J. Membrane Biol. 140, 39–46 (1994)

The Journal of
Membrane
Biology

Renal Cortical Basolateral Na^+/HCO_3^- Cotransporter: II. Detection of Conformational Changes with Fluorescein Isothiocyanate Labeling

J. Stim, A.A. Bernardo, F.T. Kear, Y.Y. Qiu, J.A.L. Arruda

Section of Nephrology, M/C 793, University of Illinois at Chicago and West Side VAMC, 840 South Wood Street, Chicago, Illinois 60612-7315

Received: 16 August 1993/Revised: 4 February 1994

Abstract. Fluorescein isothiocyanate (FITC) fluorescently labels amino groups and has been useful in detecting conformational changes in transport proteins through quenching or enhancement of the fluorescence signal upon exposure of protein to substrates. Solubilized renal basolateral membrane proteins, enriched in Na^+/HCO_3^- cotransporter activity, were reconstituted into liposomes and treated with FITC or its nonfluorescent analogue PITC (phenyl isothiocyanate). In the absence of Na^+ and HCO_3^-, incubation of proteoliposomes with PITC or FITC significantly inhibited cotransporter activity. However, in the presence of Na^+ and HCO_3^- during labeling both agents failed to inhibit cotransporter activity, indicating that these probes interact specifically with the cotransporter. In the presence of the substrates Na^+ and HCO_3^-, PITC binds covalently to amino groups unprotected by substrates leaving the Na^+/HCO_3^- cotransporter available for specific labeling with FITC. Addition of $NaHCO_3$ to FITC-labeled proteoliposomes resulted in a concentration-dependent enhancement of the fluorescence signal which was inhibited by pretreatment with 4,4′-diisothiocyanostilbene 2′,2-disulfonic acid (DIDS) prior to FITC labeling. SDS PAGE analysis of FITC-treated proteoliposomes showed the presence of two distinct fluorescent bands (approximate MW of 90 and 56 kD). In the presence of substrates, the fluorescence intensity of these bands was enhanced as confirmed by direct measurement of gel slice fluorescence. Thus, FITC detects conformational changes of the Na^+/HCO_3^- cotransporter and labels proteins which may represent the cotransporter or components of this cotransporter.

Correspondence to: J.A.L. Arruda

Key words: FITC — Protein labeling — Disulfonic stilbenes — Fluorescence — Proteoliposomes — Protein conformation

Introduction

An electrogenic Na^+/HCO_3^- cotransporter across the basolateral membrane of proximal convoluted tubules has been described in amphibian and mammalian kidney [1, 2, 4, 5] and functions as the main system for HCO_3^- transport from cell to blood. Na^+/HCO_3^- cotransporter activity is demonstrable in basolateral membrane vesicles isolated from rabbit renal cortex [1, 2] and was partially purified from these membranes [3]. Partial purification has allowed further characterization of the Na^+/HCO_3^- cotransporter and its substrate binding sites.

Fluorescein isothiocyanate (FITC) labeling of transport proteins has been used to identify protein subunits and to study substrate-transport protein interactions [6, 7, 9, 10–13, 15, 18, 19]. Phenyl isothiocyanate (PITC) labeling of transporter in the presence of substrates prior to FITC labeling enhances the specificity of FITC labeling by occupying potentially reactive amino groups with a nonfluorescent compound while leaving amino groups at substrate binding sites available for reaction with FITC. The isothiocyanates covalently bind to uncharged α-amino groups, and may also bind to the ε-amino group of the lysine residue under alkaline conditions [9, 15]. The presence of FITC at or near substrate binding sites can then be used to study substrate-induced conformational changes in the transport protein by monitoring changes in the fluorescent yield of bound FITC. The purpose of this study was to label substrate binding sites of the renal basolateral

Na^+/HCO_3^- cotransporter with FITC in order to analyze substrate-induced conformational changes of the protein and to try to identify components of the cotransporter through fluorescently labeled protein bands on SDS polyacrylamide gel electrophoresis.

Materials and Methods

Materials

^{22}Na was purchased from Amersham. L-α phosphatidyl choline, octylglucoside, Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide, FITC, PITC, and DIDS were bought from Sigma. General laboratory chemicals of analytical grade were obtained from Sigma or Fisher.

Preparation of Membranes and Proteoliposomes

Renal cortical basolateral membrane vesicles were prepared from New Zealand White rabbits by means of differential and gradient centrifugation with ionic precipitation as described previously [17]. This procedure results in highly purified basolateral membranes enriched on the average of 12- to 14-fold in Na-K-ATPase activity compared with homogenates, and with less than 5% cross contamination with brush border membranes.

The proteoliposomes were prepared as previously described [3]. Briefly, one part purified basolateral membrane vesicles (5 mg/ml) was incubated with 1.25 parts 2% *n*-octyl β-D-glucopyranoside (octylglucoside) in 50 mM HEPES and 250 mM mannitol (buffer A), pH 7.2, on ice for 15 min. The concentration of the detergent octylglucoside was 1.1% and the protein concentration was 2.2 mg/ml. The protein-detergent mixture was spun for 30 min at 110,000 × *g*. The supernatant was recovered and concentrated using Centriprep 10 (Amicon). The protein (2.5 mg/ml) was reconstituted into liposomes by mixing 1.6 part of protein (v/v) with one part of L-α-phosphatidylcholine (35 mg/ml) which was sonicated for 10 min. The combination of proteins and lipids was dialyzed for 18 hr in buffer A using a membrane restricting the passage of 6–8 kD molecules.

Effect of PITC Treatment on Basolateral Membrane Vesicle Na^+/HCO_3^- Cotransporter Activity

Basolateral membrane vesicles were treated with 2 mM PITC in 50 mM Tris-base, 2 mM EDTA, pH 9.2 for 30 min at room temperature. The reaction was stopped by a 10-fold addition of buffer and the preparation was centrifuged at 38,000 × *g* for 30 min, 4°C to remove unreacted PITC. The PITC-treated membranes were resuspended in 100 μl of loading medium consisting of 200 mM sucrose, 50 mM HEPES, 1 mM Mg gluconate, pH 7.5. Control basolateral membranes had been incubated in loading medium instead of PITC. Na^+/HCO_3^- cotransporter activity of control and PITC-treated basolateral membrane vesicles was measured by the rapid filtration ^{22}Na uptake assay as previously described [14]. Na^+/HCO_3^- cotransporter activity was defined as ^{22}Na uptake (nmol/mg protein/3 sec) in the presence of 25 mM HCO_3 minus the uptake in the presence of 25 mM gluconate. Uptakes were performed at room temperature using 10 μg of membrane proteins.

Effect of PITC and FITC Treatment on Proteoliposome Na^+/HCO_3^- Cotransporter Activity

Proteoliposomes were exposed to 2 mM PITC for 40 min at 25°C with agitation, either in the presence or absence of 100 mM $NaHCO_3$. The reaction was stopped by addition of a 10-fold volume of cold Solution M (50 mM HEPES/Tris base, 250 mM mannitol, pH 7.5). Unreacted PITC and $NaHCO_3$ were removed by centrifugation at 115,000 × *g* for 30 min at 4°C followed by resuspension of the pellet in cold Solution M and recentrifugation. Control proteoliposomes were not treated with PITC. Aliquots of the proteoliposomes treated with PITC in the presence of $NaHCO_3$ (substrate protected) were further exposed to 300 μM FITC in the dark for 40 min at 25°C either in the presence or absence of 100 mM $NaHCO_3$. All further processing occurred in the dark. The reaction was stopped by the addition of a 10-fold volume of cold Solution M and unreacted FITC and $NaHCO_3$ were removed as previously described for the PITC step. The final pellet was resuspended in 100 μl of Solution M. Na^+/HCO_3^- cotransport activity of control, PITC-treated, and FITC-treated proteoliposomes was measured as previously described for membrane vesicles, except for the replacement of loading medium with Solution M.

Fluorescence Measurement of FITC-treated Proteoliposomes

Proteoliposomes were treated with 2 mM PITC in the presence of 100 mM $NaHCO_3$ as previously described. Subsequently, the PITC-treated proteoliposomes were exposed to 300 μM FITC in the presence or absence of 100 mM $NaHCO_3$ for 40 min at 25°C. In some experiments, Na^+/HCO_3^- cotransporter-enriched solubilized protein was treated with 2 mM DIDS for 1 hr at 25°C prior to incorporation into liposomes. Excess DIDS was removed by dilution in Solution M followed by centrifugation and resuspension of the pellet. PITC-treated proteoliposomes exposed to FITC in the absence of $NaHCO_3$ were washed/resuspended in Solution M, pH 8.5 while those exposed to FITC in the presence of $NaHCO_3$ were washed/resuspended in Solution M, pH 7.5. Final pH of both treatments after 40 min was approximately 8.5. The FITC reaction was stopped with a 10-fold volume of cold Solution M, pH 7.5 and unreacted FITC and $NaCO_3$ were removed as previously described. The final pellet was resuspended in 100 μl of Solution M, pH 7.5 and the protein concentration was determined. Fifty micrograms of FITC-treated proteoliposomes were suspended in a cuvette with 50 mM HEPES/Tris base, pH 7.5 and the fluorescence was monitored using a Perkin-Elmer 650-40 fluorescence spectrophotometer set in the ratio mode with excitation wavelength of 495 nm, emission wavelength of 525 nm and slit widths of 4 nm. Fluorescence was measured by continuous graph tracing and, after a stable baseline was established, substrates were added with agitation from 1 M stock solutions ($NaHCO_3$, Na gluconate, $KHCO_3$, K gluconate) to bring the total volume to 2 ml. For each substrate concentration, dilutional effect on fluorescence was determined by the addition of corresponding volumes of buffer instead of substrate. pH was measured using a standard pH electrode after final fluorescence measurement. The change in fluorescence intensity upon addition of substrate or buffer was quantitated by dividing the change in fluorescence by the initial baseline and multiplying by 100 ($\pm\Delta$ F/F × 100). A positive value indicates enhancement while a negative value indicates quenching.

SDS-Polyacrylamide Gel Electrophoresis

Proteoliposomes exposed to 300 μm FITC in the presence and absence of 100 mM $NaHCO_3$ were prepared and dialyzed overnight in Solution M, pH 7.5. Treated proteoliposomes were centrifuged at 115,000 × *g*, 30 min, 4°C and resuspended in 100 μl Solution M. Aliquots (30–40 μg) were vortexed with standard sample buffer for at least 2 min, and electrophoresed on 10% polyacrylamide slab gels using the method of Laemmli [8]. Parallel sets of samples were run for Coomassie Blue staining. Fluorescent bands were visualized with UV light. The gel was cut into sequential 4 mm slices and protein was eluted by mashing each slice in 1.3–1.5 ml 2.5% SDS, 10 mM Tris base, pH 9.2 and incubating overnight at room temperature. After centrifugation, the supernatant was measured for fluorescence using excitation wavelength of 495 nm, emission wavelength of 525 nm, and 4 nm slit widths.

Analysis of Data

Results are presented as mean ± SEM and the *t*-test for paired or unpaired data was used to analyze the results whenever appropriate.

Results

PITC and FITC Inhibition of Renal Basolateral Na^+/HCO_3^- Cotransporter Activity

Figure 1 shows the effect of PITC and FITC on Na^+/HCO_3^- cotransporter in basolateral membranes or in proteoliposomes. The results were normalized by expressing the values as a percentage of control values. The left panel shows that PITC treatment of basolateral membranes decreased HCO_3^--dependent ^{22}Na uptake, a measure of Na^+/HCO_3^- cotransporter activity, by 66% from 2.45 ± 0.66 to 0.84 ± 0.28 nmol/mg protein/3 sec, $P < 0.025$. PITC treatment did not affect ^{22}Na uptake in the presence of gluconate (*not shown*), indicating that PITC does not have an effect on diffusive ^{22}Na uptake but directly affects the cotransporter activity.

The right panel of Fig. 1 shows the effect of PITC or FITC on Na^+/HCO_3^- cotransporter activity in proteoliposomes. As expected, the activity of the Na^+/HCO_3^- cotransporter was enriched in proteoliposomes compared to the original basolateral membranes, thus allowing easier detection of the effect of isothiocyanates on the cotransporter. PITC decreased Na^+/HCO_3^- cotransporter activity significantly by 60% from 75.1 ± 21.5 to 30.5 ± 9.6 nmol/mg protein/3 sec, $P < 0.05$. When the proteoliposomes were treated with PITC in the presence of 100 mM Na^+/HCO_3^-, PITC failed to inhibit the Na^+/HCO_3^- cotransporter activity (control 75.1 ± 25.8 *vs.* $NaHCO_3$ + PITC 79.9 ± 17.6 nmol/mg protein/3 sec, NS).

In an additional series of experiments, we examined the interaction of PITC, $NaHCO_3$ and FITC on the inhibition of Na^+/HCO_3^- cotransporter activity by FITC (Fig. 1, right panel). The proteoliposomes were treated with PITC in the presence of 100 mM $NaHCO_3$ to protect the cotransporter sites. Removal of $NaHCO_3$ and PITC followed by treatment with FITC resulted in 76% inhibition of the Na^+/HCO_3^- cotransporter compared to control values (control 75.1 ± 21.5 *vs.* FITC 18.1 ± 4.6 nmol/mg protein/3 sec, $P < 0.05$). If the proteoliposomes were treated with FITC in the presence of 100 mM $NaHCO_3$, then the effect of FITC to inhibit the Na^+/HCO_3^- cotransporter was partially blocked (control 75.1 ± 21.5, FITC 18.1 ± 4.5, $NaHCO_3$ + FITC 48.2 ± 12.9 nmol/mg protein/3 sec). These data demonstrate that PITC or FITC inhibits Na^+/HCO_3^- cotransporter activity, and that the effect of these agents to inhibit the activity of this cotransporter is prevented or blunted by the presence of $NaHCO_3$ prior to treatment of the membranes or proteoliposomes.

The above results are compatible with the hypothesis that PITC and FITC interact with the cotransporter, and that the presence of $NaHCO_3$ protects against inhibition by occupying sites near the Na^+ and HCO_3^- substrate sites in the cotransporter, thus preventing the

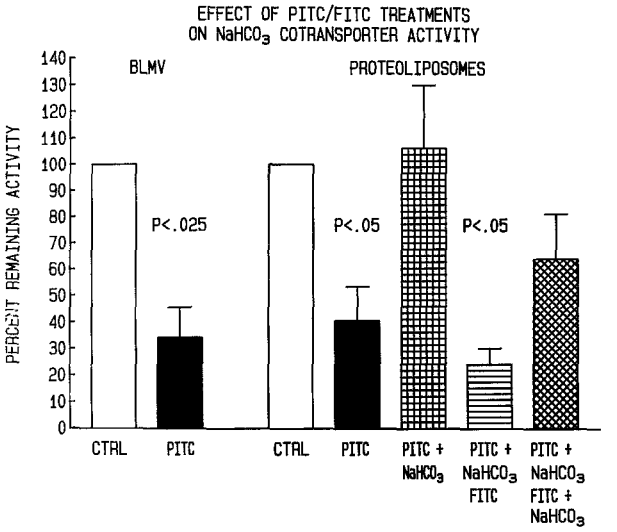

Fig. 1. Effect of PITC and FITC treatment on HCO_3^--dependent ^{22}Na uptake by basolateral membranes or octylglucoside-solubilized protein reconstituted in proteoliposomes. The left panel shows the effect of PITC treatment on Na^+/HCO_3^- cotransporter activity in basolateral membranes compared to untreated controls. The right panel shows the effect of PITC treatment (filled bars) on Na^+/HCO_3^- cotransporter activity in proteoliposomes compared to untreated control (open bars). The proteoliposomes were treated with PITC either in the presence (squared bars) or absence (filled bars) of $NaHCO_3$. Subsequent treatment of proteoliposomes that were exposed to PITC in the presence (squared bars) of $NaHCO_3$ with FITC either in the presence (cross-latched bars) or absence of $NaHCO_3$ (bars with horizontal lines) was then studied.

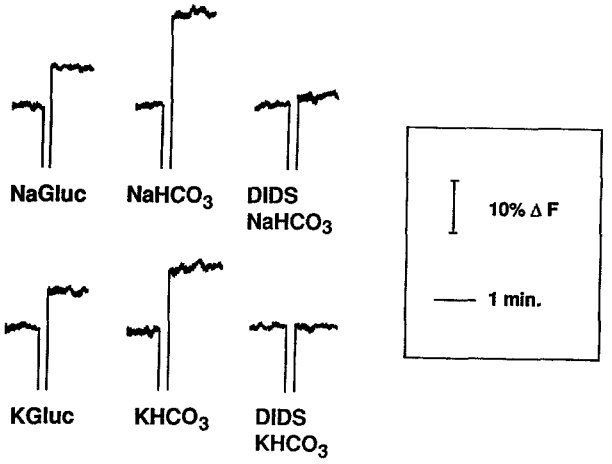

Fig. 2. Substrate effects on the fluorescence of FITC-treated proteoliposomes. Representative tracings are depicted showing the effect of 50 mM Na gluconate, K gluconate, $NaHCO_3$ or $KHCO_3$ on the fluorescence of FITC-treated proteoliposomes enriched in Na^+/HCO_3 cotransporter activity. Substrate was added during the gap in the tracing. All proteoliposomes had been treated with PITC in the presence of $NaHCO_3$, followed by FITC in the presence of $NaHCO_3$. The right panel shows the effect of pretreating octylglucoside-solubilized protein with 2 mM DIDS prior to PITC and FITC treatments on the fluorescence enhancement caused by $NaHCO_3$ and $KHCO_3$.

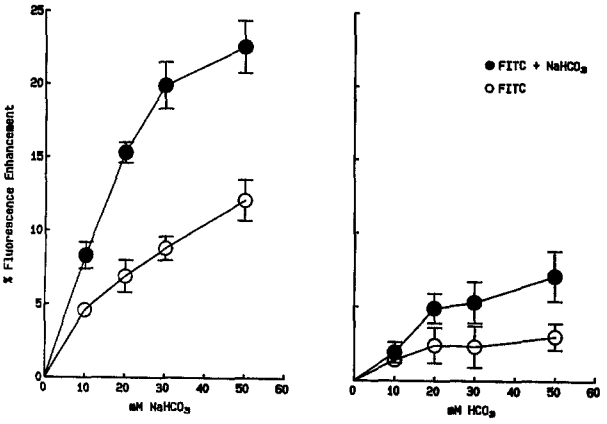

Fig. 3. The enhancement of fluorescence in FITC-treated proteoliposomes as a function of $NaHCO_3$ concentration. Proteoliposomes were treated with PITC in the presence of $NaHCO_3$ and then treated subsequently with FITC either in the presence or absence of $NaHCO_3$. After removal of excess reagents, fluorescence was measured before and after the addition of various concentrations of $NaHCO_3$ (left panel). The right panel shows the same data but corrected for the effect of Na^+ by subtracting the fluorescence enhancement due to Na gluconate.

binding of PITC or FITC to the cotransporter. These findings suggested that FITC binds to the cotransporter and thus may serve as a fluorescent label to monitor conformational changes as Na^+ and/or HCO_3^- interact with the cotransporter.

Effect of Na^+ or HCO_3^- on the Fluorescence of FITC-treated Proteoliposomes

Figure 2 shows the fluorescence of FITC-treated proteoliposomes. These proteoliposomes were pretreated with $NaHCO_3$ with PITC, and then with $NaHCO_3$ and FITC. The $NaHCO_3$ was then removed along with the excess FITC. The fluorescence recorded in Fig. 2 should reflect labeling of the Na^+/HCO_3^- cotransporter. We tested the ability of either Na^+ or HCO_3^- to interact with the cotransporter and to detect conformational changes of the cotransporter as assessed by changes in FITC fluorescence. Not shown is the finding that addition of sucrose or water caused a decrease in fluorescence, presumably through a dilutional effect. Addition of different salts, such as 50 mM NaCl, 50 mM Na gluconate, or 50 mM Na isethionate led to an enhancement of baseline fluorescence by approximately 10%. That salts containing Na^+ or no Na^+ leads to a comparable enhancement of fluorescence suggests that this effect is nonspecific and probably related to ionic strength.

$NaHCO_3$, on the other hand, caused a greater increase in baseline fluorescence than Na gluconate or other non-HCO_3^- salts by approximately 10% (Fig. 2). $KHCO_3$ also caused an enhancement in fluorescence although of smaller magnitude than that elicited by $NaHCO_3$ (Fig. 2). These results suggested that HCO_3^- causes an enhancement of fluorescence and that the presence of this anion seems to have an effect additive to Na^+. The results of the addition of 20 mM $NaHCO_3$ or $KHCO_3$ on fluorescence of FITC-treated proteoliposomes were normalized for the effect of the cation by subtracting the results from those obtained with the same concentration of Na gluconate or K gluconate. $NaHCO_3$ caused a greater net increase in fluorescence than $KHCO_3$ (5.3 ± 0.3 *vs.* $3.6 \pm 0.3\%$, $P < 0.05$).

It should be noted that the addition of increasing amounts of HCO_3^- increased the pH of the proteoliposome-buffer system in the cuvette, which typically increased 0.25–0.30 units above the buffer pH of 7.50 after the addition of 30 mM HCO_3^-. The fluorescence intensity of FITC in solution was measured over a pH range of 6.0 to 8.5 and plateaued at pH 7.5 (*data not shown*), suggesting that pH effects on the fluorescence of FITC-labeled proteoliposomes were minimal at pH 7.5 and above. To further exclude the possibility that the effect of $NaHCO_3$ to enhance FITC fluorescence was secondary to changes in pH, the pH of the $NaHCO_3$ stock solution was maintained constant at 7.5 by bubbling it with 10% CO_2. The maintenance of the pH at this level was verified by measuring the pH of the cuvette contents at the end of the experiment, which took less than 3 min to complete. Under these conditions, addition of $NaHCO_3$ caused the same degree of fluorescence enhancement, suggesting that the effect of

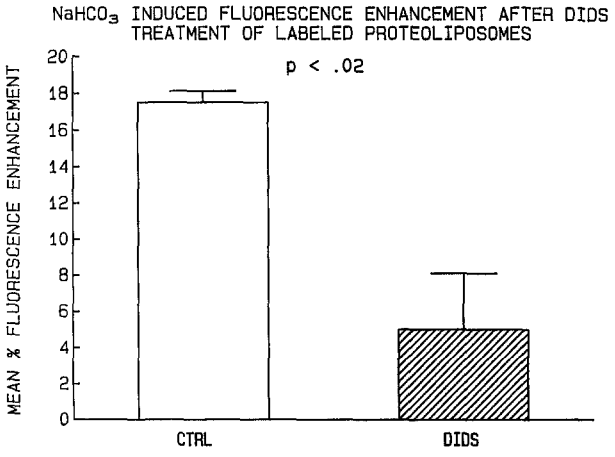

Fig. 4. The effect of DIDS on the fluorescence of FITC-treated proteoliposomes. Octylglucoside-solubilized protein enriched in Na^+/HCO_3^- cotransporter activity was treated with 2 mM DIDS for 1 hr *vs.* no treatment. Protein was reconstituted into liposomes and then treated with PITC in the presence of $NaHCO_3$ followed by FITC in the presence of $NaHCO_3$. Fluorescence was measured before and after addition of 50 mM $NaHCO_3$ and the percent increase in fluorescence was calculated.

$NaHCO_3$ was not secondary to pH. In addition, 20 mM $NaHCO_3$, a concentration which caused only 0.1 pH unit change, elicited a significant increase in fluorescence (*see above*).

Figure 3 shows the enhancement of FITC fluorescence by different concentrations of $NaHCO_3$. The membranes were labeled with FITC in the absence and presence of $NaHCO_3$ (to protect the substrate sites). In the membranes labeled with FITC in the presence of $NaHCO_3$, the enhancement of fluorescence by $NaHCO_3$ was greater than that observed in membranes labeled in the absence of $NaHCO_3$. A concentration-dependent increase in fluorescence enhancement occurred which began to plateau at a $NaHCO_3$ concentration of 30 mM and exhibited an apparent K_m of 15 mM. These results are in agreement with the results of Fig. 1 showing that the presence of $NaHCO_3$ prior to labeling with FITC protects the binding sites for Na^+ and HCO_3^-. These results are also in agreement with gel electrophoresis results (*see* Fig. 5 below), showing that the presence of $NaHCO_3$ during labeling with FITC enhances the fluorescence intensity of labeled bands.

Disulfonic stilbenes inhibit the activity of the Na^+/HCO_3^- cotransporter. We chose to examine the effect of DIDS on the enhancement of FITC fluorescence by $NaHCO_3$. DIDS was chosen because it does not interfere with the fluorescence of FITC. The solubilized protein was treated either with the vehicle or 2 mM DIDS for one hour. This period of time has been shown to be sufficient for binding of DIDS to the Na^+/HCO_3^- cotransporter [16]. The solution containing DIDS was then washed and the membranes were labeled with PITC followed by FITC. Figure 2 shows that DIDS treatment inhibits the enhancement of fluorescence elicited by 50 mM $NaHCO_3$ or by $KHCO_3$. Figure 4 summarizes the effects of DIDS on the enhancement of FITC fluorescence by $NaHCO_3$. It is clear that DIDS significantly blunts the effect of $NaHCO_3$ to enhance fluorescence. These results strongly suggested that the effect of $NaHCO_3$ or $KHCO_3$ to enhance FITC fluorescence is the result of a specific interaction with the $NaHCO_3$ cotransporter.

Effect of $NaHCO_3$ on Fluorescence Labeling of Membrane Proteins by FITC

Solubilized basolateral membrane proteins were labeled with FITC in the presence or absence of 100 mM $NaHCO_3$. The protein was then subjected to SDS-PAGE and the fluorescence of gel slices was determined. It is clear that in the absence of $NaHCO_3$, there is some protein labeling by FITC (Fig. 5). This labeling is strikingly enhanced in the presence of $NaHCO_3$ with the particular enhancement of a 56 kD band. The 90 and 34 kD bands also showed fluorescence enhancement. The Coomassie Blue staining showed no difference qualitatively in staining intensity between the two FITC treatments, suggesting that the increased fluorescence was due to increased FITC labeling of these bands. The right panel shows the SDS-PAGE analysis of FITC-solubilized proteins. It is clear that several bands are present in SDS gel while three bands are prominently labeled with FITC.

Discussion

The presence of a fluorescent probe at substrate binding sites of a transport protein may serve as a sensitive monitor of substrate-induced conformational change of the protein through alterations in the fluorescence of the probe. Isothiocyanates covalently bind to α-amino groups, and under alkaline conditions, may also bind to the ε-amino group of lysine residues [9, 15]. Through the initial labeling of nonspecific amino groups with a nonfluorescent isothiocyanate (PITC) in the presence of substrates to protect substrate binding sites, the fluorescent isothiocyanate (FITC) can then specifically label substrate binding sites. This technique has been successfully utilized to study substrate-transporter interactions in the intestinal and renal brush border Na-glucose cotransporters [10, 19], and the intestinal Na-proline cotransporter [18]. Other transport proteins including the Na-K-ATPase [7], the gastric H-K-ATPase [6], and the sarcoplasmic reticulum Ca-ATPase [11–13] have been studied by fluorescent labeling with FITC. The Na^+ and HCO_3^- binding sites of the renal Na^+/HCO_3^- cotrans-

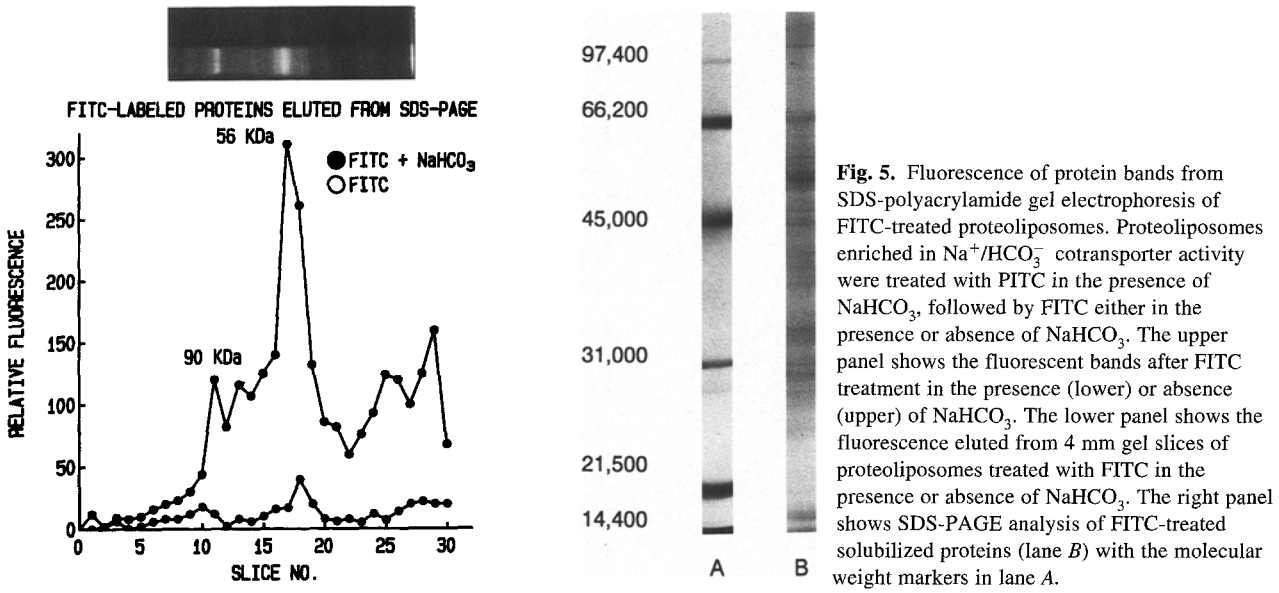

Fig. 5. Fluorescence of protein bands from SDS-polyacrylamide gel electrophoresis of FITC-treated proteoliposomes. Proteoliposomes enriched in Na^+/HCO_3^- cotransporter activity were treated with PITC in the presence of $NaHCO_3$, followed by FITC either in the presence or absence of $NaHCO_3$. The upper panel shows the fluorescent bands after FITC treatment in the presence (lower) or absence (upper) of $NaHCO_3$. The lower panel shows the fluorescence eluted from 4 mm gel slices of proteoliposomes treated with FITC in the presence or absence of $NaHCO_3$. The right panel shows SDS-PAGE analysis of FITC-treated solubilized proteins (lane *B*) with the molecular weight markers in lane *A*.

porter were specifically labeled with FITC after PITC exposure to begin characterization of this cotransporting protein and to identify its components.

That the PITC and FITC were indeed bound at substrate binding sites was strongly suggested by the significant inhibition of the HCO_3^--dependent ^{22}Na uptake by PITC or FITC binding in basolateral membrane vesicles and solubilized basolateral membranes reconstituted into proteoliposomes. Although it could be argued that the effect of PITC and FITC on the Na^+/HCO_3^- cotransporter in the proteoliposomes could be related to the fact that the agents interfered with the efficiency of reconstitution, this is unlikely because these agents had similar effects on the native membranes as they did on the proteoliposomes. The finding that the presence of $NaHCO_3$ prior to the exposure of the membranes to PITC or FITC protected against the inhibitory effect of PITC or FITC on Na^+/HCO_3^- cotransporter activity is strong evidence that these agents interacted specifically with the cotransporter. The ability of substrates to protect the transport protein from inactivation by isothiocyanates has been described in other transporting systems, including the Na-K-ATPase, Ca-ATPase, and Na-glucose cotransporter [7, 10, 11].

Since FITC labels substrate binding sites, the changes in fluorescence elicited by the substrate should reflect changes in the microenvironment of substrate binding sites. In transport proteins labeled with FITC, the conformational changes elicited by exposure to substrates have caused either quenching or enhancement of fluorescence of the fluorescein label. In the FITC-labeled intestinal Na-glucose cotransporter, a Na-induced quenching of fluorescence was observed which was saturable and concentration dependent, reflecting a Na^+-induced conformational change in the transporter [10]. Interestingly, this was not observed in the FITC-labeled renal Na-glucose cotransporter, in which the addition of Na^+ did not cause quenching of fluorescence but did cause an increase in the fluorescent labeling of the cotransporter as revealed by SDS-PAGE [19]. The FITC-labeled Na-K-ATPase showed K^+-induced fluorescence quenching that was recovered by the addition of Na^+ [15]. The FITC-labeled sarcoplasmic reticulum Ca-ATPase responded to Ca^{2+} with quenching of the labeled fluorescence, while vanadate exposure resulted in fluorescence enhancement [13]. Thus, it becomes clear from our study, as well as other studies [6, 7, 10–13, 18, 19], that specific binding of FITC to a cotransporter protein may be associated with a decrease, an increase, or no change in fluorescence when the substrate interacts with the labeled transporter. Although the explanation for this phenomenon is not completely clear it may be related to conformational changes of the cotransporter protein as it interacts with the substrates which may result in exposure of FITC-labeled amino acids (resulting in enhancement of fluorescence) or masking of the same FITC labeled amino acids (resulting in quenching of fluorescence).

Our results with the Na^+/HCO_3^- cotransporter showed that addition of HCO_3^-, and to a lesser degree Na^+, induced an enhancement of fluorescence in FITC-labeled proteoliposomes which was maximal when substrate sites were protected during FITC treatment. Addition of Na^+ also caused an enhancement of fluorescence that was not different from the enhancement observed in the presence of K^+, suggesting that Na^+ per se did not have a specific effect. However, the combined effect of Na^+ and HCO_3^- was greater than the ef-

fect of HCO_3^- alone, suggesting the presence of HCO_3^- is necessary for Na^+ to elicit its effect. That this effect of HCO_3^- is not due to pH changes was shown by the fact that the fluorescence enhancing effect of HCO_3^- was undiminished when pH was maintained constant at 7.50 by the bubbling of CO_2 into the stock solution. Additionally, the HCO_3^- effect was present at low concentrations of HCO_3^- (<20 mM) where pH increment was minimal. Furthermore, that DIDS prevents the effect of HCO_3^- to enhance fluorescence without altering its effects on pH clearly indicates that the effect of HCO_3^- results from interaction with the cotransporter rather than from a nonspecific effect of pH.

Our results show that pretreatment of solubilized protein with DIDS prior to PITC/FITC labeling resulted in the inhibition of the $NaHCO_3$-induced fluorescence enhancement. DIDS is an isothiocyanate compound which covalently binds amino groups, as PITC and FITC do. Due to the presence of two reactive moieties, DIDS may cause protein crosslinking and this could inhibit incorporation of proteins into liposomes. We feel, however, that the explanation for the DIDS effect is not due to crosslinking but to DIDS competing with FITC for binding sites. That fluorescein-labeled protein is indeed incorporated into liposomes is evidenced by the presence of $NaHCO_3$-induced fluorescence enhancement, albeit significantly lower than in the absence of DIDS pretreatment. DIDS exposure after PITC/FITC labeling had no effect on $NaHCO_3$-induced fluorescence enhancement, suggesting that DIDS and FITC do compete for the same binding sites.

The results from the SDS-PAGE of FITC-labeled proteoliposomes reinforce that FITC was bound to specific protein bands which may represent the Na^+/HCO_3^- cotransporter or subunits of the cotransporter. FITC-treated proteoliposomes without substrate protection show faint labeling of a 56 kD band which is maximally labeled with FITC when substrate sites are protected. Although other fluorescent bands are evident in the substrate-protected FITC-labeled proteoliposome lane, background fluorescence appears to be increased which may be due to FITC lysis, and this could contribute to the fluorescence of individual bands. However, the significance of the 56 kD band lies with its dramatic fluorescence above background for both FITC preparations and suggests that this is the Na^+/HCO_3^- cotransporter. As shown in the preceding paper [3], partial purification of the Na^+/HCO_3^- cotransporter shows an enhancement of a protein doublet of 56 kD, a protein band clearly labeled by FITC in SDS-PAGE.

It is unlikely that the enhancement of FITC fluorescence by $NaHCO_3$ in the experiments represents binding to Na-K-ATPase since solubilization of the membranes with octylglucoside resulted in loss of Na-K-ATPase activity of basolateral membranes (*data not shown*). Furthermore, as indicated earlier, the quenching of fluorescence by K^+ in FITC-labeled Na-K-ATPase is quite unlike the effects of K^+ on the fluorescence of our FITC-labeled Na^+/HCO_3^- cotransporter.

Taken together, our findings suggest that the binding of Na^+ and HCO_3^- to their binding sites may induce conformational changes in the cotransporter which enhance the exposure of reactive amino groups to FITC and facilitate the FITC binding process. It may be speculated that these reactive amino groups may be located in the vicinity of substrate binding sites and that binding of substrates may reversibly alter the aqueous environment around these groups to such an extent that the fluorescent yield of an attached fluorescein molecule is increased, resulting in the fluorescence enhancement seen upon the addition of $NaHCO_3$. Another reason for the greater fluorescence enhancement observed in proteoliposomes labeled with FITC in the presence of $NaHCO_3$ is the unhindered access of Na^+ and HCO_3^- to their binding sites, which may then cause conformational changes that are necessary for full cotransporter activity, shown to be relatively unhindered by substrate protected FITC labeling.

In conclusion, isothiocyanates interact specifically with the Na^+/HCO_3^- cotransporter and the presence of substrates (Na^+ and HCO_3^-) protect against the inhibition caused by FITC or PITC. Interaction of Na-HCO_3 with the FITC-labeled cotransporter elicits changes in fluorescence suggestive of conformational changes in the protein. In the presence of $NaHCO_3$, FITC labeling shows enhanced fluorescence of certain protein bands, especially of a 56 kD band which may represent the cotransporter or active component thereof.

The authors thank Carmen Hill and Clary Olichwier for excellent secretarial assistance in the preparation of this manuscript.

This work was supported by the Merit Review Program from the Veterans Administration Central Office (J.A.L.A.), and the National Kidney Foundation of Illinois (A.A.B.).

References

1. Akiba, T., Alpern, R.J., Eveloff, J., Calamina, J., Warnock, D.G. 1986. Electrogenic sodium/bicarbonate cotransport in rabbit renal cortical basolateral membrane vesicles. *J. Clin. Invest.* **78:** 1472–1478
2. Alpern, R.J., Chambers, M. 1986. Cell pH in the rat proximal convoluted tubule: regulation by luminal and peritubular pH and sodium concentration. *J. Clin. Invest.* **78:**502–510
3. Bernardo, A.A., Kear, F.T., Ruiz, O.S., Arruda, J.A.L. 1994. Renal cortical basolateral Na^+/HCO_3^- cotransporter: I. Partial purification and reconstitution. *J. Membrane Biol.* **140:**31–37
4. Boron, W.F., Boulpaep, E.L. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO_3^- transport. *J. Gen. Physiol.* **81:**53–94
5. Grassl, S.M., Aronson, P.S. 1986. Na^+/HCO_3^- co-transport in basolateral membrane vesicles isolated form rabbit renal cortex. *J. Biol. Chem.* **261:**8778–8783

6. Jackson, R.J., Mendlein, J., Sachs, G. 1983. Interaction of fluorescein isothiocyanate with the $(H^+ + K^+)$-ATPase. *Biochim. Biophys. Acta* **731:**9–15
7. Karlish, S.J.D. 1980. Characterization of conformational changes in (Na,K) ATPase labeled with fluorescein at the active site. *J Bioenerg. Biomembr.* **12:**111–136
8. Laemmli, M.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature* **227:**680–685
9. Mitchison, C., Wilderspin, A.F., Trinnaman, B.J., Green, N.M. 1982. Identification of a labelled peptide after stoichiometric reaction of fluorescein isothiocyanate with the Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum. *FEBS Lett.* **146:**87–92
10. Peerce, B.E., Wright, E.M. 1984. Conformational changes in the intestinal brush border sodium-glucose cotransporter labeled with fluorescein isothiocyanate. *Proc. Natl. Acad. Sci. USA* **81:** 2223–2226
11. Pick, U. 1981. Interaction of fluorescein isothiocyanate with nucleotide-binding sites of the Ca-ATPase from sarcoplasmic reticulum. *Eur. J. Biochem.* **121:**187–195
12. Pick, U., Bassilian, S. 1981. Modification of the ATP binding site of the Ca^{2+}-ATPase from sarcoplasmic reticulum by fluorescein isothiocyanate. *FEBS Lett.* **123:**127–136
13. Pick, U., Karlish, S.J.D. 1982. Regulation of the conformational transition in the Ca-ATPase from sarcoplasmic reticulum by pH, temperature, and calcium ions. *J. Biol. Chem.* **257:**6120–6126
14. Ruiz, O.S., Arruda, J.A.L., Talor, Z. 1989. Na-HCO_3 cotransport and Na-H antiporter in chronic respiratory acidosis and alkalosis. *Am. J. Physiol.* **256:**F414–420
15. Sen, P.C., Kapakos, J.G., Steinberg, M. 1981. Modification of $(Na^+ + K^+)$-dependent ATPase by fluorescein isothiocyanate: Evidence for the involvement of different amino groups at different pH values. *Arch. Biochem. Biophys.* **211:**652–661
16. Soleimani, M., Aronson, P.S. 1989. Effects of acetazolamide on Na^+-HCO_3^- cotransport in basolateral membrane vesicles isolated from rabbit renal cortex. *J. Clin. Invest.* **83:**345–351
17. Talor, Z., Richison, G., Arruda, J.A.L. 1985. High-affinity calcium binding sites in luminal and basolateral renal membranes. *Am. J. Physiol.* **248:**F472–F478
18. Wright, E.M., Peerce, B.E. 1984. Identification and conformational changes of the intestinal proline carrier. *J. Biol. Chem.* **259:**14993–14996
19. Wu, J.S.R., Lever, J.E. 1987. Monoclonal antibodies that bind the renal Na^+/Glucose symport system. 2. Stabilization of an active conformation. *Biochemistry* **26:**5790–5796