM), 32×10^3 ; FITC and fluorescein *N*-acetylimidazole (FNAI), 73×10^3 ; and eosin isothiocyanate (EITC) and eosin *N*-acetylimidazole (ENAI), 83×10^3 M⁻¹·cm⁻¹. Similar values were found for EITC and ENAI at pH 5.5 (50 mM 4-morpholineethanesulfonic acid/Tris), where the fluorescein derivatives had an extinction coefficient of 27×10^3 . In the organic solvent corresponding to the composition of the fluorescent fractions from HPLC (Fig. 3)—i.e., 85% 6 M formic acid, 0.13 M triethylamine, H₂O and 15% 4 M formic acid, 0.09 M triethylamine, 72% propanol, H₂O—the fluorescein and eosin derivatives had extinction coefficients of 10×10^3 M⁻¹·cm⁻¹.

Experiments were performed in 50 mM Tris·HCl/2 mM EDTA, pH 7.5, on an SLM SPF500 spectrofluorometer at 22°C. Protein concentrations were maintained between 10 and 20 μg/ml with absorbances <0.02 to minimize inner filter effects and probe self-quenching. Sodium quenching of fluorescent isothiocyanate derivatives was also measured as described elsewhere (3, 10).

Abbreviations: FITC, fluorescein isothiocyanate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ANAI, 2-anthracene *N*-acetylimidazole; ENAI, eosin *N*-acetylimidazole; FNAI, fluorescein *N*-acetylimidazole; AITC, 2-anthracene isothiocyanate; EITC, eosin isothiocyanate; IPM, 3-(4-isothiocyanatophenyl)-7-diethyl-4-amino-4-methylcoumarin.

Fluorescence energy transfer (E) was determined from: (i) the decrease in donor quantum yield in the presence of acceptor,

$$E = 1 - \frac{\phi_{DA}}{\phi_D}, \quad [1]$$

and rhodamine b in ethanol as standard with a quantum yield of 0.97. ϕ_{DA} is the quantum yield of the donor in the presence of acceptor, and ϕ_D is that in the absence of acceptor; and (ii) the sensitized fluorescence of the acceptor,

$$E = \frac{F'_{DA}(\lambda_D\lambda_A)A_{DA}(\lambda_A)I(\lambda_A)}{F_D(\lambda_A\lambda_A)A_{DA}(\lambda_D)I(\lambda_D)}, \quad [2]$$

where F'_{DA} is corrected fluorescence of acceptor, A_{DA} is absorbance, F_D is fluorescence of donor, and λ_A and λ_D are wavelength of acceptor or donor emission or absorbance. $I(\lambda_A)/I(\lambda_D)$ is a correction factor for differential responses of the spectrofluorometer at the acceptor and donor absorption bands. F'_{DA} is related to F_{DA} (the observed fluorescence) by

$$F'_{DA}(\lambda_D\lambda_A) = F_{DA}(\lambda_D\lambda_A) - F_A(\lambda_A\lambda_A) - F_D(\lambda_D\lambda_A) \frac{F_{DA}(\lambda_D\lambda_D)}{F_D(\lambda_D\lambda_A)}, \quad [3]$$

where $F_{DA}(\lambda_D\lambda_A)$, $F_D(\lambda_D\lambda_A)$, and $F_A(\lambda_A\lambda_A)$ are the fluorescence intensity at the acceptor emission wavelength with excitation at the donor wavelength with acceptor and donor present, with only donor, and with only acceptor, respectively. $F_{DA}(\lambda_D\lambda_D)$ and $F_D(\lambda_D\lambda_D)$ are the fluorescence intensities with excitation at the donor wavelength and emission at the donor wavelength of the donor and acceptor labeled and donor labeled protein, respectively.

Distances between fluorophores were determined by using

$$R_{2/3} = R_0(1/E - 1)^{1/6} \text{ \AA}, \quad [4]$$

where R_0 is the distance between fluorophores at 50% energy transfer or the Förster critical distance, and $R_{2/3}$ is the distance between fluorophores assuming a random orientation of fluorophores. Corrections for direct acceptor excitation and light scatter were made by using the procedure described by Kella *et al.* (11). R_0 was determined by using the formula

$$R_0 = 9.79 \times 10^3 (Q_D K^2 J n^{-4})^{1/6} \text{ \AA}, \quad [5]$$

where Q_D is the quantum yield of the donor, K^2 is the orientation factor, which was assumed to be random—i.e., $2/3$ —and n is the refractive index of the medium, 1.4. J , the overlap integral for the donor emission and the acceptor excitation, was determined over 1.6- to 2.0-nm intervals, by using the formula

$$J = \frac{\sum_{\lambda} F_D(\lambda) E_A(\lambda) \lambda^4 \Delta\lambda}{\sum_{\lambda} F_D(\lambda) \Delta\lambda}. \quad [6]$$

Binding of fluorescent derivatives to non-cotransporter sites was minimized by pretreatment with phenyl isothiocyanate in the presence of Na^+ and glucose or pretreatment with *N*-acetylimidazole in the presence of Na^+ as described (3, 4). Residual non-cotransporter fluorescent probe was examined for energy transfer as follows. By using changes in quantum yield in the presence of acceptor, none of the noncotransporter Na or glucose site probes could donate to the

cotransporter sites nor was any change in quantum yield seen with donation or acceptance from cotransporter sites to non-cotransporter sites. Similarly, with both sites protected, the quantum yield of non-cotransporter FNAI decreased 2% or less, whereas there was no change measurable with FITC as donor to ENAI. These results suggest that only cotransporter sites are affecting changes in quantum yield or sensitized acceptor fluorescence.

All chemicals and CHAPS were purchased from Sigma and were reagent grade or better. HPLC solvents were purchased from Pierce; electrophoresis supplies were from Bio-Rad; fluorescent isothiocyanate derivatives were from Molecular Probes (Eugene, OR). Fluorescent *N*-acetylimidazole derivatives were synthesized in the laboratory (4).

RESULTS

Under appropriate experimental conditions, FITC labels a single 75-kDa polypeptide of intestinal brush border membranes (2, 3). This labeling inhibits Na^+ -dependent glucose uptake and Na^+ -dependent phlorizin binding competitively with respect to glucose and phlorizin, suggesting that FITC binds at or near the glucose site. We have also shown that the Na^+ site responsible for Na^+ activation of glucose and phlorizin binding and the Na^+ -induced conformational change involves Na^+ -protectable tyrosine residues (4). These tyrosine residues have been identified on NaDodSO₄ gels by using covalent fluorescent *N*-acetylimidazole derivatives (4). However, unlike FITC, which labels one polypeptide band, five polypeptide bands are labeled with FNAI, one being the 75-kDa band that binds FITC. We have suggested that this 75-kDa polypeptide band contains the Na^+ site of the glucose carrier. Of the five bands labeled with FNAI, the 75-kDa polypeptide contains 30% of the total label (4).

We have further purified the cotransporter in an effort to verify that this 75-kDa polypeptide contains both substrate sites. By using chromatofocusing, one band centered around pH 5.3 contains the FITC and ENAI (Fig. 1). NaDodSO₄ gel electrophoresis of the pI 5.3 fraction indicates that a single 75-kDa polypeptide band is labeled with both substrate-site probes (data not shown). In the absence of *N*-acetylimidazole, this pI 5.3 fraction shows a Na^+ -selective FITC fluorescence quench, indicating a conformational change like the intact vesicles (data not shown). In addition, the unlabeled pI 5.3 fraction shows enriched Na^+ -dependent phlorizin binding (unpublished data), and in the case of renal brush borders, this fraction exhibited Na^+ -dependent glucose transport in proteoliposomes (7). These results indicate that all of the partial reactions of the native cotransporter are contained in this fraction and suggest that both substrate sites are located on the 75-kDa polypeptide.

The Na^+ -specific quenching of fluorescent isothiocyanate derivatives was used to monitor binding of label to the glucose site. With all of the isothiocyanate derivatives used, 100 mM Na^+ produced a specific quenching of fluorescence that varied from 17% with AITC to 22% with FITC (12). The chromatofocused pI 5.3 protein showed similar Na^+ -specific quenching of AITC or FITC (data not shown). Inhibition of Na^+ -specific fluorescent isothiocyanate quenching by fluorescent *N*-acetylimidazole derivatives (3, 4) was used to monitor labeling of the Na^+ site. Inhibition varied from 88% with ANAI to 95% with the fluorescein and eosin derivatives.

The stoichiometry of Na^+ -site probe to glucose-site probe was determined by using three independent criteria. The first method involved determining the substrate-sensitive binding of the specific probes to the brush border membrane vesicles. Of the five polypeptide bands labeled with ENAI in a Na^+ -sensitive manner (Fig. 6, ref. 4), only one appears to be chromatofocused with the FITC polypeptide. Fig. 1 compares the chromatofocusing elution profile of FITC-labeled

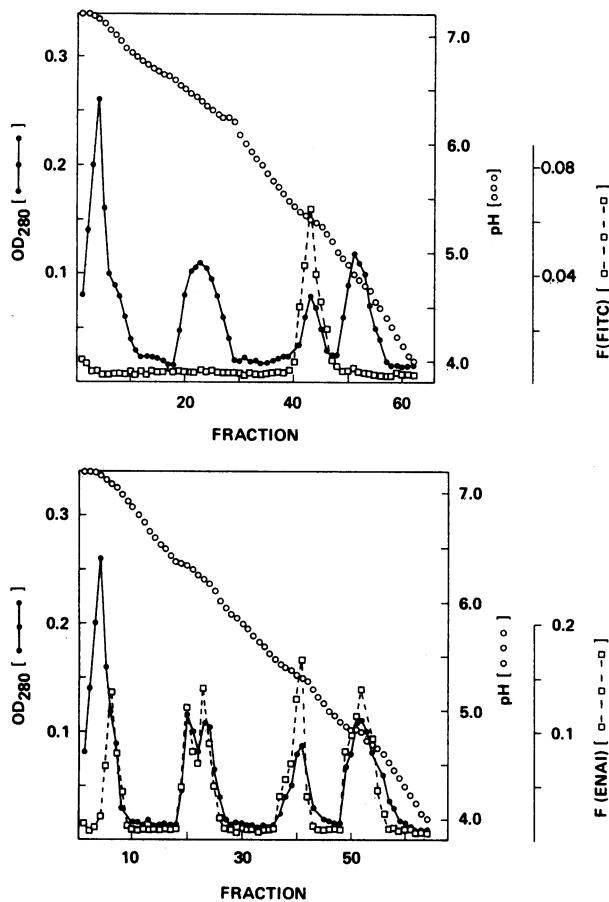


FIG. 1. Chromatofocusing elution profile of fluorescently labeled membranes. Sixty milligrams of fluorescently labeled membranes was solubilized in CHAPS/25 mM imidazole-HCl, pH 7.4, and chromatofocused between pH 7.4 and pH 4.0. (Upper) Elution profile of FITC-labeled membranes. (Lower) Elution profile of ENAI-labeled membranes. F, fluorescence. Membranes from the same preparation were used for both experiments.

vesicles (Upper) to the elution profile of ENAI-labeled vesicles (Lower). The areas under the pH 5.3 peaks are identical, showing a ratio of 0.97 ± 0.06 for the area under the FITC peak/area under the ENAI peak normalized for protein. Of the total ENAI, the pH 5.3 peak contains $33\% \pm 2\%$ (mean \pm SD; $n = 2$).

The second method of stoichiometry determination involved following the binding of ENAI to the chromatofocused pH 5.3 fraction. Fig. 2 shows the binding of ENAI to the solubilized pH 5.3 fraction following pretreatment with *N*-acetylimidazole in the presence of Na^+ and without *N*-acetylimidazole pretreatment. A single exponential is seen with a rate constant of 0.146 sec^{-1} . Similar results seen with or without *N*-acetylimidazole pretreatment indicate a single class of ENAI binding sites following chromatofocusing.

The third method involved isolation of the pH 5.3 fraction and proteolytic digestion of the proteins with chymotrypsin followed by HPLC to examine the polypeptide fragments for fluorescence. With either trypsin or chymotrypsin, a variety of fragments was seen; however, only one FITC-containing fragment and only one ENAI-containing fragment were seen. Fig. 3 shows the results of chymotryptic digestion for 3 hr at 37°C . These results suggest that the stoichiometry of isothiocyanate derivative to *N*-acetylimidazole derivative is 1:1.

The fluorescence energy transfer distance between isothiocyanate derivative bound at or near the glucose site and *N*-acetylimidazole derivative bound at or near the Na^+ site was determined using donor quantum yield in the presence

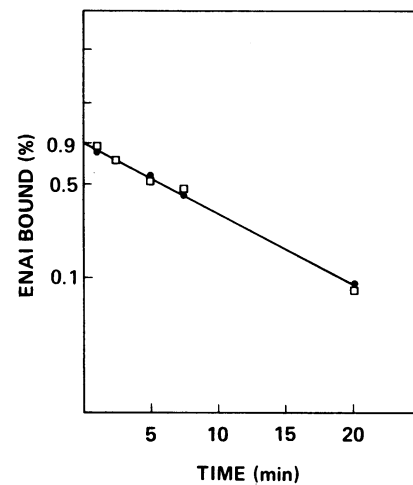


FIG. 2. Binding of ENAI to chromatofocused pi 5.3 fraction. Five hundred milligrams of pi 5.3 protein was labeled with $50 \mu\text{M}$ ENAI in 50 mM potassium phosphate buffer at pH 7.4. At the indicated time, the reaction was terminated and unbound ENAI was removed by the method of Penefsky (13). Bound ENAI was determined as described (4). \bullet , No pretreatment; \square , ENAI binding following pretreatment with 2 mM *N*-acetylimidazole in the presence of 100 mM NaCl for 30 min at room temperature.

and absence of acceptor or sensitized acceptor fluorescence. The results with glucose site probe as energy donor are shown in Table 1. Both preparations of glucose carrier resulted in similar energy transfer distances from the glucose site to the Na^+ site. The average distance between fluorophores is 35 \AA for both membranes and the solubilized chromatofocused pi 5.3 fraction. Similar results with both preparations and two donor/acceptor pairs with membranes and two donor/acceptor pairs with pi 5.3 chromatofocused fraction suggest that our approximation of a 1:1 stoichiometry is a good one. If there were multiple donor/acceptor pairs contributing to the observed transfer, one would predict smaller distances for the least efficient donor/acceptor pairs, since these pairs may not accept donation from distant donors. This is not the case.

Fluorescence energy transfer with the Na^+ -site probe as donor is shown in Table 2. The apparent discrepancy be-

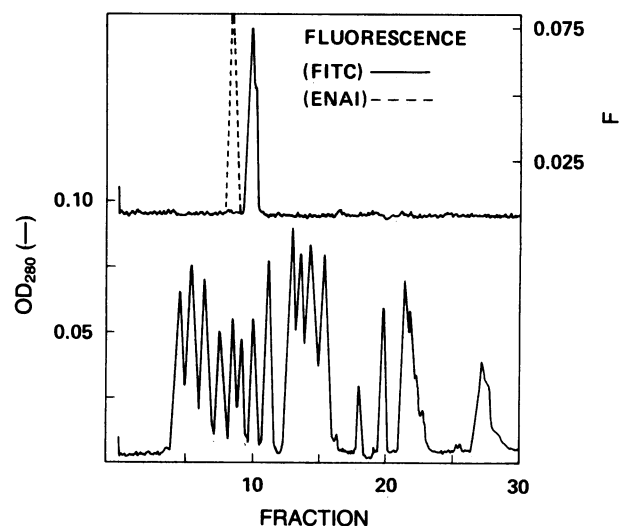


FIG. 3. HPLC of pi 5.3 fraction following chymotryptic digestion. HPLC was performed by using a reverse-phase C_{18} column and a linear gradient of formic acid, triethylamine, H_2O /formic acid, triethylamine, propanol. Optical density at 280 nm and FITC or ENAI fluorescence (F) were recorded simultaneously.

Table 1. Energy transfer between fluorophores: Glucose site to Na⁺ site

Parameter	Donor/acceptor			
	Membranes		Partially purified protein	
	FITC/ ENAI	IPM/ FNAI	FITC/ ENAI	IPM/ FNAI
λ_D , nm	520	458	520	458
λ_A , nm	516	490	516	490
ϕ_D	0.47	0.46	0.45	0.46
ϕ_A	0.11	0.14	0.9	0.15
ϕ_{DA}/ϕ_D	0.23	0.30	0.20	0.33
$J \times 10^{-15}$, cm ³ ·M ⁻¹	130	65	130	65
R_0 , Å	46	39	46	39
Efficiency	0.78	0.70	0.80	0.67
$R_{2/3}$, Å	37	34	37	35

λ_D , donor emission wavelength maximum; λ_A , acceptor excitation wavelength maximum; ϕ_D , quantum yield of donor in absence of acceptor; ϕ_A , quantum yield of donor in presence of acceptor; J , overlap integral; R_0 , distance at which energy transfer is 50%; $R_{2/3}$, distance between probes using an orientation factor of 2/3.

tween the observed efficiencies of fluorescence energy transfer using FITC and FNAI as donor is the result of four polypeptides containing the *N*-acetylimidazole derivative that are unaffected by acceptor addition. That these proteins are separate and distinct from the cotransporter is suggested by their different NaDodSO₄/polyacrylamide molecular masses and apparently different isoelectric points. That non-cotransporter Na⁺-site probes do not contribute to the observed fluorescence energy transfer is suggested by column 3 of Table 2. By using the sensitized fluorescence of the acceptor as a means of determining fluorescence transfer efficiency, an efficiency of 77% is seen, similar to the results with isothiocyanate derivatives as donors. If the *N*-acetylimidazole derivatives are corrected for contributing only one-third of the total fluorescence, the efficiency of transfer is in good agreement with Table 1 and sensitized fluorescence emission data. These results suggest that the Na⁺ and glucose sites are located on the same 75-kDa polypeptide with an approximate isoelectric point of 5.6–5.3 and are ≈35 Å apart.

DISCUSSION

Fluorescence energy transfer as a means of estimating distances between fluorophores has provided topographical estimates of a number of membrane proteins. These include the sarcoplasmic reticulum Ca²⁺-ATPase (14), rhodopsin

 Table 2. Energy transfer between fluorophores: Na⁺ site to glucose site

Parameter	Donor/acceptor		
	FNAI/EITC	ANAI/IPM	FNAI*/EITC
λ_D , nm	522	382	
λ_A , nm	522	380	
$J \times 10^{-15}$, cm ³ ·M ⁻¹	105	13	
R_0 , Å	45	30	
Efficiency			
Observed	0.27	0.106	0.77
Corrected	0.82	0.32	
$R_{2/3}$, Å	35	34	37

Corrected efficiencies are corrected for nonstoichiometric binding of FNAI to the Na-glucose cotransporter. FNAI binding to the cotransporter is 33% of the total FNAI bound to membranes.

*Distance determined by sensitized acceptor fluorescence.

(15), the *N,N'*-dicyclohexylcarbodiimide binding protein of mitochondria (16), and the acetylcholine receptor (17, 18).

Assumptions were made in our estimates of the distance between the fluorophores. These are that the fluorophores have a random, rapidly rotating orientation with respect to each other—i.e., the orientation factor $K^2 = 2/3$ —and the stoichiometry between donor/acceptor pairs is 1.

The orientation assumption introduces a degree of uncertainty into the distance calculations, the seriousness of which depends on whether the true K^2 is close to 4 or 0 (19). We have determined the limiting anisotropy for FNAI, EITC, and FITC in 50% propylene glycol. All three probes have polarizations of <0.25 (data not shown). The error introduced by assuming a random distribution with polarization values of <0.3 is <10% (20). We have also reported that pyrene isothiocyanate bound at the glucose site has limited solvent accessibility based on quenching studies with the heavy atom quencher Tl⁺ (12). These results are consistent with pyrene isothiocyanate located in a membrane crevice rather than freely rotating on the protein surface.

The second assumption regarding donor/acceptor pair stoichiometry has been examined by using four approaches. The first involves purification of the cotransporter by chromatofocusing and comparison of the fluorophores. The ratio of FITC/ENAI on the partially purified protein is 1. This is consistent with a 1:1 stoichiometry. The purified protein was also examined for ENAI binding following chromatofocusing. The pI 5.3 fraction gives identical binding rate constants with and without pretreatment with Na⁺ and ENAI, suggesting a single class of ENAI binding sites that are Na⁺ protectable. The membrane preparation was also examined for ENAI inhibition of transport and a single rate constant of 0.4 sec⁻¹ was determined. Since no energy transfer to FITC is observed if Na⁺ is included during exposure to ENAI, it appears that only one class of tyrosines is responsible for ENAI binding in both preparations.

The chromatofocused cotransporter was also examined by proteolytic digestion using fluorescent *N*-acetylimidazole derivatives as markers of the Na⁺ site and fluorescent isothiocyanate derivatives as markers of the glucose site. Of the 18 polypeptide fragments seen by HPLC, only 1 fragment contains FITC, whereas a second contains ENAI. Although the molecular masses of these fragments is yet unknown, the agreement between chymotryptic and tryptic digestion profiles supports a 1:1 stoichiometry. Since protein was digested without denaturation, these results are also consistent with substrate site separation.

The final method of stoichiometry analysis involved the use of four different donor/acceptor pairs and two methods of distance determination. The use of a number of donor/acceptor pairs of varying efficiencies has been cited as useful in answering questions of stoichiometry (21–24). This approach to stoichiometry stems from theoretical considerations that the more efficient pair should result in a larger distance estimate than the lesser transfer efficient pairs when the stoichiometry is different from the predicted value. With membrane vesicle determinations, the critical distance, R_0 varied from 30 Å to 46 Å, with only an 8% variation in the distance between fluorophores compared to a 5% variation with the pI 5.3 fraction. These results are in good agreement with a 1:1 stoichiometry in both preparations.

Our results suggest that both substrate sites are located on a single 75-kDa polypeptide based on fluorescent labeling with probes of a single molecular mass band (4) and pI 5.3 protein. Although the distance between fluorophores of 35 Å contains a number of assumptions, it seems likely that this value is within 10% of the true value. On this basis, we suggest that the apparent Na⁺ binding site is 40 Å to 30 Å away from the apparent glucose binding site. These results may have significant implications on the carrier mechanism.

Since the carrier undergoes a Na⁺-induced conformational change, our results suggest that the change is relatively extensive since the sites are far removed from each other. The results also imply that a single channel mechanism is unlikely since the apparent binding sites are far away from each other.

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