# The $Na^+/H^+$ Exchanger Cytoplasmic Tail: Structure, Function, and Interactions with Tescalcin†

Xiuju Li,‡ Yongsheng Liu,‡ Cyril M. Kay,§ Werner Müller-Esterl,|| and Larry Fliegel*,‡

*Department of Biochemistry, Faculty of Medicine, CIHR Membrane Protein Research Group, and Protein Engineering Network of Centers of Excellence, University of Alberta, 347 Medical Science Building, Edmonton, Alberta, Canada T6G 2H7, and Institute for Biochemistry II, University Hospital Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany*

*Received November 11, 2002; Revised Manuscript Received May 5, 2003*

ABSTRACT: We characterized the regulatory cytoplasmic tail of the $Na^+/H^+$ exchanger using a histidine-tagged protein containing the C-terminal 182 amino acids (His182). Both tescalcin and calmodulin, two $Na^+/H^+$ exchanger binding proteins, bound to the His182 protein. Cascade blue was used to label the His182 protein. Calcium caused an increase in fluorescence, suggesting exposure of the label on the protein to a more hydrophilic environment. Decreasing external pH caused a transient increase in cascade blue fluorescence, followed by a decrease in fluorescence of the cascade blue labeled $Na^+/H^+$ exchanger C-terminus. Tescalcin caused a decrease in fluorescence by labeled His182 protein, and calcium reversed this effect. Expression of tescalcin in vivo inhibited activity of the $Na^+/H^+$ exchanger when there was an intact C-terminus of the protein. We examined the CD spectra of His182 in the presence and absence of tescalcin. The C-terminal amino acids demonstrated a very small amount of α-helical structure and much more $\beta$-sheet and $\beta$-turn. This was not greatly affected by the presence of tescalcin, but calcium caused an increase in the amount of $\beta$-structure and a decrease in the unstructured proportion of the protein. Sedimentation equilibrium analysis demonstrated that the C-terminal 182 amino acids exist predominantly as a monomer. The results suggest that the C-terminus of the $Na^+/H^+$ exchanger exists primarily as a monomeric protein that binds regulatory tescalcin and can change conformation depending on pH and calcium. Conformation changes in this region of the protein may be responsible for altering the pH sensitivity of the intact $Na^+/H^+$ exchanger.

The regulation of intracellular pH is essential for growth of eukaryotic cells and for a variety of different cellular functions. The $Na^+/H^+$ exchanger is a ubiquitous membrane protein that plays a key role in intracellular pH regulation. Eight isoforms of the $Na^+/H^+$ exchanger exist. The first isoform discovered (NHE1)[1] is universally present in the plasma membrane of mammalian cells and functions to remove one intracellular proton in exchange for an extracellular sodium. The protein is responsible for shifts in intracellular pH that can stimulate changes in the growth of cells. $Na^+/H^+$ exchange is also involved in movement of sodium in response to osmotic shrinkage (*1*). The $Na^+/H^+$ exchanger is significant in several disease states in the myocardium. It is intimately involved in the damage that occurs to the myocardium during ischemia and reperfusion (*2*, *3*), and recent evidence has suggested that the $Na^+/H^+$ exchanger plays an important role in myocardial hypertrophy (*4*, *5*).

The mammalian NHE1 isoform of the $Na^+/H^+$ exchanger consists of an amino-terminal 500 amino acids plus a carboxyl-terminal cytoplasmic domain of about 315 amino acids. The amino-terminal domain contains 12 integral membrane segments plus several intervening loops that may be associated with the lipid bilayer (*6*). The amino-terminal domain is responsible for transport of ions while the C-terminal domain is regulatory. The tail mediates growth factor activation of the membrane domain of the $Na^+/H^+$ exchanger. The $Na^+/H^+$ exchanger is maximally active at low intracellular pH (pH <6.5). Its activity declines as the intracellular pH increases. Hormonal activation of the $Na^+/H^+$ exchanger acts through the tail to shift the pH dependence into a more alkaline range. The cytoplasmic, C-terminal tail of the exchanger has many subdomains that are involved in different regulatory aspects of activity. It contains a site for ATP-dependent regulation of the protein on amino acids 513 and 564 that may function through PIP2 (*7*). Further downstream located between amino acids 567 and 637 is a binding site for an inhibitory protein called calcineurin homologous protein (CHP) (*8*) that is homologous to the B subunit of calcineurin and calmodulin. Another part of the C-terminal region binds to calmodulin and contains both

† Supported by funding from the CIHR to L.F. and by the Protein Engineering Network of Centers of Excellence to C.M.K. L.F. is a Scientist of the Alberta Heritage Foundation for Medical Research.

* To whom correspondence and reprint requests should be addressed. Tel: 780-492-1848. Fax: 780-492-0886. E-mail: lfliegel@ualberta.ca.

‡ Department of Biochemistry, Faculty of Medicine, CIHR Membrane Protein Research Group, University of Alberta.

§ Protein Engineering Network of Centers of Excellence, University of Alberta.

|| Institute for Biochemistry II, University Hospital Frankfurt.

[1] Abbreviations: BCECF-AM, 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM; CB, cascade blue; CHP, calcineurin homologous protein; GST, glutathione *S*-transferase; His182, histidine-tagged C-terminal 182 amino acids of the $Na^+/H^+$ exchanger; NHE1, $Na^+/H^+$ exchanger isoform 1.

10.1021/bi027143d CCC: $25.00 
Published on Web 05/31/2003

high- and low-affinity calmodulin binding sites (amino acids 636−656 and 657−700, respectively). Deletion of the high-affinity binding site yields an "activated" protein (*9*). Recently, a calcium binding protein, tescalcin, has also been shown to bind to the C-terminus of the $Na^+/H^+$ exchanger. Tescalcin has homology with CHP and calcineurin binding protein. Coprecipitation and immunofluorescence experiments demonstrated that tescalcin is associated with the NHE1 isoform of the $Na^+/H^+$ exchanger in vivo; however, the location of where tescalcin binds to the $Na^+/H^+$ exchanger was not elucidated. Tescalcin has tissue-specific expression and was strongly expressed in the myocardium (*10*).

In this study we characterize structural and functional aspects of the cytoplasmic domain of the NHE1 isoform of the $Na^+/H^+$ exchanger. We characterize basic structural aspects of this domain and its interactions with the regulatory protein tescalcin and the resultant physiological effects. Our study is the first examination of the structural and related functional aspects of this domain of the $Na^+/H^+$ exchanger and the first demonstration of a physiological role of the regulatory protein tescalcin.

## MATERIALS AND METHODS

*Materials*. Restriction enzymes, *Escherichia coli* BL21-SI, pDest 14, pDest 17, pDest 40, lipofectamine 2000, and related Gateway cloning items were from Life Technologies, Inc. (Rockville, MD). pGEX-3X, glutathione−Sepharose 4B, and protein A−Sepharose CL-4B were from Pharmacia Biotech AB (Uppsala, Sweden). Glutathione and thrombin were from Sigma (St. Louis, MO). Rabbit anti-His-tag antibody was purchased from BioWorld, Dublin, OH. Conjugated antibodies were from Jackson ImmunoResearch (Mississauga, Ontario, Canada). Ni-NTA−agarose resin was from Qiagen (Valencia, CA). Cascade blue was obtained from Molecular Probes, Inc. The calmodulin-S-tag expression vector was a generous gift of Dr. B. A. Magnuson, University of Maryland, College Park, MD. S-protein coupled to horseradish peroxidase was obtained from Novagen (Madison, WI).

*Expression and Purification of Fusion Proteins*. The carboxyl-terminal 182 amino acids of the human $Na^+/H^+$ exchanger (NHE1) were expressed as fusion protein with a C-terminal hexahistidine tag (His182) using the plasmid pDest 14 and the Gateway cloning system. In this case the *E. coli* BL21-SI strain was induced with 0.3 M NaCl for 3 h. The His182 protein was harvested using standard conditions, and the protein was purified from the supernatant of *E. coli* via Ni-NTA affinity chromatography as described by the manufacturer (Qiagen). Further purification of the His182 protein was achieved by reverse-phase HPLC. Tescalcin (*10*) was produced and purified as an amino-terminal hexa-His-tagged fusion protein using the plasmid pDest 17 and the Gateway cloning system. The cDNA was amplified using PCR and subcloned so as to produce a protein product identical to the sequence of tescalcin described earlier (*10*) with the exception of the addition of the following sequence MSYYHHHHHHLESTSS- on the amino terminus. CHP (calcineurin homologous protein) was obtained from Dr. D. Barber and was used as a template in PCR to produce a histidine-tagged protein (*8*). The entire coding region of CHP was expressed as a fusion protein with an N-terminal hexahistidine tag using the plasmid pDest 17 and the Gateway cloning system. It was purified as described above with Ni-NTA affinity chromatography, and the identity was confirmed by immunoreactivity with anti-His antibody.

*Circular Dichroism (CD)*. All of the CD spectra were collected on a Jasco J-720 spectrophotometer, which was calibrated using a 0.06% solution of ammonium *d*-camphor-10-sulfonate. The temperature in the sample chamber was maintained using a Lauda RM6 low-temperature circulator. Data were collected using the J700 for Windows standard analysis software, version 1.10.00 (copyright 1992−1994 Jasco Corp.), using a Pentium II processor running Windows98 (4.10.222A). Each scan was the average of eight data sets collected every 0.05 nm at 50 nm/min. The scans were baseline corrected, and noise reduction was performed using the Jasco software. The Contin program was then used to perform the structural analysis. All scans were done at 25 °C using a 0.02 cm path length cell. Spectra were analyzed by the Contin program, version 1.0, of Provencher and Glöckner (*11*).

*Sedimentation Equilibrium Measurements*. Sedimentation equilibrium experiments were carried out at 20 °C in a Beckman XL-I analytical ultracentrifuge using absorbance optics following the procedures described by Laue and Stafford (*12*). Aliquots (110 $\mu$L) of the sample solution were loaded into six-sector CFE sample cells, allowing three concentrations of the sample to be run simultaneously. Runs were performed at a minimum of two different speeds, and each speed was maintained until there was no significant difference in $r^2/2$ versus absorbance at 280 nm. Scans were taken 2 h apart to ensure that equilibrium was achieved.

The sedimentation equilibrium data were evaluated using the NONLIN program, which incorporates a nonlinear least-squares curve-fitting algorithm described by Johnson et al. (*13*). This program allows the analysis of both single and multiple data files. Data can be fit to either a single ideal species model or models containing up to four associating species, depending on which parameters are permitted to vary during the fitting routine.

The protein's partial specific volume and the solvent density were estimated using the SEDNTERP program, which incorporates calculations detailed by Laue et al. (*14*).

If the sample data fit a multiple species model, the raw $K_a$ in absorbance must be converted to a molar $K_a$ using the equation (*15*):

$$K_{conc} = K_{abs}\frac{(\epsilon l)^{n-1}}{n}$$

where $K_{conc}$ is the association constant in molar concentration terms depending on the stoichiometry, $K_{abs}$ is the absorbance association constant from the fit, $\epsilon$ is the molar extinction coefficient, $l$ is the path length of the centerpiece in centimeters, and $n$ is the stoichiometry of the larger association species.

*Fluorescence Labeling*. Proteins were labeled with cascade blue (CB) acetyl azide by use of a FluroTag FITC conjugation kit as recommended by the manufacturer. Cascade blue (1.1 mg/mL) was dissolved in 100 mM carbonate/bicarbonate buffer (pH 9.0). Six hundred micrograms of purified His182 protein in a 100 mM sodium carbonate/bicarbonate buffer

(pH 9.0) was used directly for labeling. The dye was added dropwise to the protein mixture with constant stirring. The reaction was incubated in the dark for 2 h at room temperature with constant stirring. Labeled proteins were separated from the free dye on a Sephadex G-25 column (8 mL, bed height 5 cm) previously equilibrated with PBS. The reaction mixture was applied, and fractions were eluted with PBS. The fluorescence of each fraction was determined with an excitation wavelength of 385 nm and an emission wavelength of 430 nm with a Shimadzu RF5000 spectrophotometer. Fractions containing labeled protein were combined and used for protein−protein interaction studies and examination of conformational changes of the carboxyl terminus of the $Na^+/H^+$ exchanger.

*Fluorescence Measurements*. Fluorescence measurements were performed at an excitation wavelength of 385 nm (slit width 10 nm) and an emission wavelength of 430 nm (slit width 10 nm) at room temperature using a Shimadzu RF5000 spectrophotometer. Fluorescence intensity was measured with constant stirring in 2.5 mL of buffer containing 10 mM MOPS, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, and 0.5 mM EGTA. Appropriate protein or ions were added to the reaction mixture, and the changes in fluorescence were monitored. Quantum yields of CB-labeled proteins or of CB were calculated from the emission spectrum (400−480 nm) obtained at an excitation wavelength of 385 nm.

*Affinity Blotting of the $Na^+/H^+$ Exchanger and Tescalcin*. To examine tescalcin binding to the $Na^+/H^+$ exchanger immobilized on nitrocellulose, 20 $\mu$g of ovalbumin or GST and the His182 proteins were separated on 12% SDS−PAGE and then transferred to nitrocellulose membranes (*16*). Nitrocellulose membranes were blocked with 10% (w/v) skim milk powder in TBS (20 mM Tris, pH 7.4, 137 mM NaCl) for 5 h at 4 °C. They were then incubated with 20 $\mu$g/mL tescalcin with 1% (w/v) skim milk powder in TBS and rocked gently overnight at 4 °C in the absence of externally added $Ca^{2+}$. Membranes were washed with TBS for 4 × 15 min at room temperature. The nitrocellulose was then incubated with rabbit anti-tescalcin antibody (1:2000) (*10*) in TBS with 1% skim milk powder for 1 h at room temperature, followed by washing for another hour with TBS. Further amplification was achieved by a subsequent incubation with goat anti-rabbit HRP antibodies. Reactive bands were visualized by the Amersham enhanced chemiluminescence system. In some cases affinity blotting was done in the presence of 0.1 mM $CaCl_2$ or in the presence of 100 $\mu$g of calmodulin, as indicated in the figure legends.

*Measurement of Intracellular pH*. NHE activity was measured fluorometrically using 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF-AM) essentially as described previously (*17*, *18*). pH regulation by the $Na^+/H^+$ exchanger was examined in (untransfected or mock transfected) CHO cells and CHO cells stably transfected with the tescalcin protein maintained as described earlier. Tescalcin was expressed using the plasmid pcDNA3.1, and transfections and selection were as described earlier (*19*). Cells were grown on glass coverslips, and the acetoxy methyl ester of 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein(BCECF-AM) was used to measure $pH_i$. Cells on the coverslips were incubated with BCECF-AM for 2−4 min, placed into a holder device, and inserted into a fluorescence cuvette at 37 °C. Ammonium chloride prepulse was used to induce transient intracellular acidification as described earlier (*19*), and the rate of recovery from an acid load was measured. The initial pH after ammonium chloride induced acidification was 6.10 ± 0.03 and did not vary significantly between controls and experimentals. Cells were grown in either serum-containing (10%) or serum-free (0.25%) medium for 20 h; then cells were treated with fresh 10% serum or in fresh medium without serum for 15 min at 37 °C prior to measurement of the rate of recovery from an acid load. For some experiments cells were grown in serum-free medium overnight and then treated with 1 unit/mL thrombin to stimulate $Na^+/H^+$ exchanger activity prior to recovery from an acute acid load.

For some experiments we examined intracellular pH regulation in AP-1 cells that were transiently transfected with a shortened $Na^+/H^+$ exchanger or a shortened $Na^+/H^+$ exchanger plus tescalcin. We have previously used AP-1 cells that are a CHO-derived cell line that are devoid of their own $Na^+/H^+$ exchanger protein (*19*). Transfection of AP-1 cells was with lipofectamine 2000 as described by the manufacturer (Life Technologies, Inc., Rockville, MD). Proton transport measurement was approximately 36 h after transfection. To make a shortened NHE1 protein, we used the Gateway cloning system. The coding region of the $Na^+/H^+$ exchanger was amplified from the plasmid pYN4+ (*19*). The C-terminal primer was designed such that amino acid 635 was the last of the $Na^+/H^+$ exchanger, leaving the protein 180 amino acids shorter than the full-length protein. The product was cloned into the pDest 40 expression vector after passage through pDonr 201.

*Production, Purification, and Affinity Blotting of Calmodulin*. Calmodulin was produced with an S-tag as described earlier (*20*). The fusion protein was produced with the S-tag and a His tag and purified using an immobilized metal affinity chromatography (nickel column) as described earlier (*20*). To examine calmodulin binding to the $Na^+/H^+$ exchanger immobilized on nitrocellulose, 20 $\mu$g of the His182 protein and purified GST were separated on 12% SDS−PAGE and then transferred to nitrocellulose membranes, and affinity blotting was essentially as described for tescalcin. The blot was incubated with an S-protein−horseradish peroxidase conjugate to visualize calmodulin binding. The reactive bands were visualized by the Amersham enhanced chemiluminescence system.

## RESULTS

*Affinity Blotting of $Na^+/H^+$ Exchanger Binding Proteins.* In initial experiments we wanted to confirm that the C-terminal 182 amino acids of the $Na^+/H^+$ exchanger were able to bind calmodulin and tescalcin, two proteins known to bind to this region of the protein. In both cases we used an affinity blotting technique (Figure 1). In the case of tescalcin, equal amounts (20 $\mu$g) of purified His182 protein or ovalbumin were run on SDS−PAGE, transferred to nitrocellulose membranes, and probed with tescalcin as described in the Materials and Methods. The expressed $Na^+/H^+$ exchanger fusion protein bound tescalcin (lane 1) while the control protein (lane 2) did not.

It was previously reported that calmodulin can bind to and regulate the activity of the NHE1 isoform of the $Na^+/H^+$ exchanger (*21*, *22*). To confirm that the His182 protein

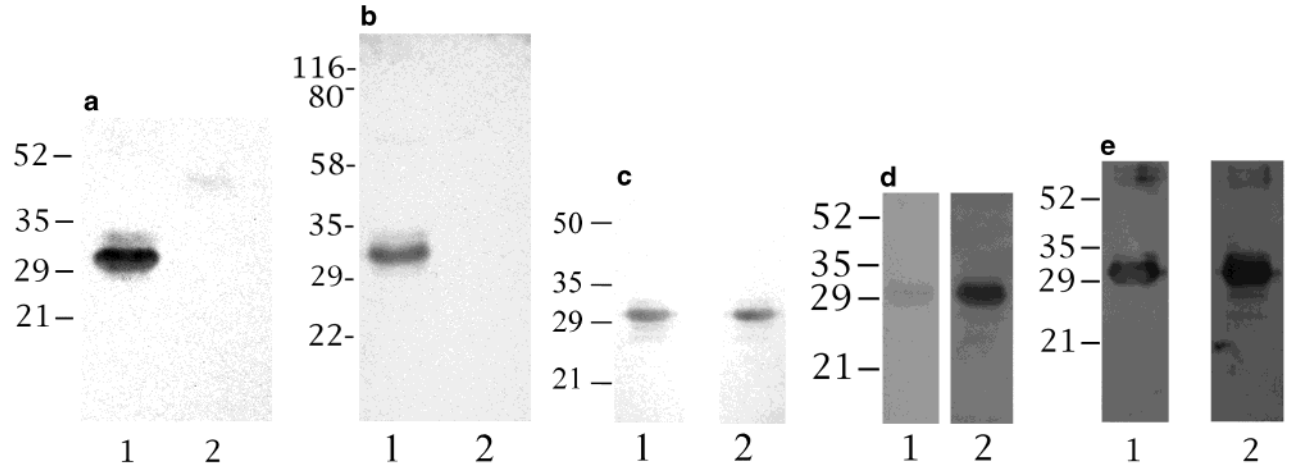


FIGURE 1: Affinity blotting assays of $Na^+/H^+$ exchanger carboxyl-terminal protein with tescalcin. Proteins were separated with SDS−PAGE and transferred to nitrocellulose. (A) Lanes 1 and 2 are 20 $\mu$g of the His182 protein and ovalbumin probed with 20 $\mu$g of tescalcin. (B) Affinity blotting assay of His182 fusion protein with calmodulin. Fusion proteins were separated with SDS−PAGE and transferred to nitrocellulose. Lanes 1 and 2 are 20 $\mu$g of the proteins His182 protein and GST, respectively. (C) Affinity blotting assay of $Na^+/H^+$ exchanger fusion protein with tescalcin in the presence or absence of calcineurin homologous protein. Lane 1 is affinity blotting with 20 $\mu$g of tescalcin. Lane 2 is affinity blotting with 20 $\mu$g of tescalcin and a 5-fold excess of calcineurin homologous protein. Results are typical of three experiments. (D) Affinity blotting of His182 protein in the presence or absence of 0.1 mM $CaCl_2$. Equal amounts of His182 protein (20 $\mu$g) were probed with tescalcin as described for panel A in the absence (lane 1) or presence (lane 2) of 0.1 mM $CaCl_2$. (Exposure times are reduced relative to panel A.) (E) Affinity blotting of the His182 protein in the presence or absence of calmodulin (100 $\mu$g). Equal amounts of simultaneously run His182 (20 $\mu$g) were probed with tescalcin as described for panel D in the presence of 0.1 mM $CaCl_2$. Lane 1 was probed in the absence of calmodulin while lane 2 was in its presence.

contained a functional calmodulin binding domain, we affinity blotted with the S-tagged calmodulin binding protein. Earlier studies have shown that a tag on this protein does not interfere with its properties and is less intrusive to protein function than labeling the protein, such as with biotinylation (*20*). The results are shown in Figure 1B. Calmodulin bound to the His182 protein but did not bind to a control protein (GST), confirming that the His182 had a functional calmodulin binding domain.

It has previously been reported that a calcineurin B homologous protein (CHP) binds to the C-terminus of the $Na^+/H^+$ exchanger and regulates the activity of the protein and the GTPase activation of the exchanger (*8*). Tescalcin is homologous to CHP (*10*), and it was of interest to investigate whether the binding site of tescalcin overlaps with that of CHP. To examine this, we tested if CHP could eliminate or reduce the binding of tescalcin to the carboxyl terminus of the $Na^+/H^+$ exchanger. The results (Figure 1C) demonstrated that the presence of CHP did not reduce the binding of tescalcin to the His182 protein. Since tescalcin is a calcium binding protein, we examined if the presence of calcium affected its binding to the $Na^+/H^+$ exchanger. The results (Figure 1D) demonstrated that calcium enhanced the binding of tescalcin to the $Na^+/H^+$ exchanger. We also examined if calmodulin affects the binding of tescalcin to the His182 protein. Since calcium enhances the binding of calmodulin to the $Na^+/H^+$ exchanger (*21*), this experiment was done in the presence of added calcium. Calmodulin did not reduce the binding of tescalcin to the $Na^+/H^+$ exchanger (Figure 1E), suggesting that the two proteins bind at different sites.

*Conformational Changes in the $Na^+/H^+$ Exchanger Carboxyl Terminus*. Cascade blue has been used earlier to characterize changes in the conformation of proteins (*23*). CB reacts with aliphatic amines of proteins and is highly fluorescent and resistant to quenching upon conjugation (*24*). The fluorescence intensity of CB changes, depending on the polarity of the solvent used. To confirm this property of CB in our system, we examined the fluorescence intensity of CB in a variety of solvents (Figure 2, Table 1). CB had a relatively high fluorescence in PBS (pH 7.4) and 10 mM MOPS (pH 7.0) but a much lower fluorescence in nonpolar solvents such as 1-butanol. These observations confirmed that CB is sensitive to the external environment and could be used to detect changes in conformation of the $Na^+/H^+$ exchanger carboxyl terminus.

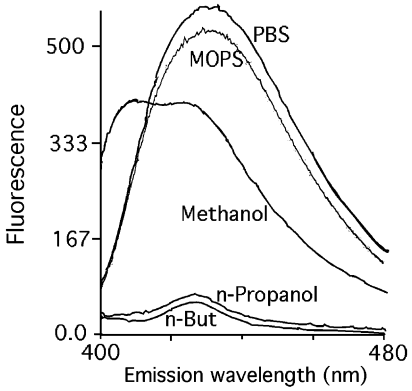


FIGURE 2: Emission spectra of cascade blue in phosphate-buffered saline (PBS), pH 7.4, 10 mM MOPS (pH 7.0), methanol, 1-propanol, and 1-butanol (n-but). The emission spectra of CB was measured in various solvents after excitation at 380 nm.

Table 1: Relative Quantum Yield of Cascade Blue (CB) and CB Coupled to His182 in Solvents of Various Polarity

| | CB | | CB−His182 | |
|---|---|---|---|---|
| solvent | $Q$ | $\lambda_{max}$ | $Q$ | $\lambda_{max}$ |
| MOPS (pH 7.0) | 1 | 430 | 1 | 430 |
| PBS (pH 7.4) | 1.05 | 432 | 0.99 | 430 |
| methanol | 0.75 | 408 | 1.03 | 408 |
| 1-propanol | 0.13 | 427 | 0.46 | 431 |
| 1-butanol | 0.10 | 428 | 0.12 | 428 |

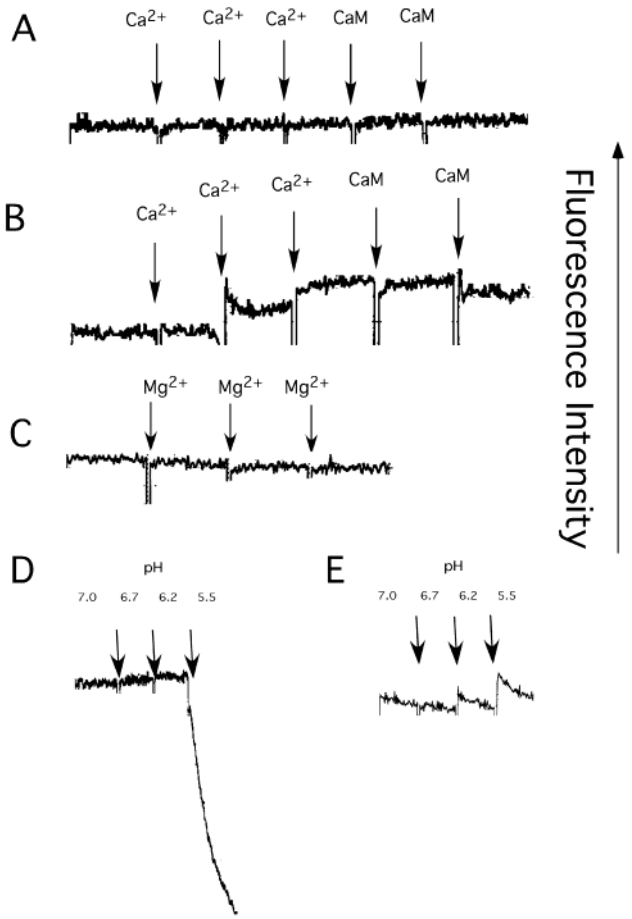


FIGURE 3: Effect of $Ca^{2+}$, $Mg^{2+}$, calmodulin (CaM), and pH on the conformation of the $Na^+/H^+$ exchanger C-terminus. The His182 protein was labeled with CB, and changes in the fluorescence intensity were measured with excitation as described in the Materials and Methods. (A and B) Increasing concentrations of $Ca^{2+}$ (1, 5, 10 mM) were added to either CB alone (A) or CB-labeled His182 protein (B). Calmodulin was added after CB addition to a final concentration of 10 $\mu$M followed by 30 $\mu$M. (C) Effect of increasing concentrations of $Mg^{2+}$ (1, 5, 10 mM) on CB-labeled His182 protein. (D and E) The effect of decreasing pH was measured on CB (C) and CB-labeled His182 protein (D). The pH was adjusted with HCl. The initial pH was 7.0 in a buffer consisting of 10 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, and 0.5 mM EGTA. This was adjusted to final pHs of 6.7, 6.2, and 5.5 as indicated by addition of HCl.

Figure 3 illustrates the effect of calcium, magnesium, and calmodulin on the conformation of the His182 protein. Addition of up to 10 mM calcium had no effect on the fluorescence intensity of CB alone (Figure 3A). In addition, addition of up to 30 $\mu$M calmodulin also did not affect the fluorescence intensity of CB. In contrast, the fluorescence intensity of the $Na^+/H^+$ exchanger C-terminus labeled with CB was sensitive to addition of calcium (Figure 3B). Calcium caused an increase in the fluorescence of the protein, indicating a possible shift of some regions to a more hydrophilic environment. Addition of the same concentrations of magnesium did not have the same effect on fluorescence, showing only a very slight decline in fluorescence of the CB-labeled $Na^+/H^+$ exchanger C-terminus (Figure 3C). Addition of calmodulin after calcium had a minor effect on fluorescence, decreasing fluorescence slightly. Because the $Na^+/H^+$ exchanger is a pH-sensitive protein and the tail could be involved in pH sensing, we also tested the effect of pH on the conformation of the His182 protein. Cascade blue alone (Figure 3D) was not affected by changes in external pH within the physiological range of 7.0−6.2. At pH 5.5 fluorescence declined dramatically, though this may have been due to precipitation of the compound. The effect of changes in external pH on CB-labeled His182 protein is shown in Figure 3E. With successive additions of lower pH buffers there was a transient increase in the fluorescence of CB-labeled His182 protein, followed by a decrease in fluorescence. This effect was reproducible and was not seen with the addition of the proteins tescalcin or calmodulin. The more acidic changes in pH caused a larger transient increase in fluorescence, followed by decreases in fluorescence.

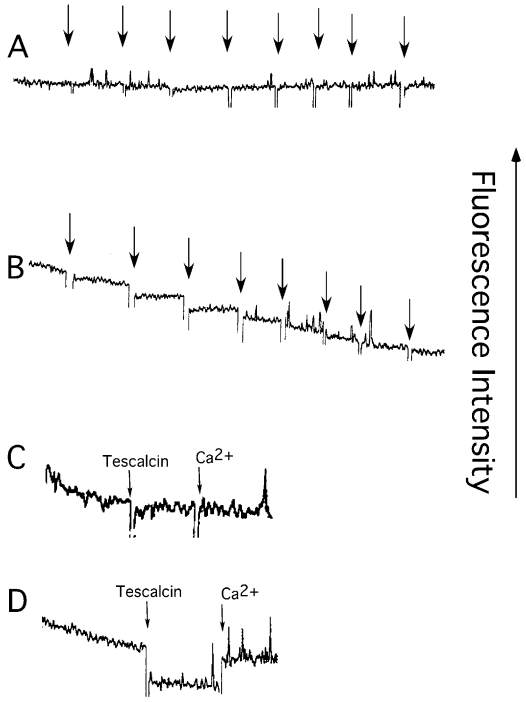


FIGURE 4: Effect of $Ca^{2+}$ and tescalcin on the conformation of the $Na^+/H^+$ exchanger C-terminus. The His182 protein was labeled with CB, and changes in the fluorescence intensity were measured with excitation as described in the Materials and Methods. (A and B) Tescalcin was added in increasing concentrations (6, 36, 96, 108, 132, 180, 270, 360 $\mu$M) to CB (A) or CB-labeled His182 protein (B). The final molar ratio of tescalcin to His182 was 2.5:1. (C and D) Effect of addition of tescalcin (15 $\mu$M) followed by $Ca^{2+}$ (5 mM) on CB alone or CB-labeled His182 protein.

We then examined the effect of tescalcin on the conformation of the $Na^+/H^+$ exchanger C-terminus. Figure 4A shows that increasing concentration of tescalcin had no effect on the fluorescence of CB that was not conjugated to protein. In contrast, tescalcin, in a concentration of 6−360 $\mu$M, caused a dose-dependent decrease in the fluorescence of the His182 protein labeled with CB (Figure 4B). The addition of calcium was able to reverse this effect (Figure 4D).

We tested to see if tescalcin had an effect on the activity of the $Na^+/H^+$ exchanger in vivo. Cells stably transfected with tescalcin were compared for their rate of recovery from an acute acid load induced by ammonium chloride. Cells were maintained in either serum-free or serum-containing media. The results are shown in Figure 5A. Expression of tescalcin caused nearly a 50% decrease in activity of the $Na^+/H^+$ exchanger in cells maintained in 10% serum. A

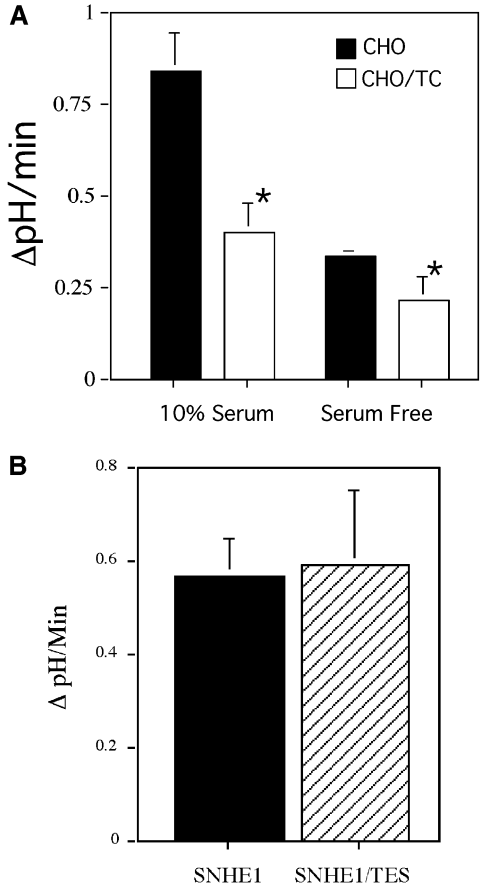


FIGURE 5: Effects of tescalcin expression on the $Na^+/H^+$ exchanger transport rate of CHO cells. (A) $Na^+/H^+$ exchanger activity was measured in mock-transfected CHO cells (CHO), or CHO cells were stably transfected with tescalcin (CHO/TC). Cells were maintained in either serum-containing (10% serum) or serum-free medium, and $Na^+/H^+$ exchanger activity was measured as described in the Materials and Methods. An asterisk indicates significantly different from the value obtained from cells not expressing tescalcin. (B) $Na^+/H^+$ exchanger activity was determined in AP-1 cells that were transiently transfected with either shortened $Na^+/H^+$ exchanger (SNHE1) or shortened $Na^+/H^+$ exchanger plus tescalcin (SNHE1/TES).

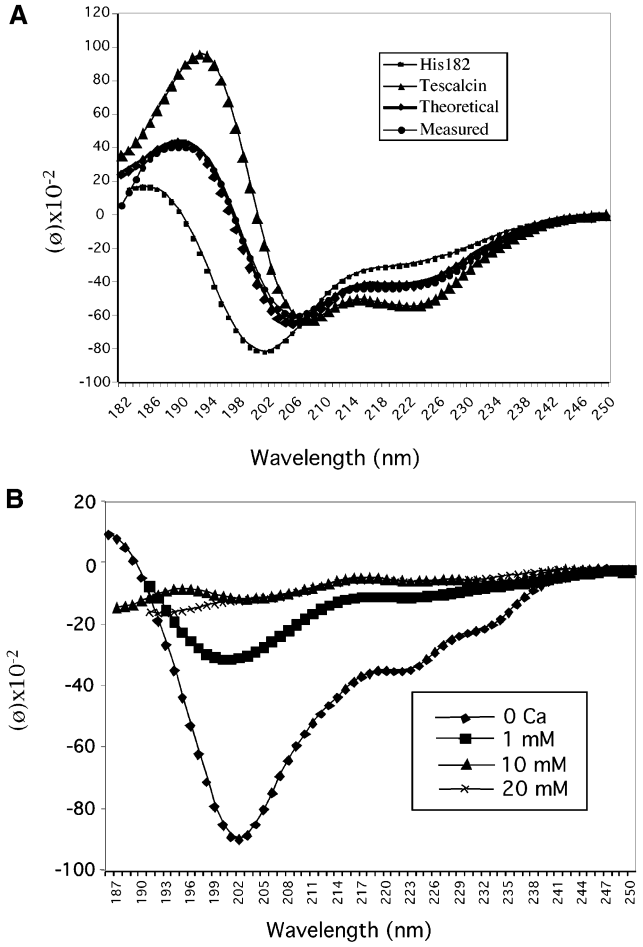


FIGURE 6: CD analysis of the $Na^+/H^+$ exchanger C-terminus and tescalcin. (A) CD spectra of purified His182 and His−tescalcin were carried out as described in the Materials and Methods. The data are plotted as molar elipticity versus wavelength for the proteins. The spectra obtained in the presence of the two proteins is illustrated as is the theoretical calculated spectra of His182 and tescalcin combined. The scan concentrations were His182, 19.2 $\mu$M, and His−tescalcin, 15.2 $\mu$M. They were mixed to final concentrations of 8.85 and 8.92 $\mu$M, respectively, for the combined scale. The theoretical spectrum is the numerical average of the His182 and His−tescalcin scans. (B) Effect of calcium on the CD spectra of the His182 protein. The CD spectra of the His182 protein were analyzed and plotted as described in (A) in either the absence or presence of 1, 10, or 20 mM $CaCl_2$.

much smaller, but significant, decrease in activity was also seen in cells treated with serum-free medium. Addition of the $Na^+/H^+$ exchanger hexamethylamiloride (3 or 10 $\mu$M) eliminated greater than 95% of the recovery from an acid load (not shown). In one set of experiments we examined if thrombin stimulation could alleviate tescalcin inhibition of $Na^+/H^+$ exchanger activity. Serum-deprived cells were treated with 1 unit/mL thrombin, and this resulted in a 42% increase in their rate of recovery from ammonium chloride induced acidosis. In cells transfected with tescalcin, thrombin stimulation increased $Na^+/H^+$ exchanger activity a similar amount (35%) despite tescalcin's inhibition of the recovery from an acid load. Thrombin did not alleviate tescalcin's inhibitory effects and did not return $Na^+/H^+$ exchanger activity to levels equivalent to non-tescalcin-treated cells (not shown).

To examine if the C-terminal 180 amino acids were critical for the regulation of NHE1 by tescalcin, we constructed a shortened $Na^+/H^+$ exchanger. We used it to examine pH regulation in transiently transfected AP-1 cells that are devoid of their own $Na^+/H^+$ exchanger (Figure 5B). There was no change in the rate of recovery from an acid load when cells transfected with $Na^+/H^+$ exchanger were compared to cells transfected with $Na^+/H^+$ exchanger plus tescalcin. These results suggested that the C-terminal 180 amino acids are necessary for inhibition of the $Na^+/H^+$ exchanger by tescalcin.

*Circular Dichroism (CD).* The CD spectra for His182, His−tescalcin, and a 1:1 molar ratio of the two proteins are portrayed in Figure 6A. Also included is a theoretical spectrum generated as the numerical average of the His182 and His−tescalcin scans. It is noted that the latter spectrum essentially superimposes the experimental spectrum, suggesting that there is no detectable conformational change, as measured by CD, associated with interaction of the two proteins. Provencher−Glöckner analysis of the far-UV CD spectra of the four scans in Figure 6 revealed the structural elements tabulated in Table 2. His182 has a preponderance of $\beta$-structure with essentially no $\alpha$-helix while tescalcin has a corresponding amount of $\beta$-structure with some 20%

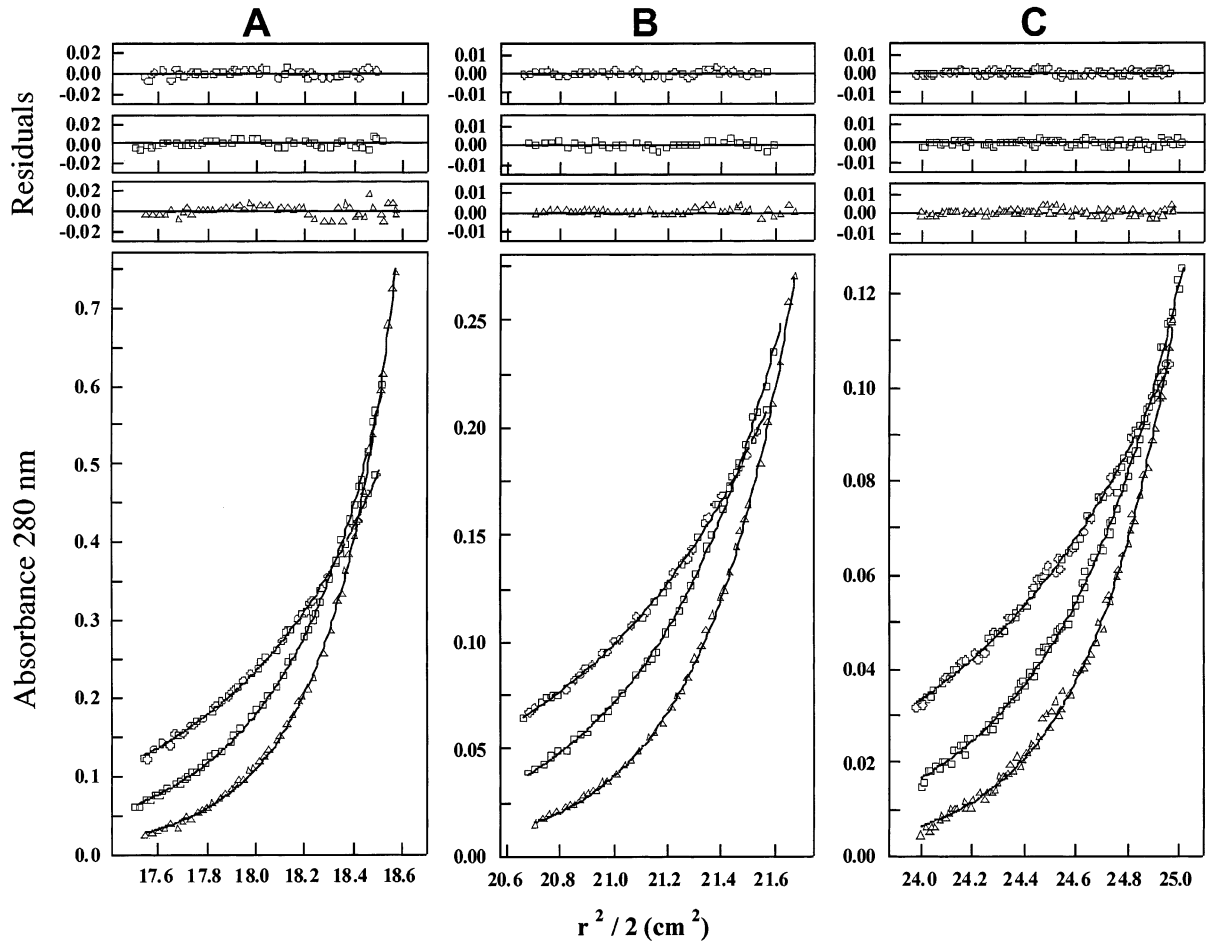


FIGURE 7: Sedimentation equilibrium of the His182 protein. Lower graphs: Radial distance versus concentration plots of His182 sedimentation equilibrium data collected from three loading concentrations (panel A, 0.67 mg/mL; panel B, 0.34 mg/mL; panel C, 0.20 mg/mL) and three rotor speeds (○, 20000 rpm; □, 26000 rpm; △, 32000 rpm) and fit globally to a monomer−dimer association model. The symbols represent the measured data points, and the solid lines represent the theoretical fit lines to the model. Upper graphs: Residual plots from the fitting of the measured data points to the model. The goodness of the fit is reflected in the random distribution of the residuals.

Table 2: Contin Analysis of the Secondary Structural Elements in His182, Tescalcin, and a 1:1 Molar Ratio of the Two Proteins[a]

| sample | α-helix | β-sheet | β-turn | remainder | scale |
|---|---|---|---|---|---|
| His182 (0.41) | 0.08 ± 0.006 | 0.44 ± 0.007 | 0.30 ± 0.007 | 0.18 ± 0.010 | 0.998 |
| $His_6$−tescalcin | 0.20 ± 0.010 | 0.47 ± 0.010 | 0.32 ± 0.012 | 0.02 ± 0.016 | 0.998 |
| combined | 0.12 ± 0.008 | 0.44 ± 0.008 | 0.28 ± 0.009 | 0.16 ± 0.013 | 0.999 |
| calculated | 0.14 ± 0.008 | 0.45 ± 0.008 | 0.31 ± 0.009 | 0.10 ± 0.012 | 0.998 |
| His182 (0.82) | 0.09 ± 0.005 | 0.38 ± 0.005 | 0.27 ± 0.006 | 0.26 ± 0.007 | 0.998 |
| 1 mM $Ca^{2+}$ | 0.01 ± 0.011 | 0.55 ± 0.012 | 0.37 ± 0.013 | 0.07 ± 0.018 | 0.999 |
| 10 mM $Ca^{2+}$ | 0.00 ± 0.000 | 0.61 ± 0.009 | 0.35 ± 0.010 | 0.04 ± 0.013 | 0.998 |
| His182 (50 mM sodium phosphate, pH 7.0) | 0.10 ± 0.008 | 0.40 ± 0.008 | 0.27 ± 0.010 | 0.23 ± 0.012 | 0.999 |
| His182 (50 mM sodium phosphate, pH 6.2) | 0.09 ± 0.006 | 0.49 ± 0.007 | 0.27 ± 0.007 | 0.16 ± 0.010 | 0.998 |

[a] Spectra were determined at pH 7.2 in 0.2 mM MOPS except where indicated.

α-helix, as reflected by a CD spectrum which exhibits negative peaks at 222 and 209 nm and a positive peak at 195 nm. The combined and calculated spectra reveal comparable amounts of α-helix (∼12%) and β-structure (∼72%). Table 2 summarizes the secondary structural analysis of His182 and tescalcin.

Figure 6B examines the effect of calcium on the conformation of the His182 protein. The addition of increasing amounts of calcium (1−20 mM) resulted in an increasing amount of β-structure and reduced the unstructured part of the protein and any residual α-helical structure, as summarized in Table 2. Intermediate concentrations of calcium (100−250 μM) had intermediate effects on the CD structure that seemed to vary with the preparation of protein used (not shown). A minor difference (that was not significant) was noted between the His182 protein at two protein concentrations of 0.41 and 0.82 mg/mL. We also examined the effect of temperature and pH on the CD spectra of His182. There was little change in the spectra (from 200 to 250 nM) when the temperature was varied between 20 and 80 °C (not shown). The effect of changing the pH on the CD spectra is summarized in Table 2. When the pH was reduced to 6.2,

there was only a slight increase in the amount of $\beta$-sheet and a corresponding decrease in unstructured protein; however, the difference was within the error of measurement.

*His182 Sedimentation Equilibrium Results.* Two dilutions of the His182 stock sample were made, and these three loading concentrations (0.67, 0.34, and 0.20 mg/mL) were run at 20000, 26000, and 32000 rpm at 4 °C. The nine data sets collected during the sedimentation equilibrium run were initially fit globally to a single species model, resulting in a calculated apparent average molecular weight of 23131, approximately 8% higher than the sequence molecular weight of 21325. The best global fit of all data sets was obtained by fitting to a monomer−dimer self-association model, so in the concentration range employed in these experiments the His182 appears to consist mainly of monomer, with a small amount of association to dimer.

## DISCUSSION

The cytoplasmic tail of the $Na^+/H^+$ exchanger affects the function of the membrane domain (*25*, *26*). Recently, tescalcin, a calcium binding protein, has been shown to bind to the cytoplasmic domain of the $Na^+/H^+$ exchanger (*26*). However, little is known about the mechanisms by which this and other proteins regulate the $Na^+/H^+$ exchanger. To elucidate how the cytoplasmic domain functions, we studied details of its structure and its conformational changes in response to environmental stimuli. We produced the C-terminal 182 amino acids with a small histidine tag. The protein retained its ability to bind to two proteins, tescalcin and calmodulin (Figure 1), suggesting that it retained much of its normal properties. Our results with the His182 protein (Figure 1) showed that tescalcin binds within these C-terminal 182 amino acids. The exact binding site of tescalcin is not known at this time. Competition experiments suggested that the binding site of CHP and tescalcin, and tescalcin and calmodulin, does not overlap despite some homology between the proteins. As CHP has been suggested to bind between amino acids 567 and 637 of NHE1 and since tescalcin bound His182 protein (amino acids 633−815), this result is not surprising. Calmodulin binds between amino acids 637 and 656 (*9*). However, there was no competition for binding between the two proteins, suggesting that they also bind at different sites.

The intact $Na^+/H^+$ exchanger forms dimers in cells (*27*, *28*). To examine if this is a function of the membrane domain or the cytosolic domain, we further characterized the cytosolic domain. Sedimentation equilibrium experiments showed that the cytoplasmic domain is a monomer, with a small tendency to form a dimer. These results suggest that the tendency to form dimers resides in the membrane domain or a more proximal C-terminal region.

Cascade blue was used in experiments to examine conformational changes in the C-terminal domain. $Ca^{2+}$ increased the fluorescence of the labeled $Na^+/H^+$ exchanger, indicative of conformational changes that cause exposure to a more hydrophilic environment. Calmodulin addition had a minor effect, reducing the effect of $Ca^{2+}$ (Figure 3). This effect of $Ca^{2+}$ was specific since $Mg^{2+}$ did not have the same effect. CD spectra of the protein showed that it had little $\alpha$-helical structure with a preponderance of $\beta$-structure. Addition of calcium confirmed a conformational change, causing an increase in the $\beta$-structure and a decrease in unstructured protein and residual $\alpha$-helix (Figure 6B, Table 2). We were unable to demonstrate any significant high-capacity binding of calcium to this region of the protein using $^{45}Ca^{2+}$ blotting techniques (unpublished observation) (*29*), suggesting that only a small amount of calcium is bound. Stimulation of cells with $Ca^{2+}$ ionophores has been shown to activate the $Na^+/H^+$ exchanger; however, the response is dependent on the presence of the calmodulin binding domain (*30*). Therefore, it is not clear at the present time what the direct physiological role of $Ca^{2+}$ is in vivo. It may have more of a structural role stabilizing the protein conformation. Amino acids 753−759 of the tail consist of acidic residues that could be the binding site for positively charged $Ca^{2+}$.

The concentrations of $Ca^{2+}$ used to obtain changes in structure in the $Na^+/H^+$ exchanger C-terminal domain are much higher than intracellular free calcium. Smaller amounts of calcium (i.e., 200 $\mu$M) gave smaller and much more variable effects (not shown). At this point it is unclear whether changes in intracellular free calcium play a significant role in modulation of the structure of the cytoplasmic domain in vivo. Thrombin elevates intracellular calcium and stimulates the $Na^+/H^+$ exchanger (*31*). Though thrombin did stimulate activity of tescalcin-treated cells, it did not alleviate the inhibition by tescalcin. It may be that physiological intracellular calcium has no regulatory role or may require the presence of other physiological stimulations or conditions not examined in this study, such as modification by phosphorylation. There have been reports that the C-termini of both the NHE1 and NHE3 isoforms of the $Na^+/H^+$ exchanger are extracellular in some cell types (*32*, *33*). Extracellular $Ca^{2+}$ is within the ranges of $Ca^{2+}$ used in this study.

Overall, we found that the carboxyl-terminal region had a preponderance of $\beta$-structure with little or no $\alpha$-helix. The large amount of $\beta$-structure and $\beta$-turns is suggestive of a compact configuration characterized by antiparallel $\beta$-sheets joined by $\beta$-turns. A compact structure would be in keeping with a physiological role whereby the tail modulates the membrane domain. For the *E. coli* isoform of the $Na^+/H^+$ exchanger, the membrane domain consists of two bundles of titled helices, one of which is quite compact (*34*). This arrangement is typical of membrane proteins and suggests that a regulatory cytoplasmic domain with many interacting components would need a relatively compact structure to interact with a compact bundle of helices.

Reduction of pH caused a transient increase in fluorescence of the labeled His182 protein, followed by a slow decrease to the initial level of fluorescence (Figure 3). The responsiveness of this domain to changes in pH is suggestive of a possible regulatory role in activity of the $Na^+/H^+$ exchanger. Removal of the cytoplasmic domain from the membrane domain of the protein results in a $Na^+/H^+$ exchanger that is not as responsive to changes in internal pH (*35*). The regulatory cytosolic domain affects an $H^+$ modifier site present on the membrane domain (*36*). A pH-responsive cytosolic domain might be a prerequisite for this to occur. Our study demonstrates that the cytosolic domain can alter its conformation in response to changes in pH.

In our study we demonstrate that tescalcin binds to the cytoplasmic 182 amino acids of the $Na^+/H^+$ exchanger. Addition of tescalcin to the fluroescently labeled $Na^+/H^+$ exchanger C-terminus resulted in decreased fluorescence of

the protein (Figure 4). Calcium was able to reverse the effect. Tescalcin could change the conformation of the $Na^+/H^+$ exchanger, or its binding could affect fluorescence by quenching. Theoretically, $Ca^{2+}$ could reverse the decrease in fluorescence caused by tescalcin by a direct effect on the $Na^+/H^+$ exchanger protein or indirectly through tescalcin. However, $Ca^{2+}$ caused an enhancement of tescalcin binding to the $Na^+/H^+$ exchanger in our overlay experiments (Figure 1D), suggesting that $Ca^{2+}$-induced changes in conformation were not due to dissociation of tescalcin. The $Ca^{2+}$-induced enhancement of tescalcin binding could be an important mechanism of regulation of the $Na^+/H^+$ exchanger.

To examine the effect of tescalcin on the conformation of the cytoplasmic domain in more detail, we examined CD spectra of the two proteins. The combined CD spectra of tescalcin and His182 were not different from that of the theoretical spectra of the two proteins combined. This suggests that either tescalcin has a small subtle effect on the conformation of the $Na^+/H^+$ exchanger C-terminus or that the effects that occurred in cascade blue labeled protein were masked by changes in each protein and were not detectable in the combined CD spectra.

We demonstrated that tescalcin has an inhibitory effect on $Na^+/H^+$ exchanger activity in intact cells (Figure 5). The effect was much more pronounced in the presence of serum, and there was a requirement for the presence of the C-terminal 180 amino acids of the $Na^+/H^+$ exchanger (Figure 5B). This is consistent with our in vitro experiments that demonstrated binding to this region of the protein. Our results are the first demonstration of a direct physiological effect of tescalcin on the $Na^+/H^+$ exchanger.

In summary, we have demonstrated that the C-terminal domain of the $Na^+/H^+$ exchanger interacts with other proteins and displays changes in conformation dependent on both pH and cations and possibly protein−protein interactions. pH- and cation-dependent changes in conformation may be a mechanism by which the tail regulates the membrane domain. Future studies will examine the site of the C-terminal interaction with the $Na^+/H^+$ exchanger and the mechanism by which it mediates regulation of activity.

## ACKNOWLEDGMENT

We are grateful to Dr. B. A. Magnuson for a gift of pET-32Ek/LIC containing S-tagged calmodulin, Les Hicks for the centrifuge work performed, Bob Luty for the CD work performed, and to Dr. J. Mailander and Dr. J. Dedio for providing the molecular probes for human tescalcin.

## REFERENCES

1. Shrode, L., Cabado, A., Goss, G., and Grinstein, S. (1996) in *The $Na^+/H^+$ Exchanger* (Fliegel, L., Ed.) pp 101−122, R. G. Landes Co., Georgetown, TX.
2. Karmazyn, M. (2001) *Expert Opin. Invest. Drugs 10*, 835−843.
3. Karmazyn, M., Sostaric, J. V., and Gan, X. T. (2001) *Drugs 61*, 375−389.
4. Kusumoto, K., Haist, J. V., and Karmazyn, M. (2001) *Am. J. Physiol. 280*, H738−H745.
5. Yoshida, H., and Karmazyn, M. (2000) *Am. J. Physiol. 278*, H300−H3004.
6. Wakabayashi, S., Pang, T., Su, X., and Shigekawa, M. (2000) *J. Biol. Chem. 275*, 7942−7949.
7. Aharonovitz, O., Zaun, H. C., Balla, T., York, J. D., Orlowski, J., and Grinstein, S. (2000) *J. Cell Biol. 150*, 213−224.
8. Lin, X., and Barber, D. L. (1996) *J. Biol. Chem. 93*, 12631−12636.
9. Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouyssegur, J., and Shigekawa, M. (1997) *Biochemistry 36*, 12854−12861.
10. Mailander, J., Muller-Esterl, W., and Dedio, J. (2001) *FEBS Lett. 507*, 331−335.
11. Provencher, S. W., and Glockner, J. (1981) *Biochemistry 20*, 33−37.
12. Laue, T. M., and Stafford, W. F., III (1999) *Annu. Rev. Biophys. Biomol. Struct. 28*, 75−100.
13. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) *Biophys. J. 36*, 575−588.
14. Laue, T. M., Shah, B. D., Rodgeway, T. M., and Pelletier, S. L. (1991) Computer-aided interpetation of analytical sedimentation data for proteins, in *Analytical Ultracentrifuation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., and Horton, J. C., Eds.) pp 90−125, Royal Society of Chemistry, Cambridge, U.K.
15. McRorie, D. K., and Voelker, P. (1993) Beckman Instruments, Inc., Fullerton, CA.
16. Silva, N. L. C. L., Haworth, R. S., Singh, D., and Fliegel, L. (1995) *Biochemistry 34*, 10412−10420.
17. Wang, H., Singh, D., and Fliegel, L. (1998) *Arch. Biochem. Biophys. 358*, 116−124.
18. Tang, X.-B., Kovacs, M., Sterling, D., and Casey, J. R. (1999) *J. Biol. Chem. 274*, 3557−3564.
19. Murtazina, B., Booth, B. J., Bullis, B. L., Singh, D. N., and Fliegel, L. (2001) *Eur. J. Biochem. 268*, 1−13.
20. Murray, G., Marshall, M. J., Trumble, W., and Magnuson, B. A. (2001) *BioTechniques 30*, 1036−1041.
21. Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) *J. Biol. Chem. 269*, 13703−13709.
22. Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) *J. Biol. Chem. 269*, 13710−13715.
23. Corbett, E. F., Oikawa, K., Francois, P., Tessier, D. C., Kay, C., Bergeron, J. J., Thomas, D. Y., Krause, K. H., and Michalak, M. (1999) *J. Biol. Chem. 274*, 6203−6211.
24. Whitaker, J. E., Haugland, R. P., Moore, P. L., Hewitt, P. C., Reese, M., and Haugland, R. P. (1991) *Anal. Biochem. 198*, 119−130.
25. Fliegel, L. (2001) *Basic Res. Cardiol. 96*, 301−305.
26. Khaled, A. R., Moor, A. N., Li, A., Kim, K., Ferris, D. K., Muegge, K., Fisher, R. J., Fliegel, L., and Durum, S. K. (2001) *Mol. Cell. Biol. 21*, 7545−7557.
27. Fliegel, L., Haworth, R. S., and Dyck, J. R. B. (1993) *Biochem. J. 289*, 101−107.
28. Fafournoux, P., Noel, J., and Pouysségur, J. (1994) *J. Biol. Chem. 269*, 2589−2596.
29. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) *J. Biochem. (Tokyo) 95*, 511−519.
30. Wakabayashi, S., Ikeda, T., Noel, J., Schmitt, B., Orlowski, J., Pouyssegur, J., and Shigekawa, M. (1995) *J. Biol. Chem. 270*, 26460−26465.
31. Pedersen, S. F., Jorgensen, N. K., and Hoffmann, E. K. (1998) *Pfluegers Arch. 436*, 199−210.
32. Biemesderfer, D., Degray, B., and Aronson, P. S. (1998) *J. Biol. Chem. 273*, 12391−12396.
33. Khan, I. (2001) *Arch. Biochem. Biophys. 391*, 25−29.
34. Williams, K. A. (2000) *Nature 403*, 112−115.
35. Counillon, L., and Pouyssegur, J. (2000) *J. Biol. Chem. 275*, 1−4.
36. Wakabayashi, S., Fafournoux, P., Sardet, C., and Pouyssegur, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2424−2428.

BI027143D