The secondary structure of the purified 70-kDa protein Na+/Ca2+ exchanger, functionally reconstituted into asolectin lipid vesicles, was examined by Fourier transform infrared attenuated total reflection spectroscopy. Fourier transform infrared attenuated total reflection spectroscopy provided evidence that the protein is composed of 44% α-helices, 25% β-sheets, 16% β-turns, and 15% random structures, notably the proportion of α-helices is greater than that corresponding to the transmembrane domains predicted by exchanger hydropathy profile. Polarized infrared spectroscopy showed that the orientation of helices is almost perpendicular to the membrane. Tertiary structure modifications, induced by addition of Ca2+, were evaluated by deuterium/hydrogen exchange kinetic measurements for the reconstituted exchanger. This approach was previously proven as a useful tool for detection of tertiary structure modifications induced by an interaction between a protein and its specific ligand. Deuterium/hydrogen exchange kinetic measurements indicated that, in the absence of Ca2+, a large fraction of the protein (40%) is inaccessible to solvent. Addition of Ca2+ increased to 55% the inaccessibility to solvent, representing a major conformational change characterized by the shielding of at least 93 amino acids.

The Na+/Ca2+ exchanger plays an important role in Ca2+ extrusion from myocardial cells and in Ca2+ homeostasis in cardiac muscle (1–3). Solubilization and reconstitution of the Na+/Ca2+ exchange activity has been carried out from different tissues (4–12). After purification, SDS-polyacrylamide gel electrophoresis showed two major protein bands at 160- and 120-kDa. Under reducing gel conditions and following mild chymotrypsin treatment, the intensity of the 160- and 120-kDa band decreased while a band at 70-kDa appeared (10, 12). Affinity-purified antibodies specific for the 3 polypeptides cross-reacted with each other, meaning that the three proteins were immunologically related (10).

The canine and bovine cardiac Na+/Ca2+ exchangers have been cloned and sequenced (13, 14). A hypothetical membrane topology model was proposed based on hydropathy profile analysis of the exchanger’s amino acid sequence, in which α-helical membrane-spanning segments are predicted (5 in the NH2- and 6 in the COOH-terminal portion), separated by a large hydrophilic cytoplasmic loop. The NH2 terminus is extracytoplasmic and the COOH terminus intracytoplasmic (13). Beside these predictions, the exchanger secondary and tertiary structure are poorly understood. Topological models describing polypeptide chain insertion in the membrane are all based on the assumption that membrane-spanning segments would have an α-helical structure (15, 16). Recently, the transmembrane regions of porins have been described as containing β-sheets instead of the expected α-helices (19, 20). Taken together, these data indicate that α-helices may not be the only secondary structure for protein transmembrane segments. Two Na+/Ca2+ exchange reaction mechanisms are possible for Na+/Ca2+ countertransport across the membrane: simultaneous or consecutive kinetics. Strong experimental evidence favors a consecutive ion-transport scheme for the cardiac Na+/Ca2+ exchanger (21–23). Nevertheless, a simultaneous transport mechanism is suggested for the exchanger in ferret red blood cells (24). Fig. 1 summarizes a consecutive reaction scheme for Na+/Ca2+ exchange in the forward mode, characterized by 2 different conformations (E1 and E2) (25). The secondary and tertiary structure changes implicated in the transition from the E1 to the E2 conformation of the exchanger have not been investigated yet, and the effect of ion-binding on induction or stabilization of one of the conformations remains to be elucidated.

Ca2+ regulation of the Na+/Ca2+ exchange activity was studied in cardiac sarcolemmal vesicles. The cardiac Na+/Ca2+ exchanger has an intracellular regulatory site with high Ca2+ affinity, located between amino acids 445 and 455 (26). Regulation can be summarized as follows: a Ca2+ increase inactivates Na+/Ca2+ exchange (27–29).

We have previously described the purification and characterization of the bovine heart 70-kDa exchanger polypeptide reconstituted into asolectin vesicles (30). The protein has a Na+/Ca2+ exchange activity and is oriented inside-out, i.e. with the large hydrophilic loop, between transmembrane segments, protruding to the outside of the vesicles (30). In the present study, the secondary structure of the 70-kDa protein is determined by FTIR-ATR, which is a reliable technique to estimate membrane protein secondary structure and orientation in the...
l lipid bilayer (31-34). Tertiary structure modifications were investigated by monitoring $^2\text{H}/\text{H}$ exchange rate for the reconstituted exchanger in the absence and presence of $\text{Ca}^{2+}$. FTIR-ATR spectra reveal that 44% of the protein is $\alpha$-helical, 25% $\beta$-sheets, 16% $\beta$-turns, and 15% random coils. $^2\text{H}/\text{H}$ exchange measurements show that upon $\text{Ca}^{2+}$ addition, a major conformational change occurs, characterized by a 15% reduction in solvent accessibility of the 70-kDa protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Bovine Heart Sarcolemmal Vesicles**

Highly purified sarcolemmal vesicles were isolated from bovine heart left ventricle as described previously (35, 36) with minor modifications (30).

**Purification and Reconstitution of the Na$^+/\text{Ca}^{2+}$ Exchanger Protein**

We have purified and reconstituted the Na$^+/\text{Ca}^{2+}$ exchanger using a previously published protocol (10, 30). Bio-Beads SM-2 (Bio-Rad) were used for Triton X-100 removal to favor proteoliposomes formation. When reconstituted proteins were used for ATR-IR measurements, the Bio-Beads were soaked and washed in 0.5 m M MOPS solution (pH 7.4, 37 °C) before incubation with the asolectin/proteoliposome mixture, at variance with previously described methods (30). The protein/asolectin preparation was loaded on top of the Bio-Beads columns and incubated for 30 min at room temperature. Proteoliposomes were collected by centrifugation of the columns for 1 min at 1000 × g. Aliquots were pooled, diluted in 0.5 m M MOPS (pH 7.4, 37 °C) solution, and centrifuged at 140,000 × g for 90 min (4 °C) to pellet the reconstituted protein. This last centrifugation step was repeated twice to ensure the complete removal of all ion traces remaining in the supernatant from the pelleted protein-lipid complex. Finally, proteoliposomes were resuspended in 50 μl of the same solution and stored at −20 °C until use.

**Na$^+/\text{Ca}^{2+}$ Exchange Activity Measurements**

Na$^+/\text{Ca}^{2+}$ exchange activity in reconstituted proteoliposomes was measured as Na$^+$-dependent $^{45}\text{Ca}^+$ uptake using two previously described protocols (35, 36). Briefly, in the first protocol, reconstituted vesicles (50 μl) were loaded with Na$^+$ and diluted in Ca$^{2+}$ uptake medium (140 mM KCl, 0.01 mM CaCl$_2$, 0.3 mM $^{45}\text{CaCl}_2$, 0.36 mM valinomycin) to initiate Na$^+$-dependent $^{45}\text{Ca}^{2+}$ uptake. The uptake reaction was quenched after 3 s by addition of 30 μl of 140 mM KCl, 10 mM EGTA followed by the addition of 1 ml of ice-cold 140 mM KCl, 1 mM EGTA. Samples were then filtered using 0.22-μm nitrocellulose filters (Sartorius) and filters were washed with 2 × 5 ml of ice-cold 140 mM KCl, 1 mM EGTA (10). In the second protocol, a similar procedure was used except that Ca$^{2+}$ uptake reaction was stopped after 3 s by addition of 5 ml of ice-cold 140 mM KCl, 0.1 mM EGTA and vesicles were harvested by filtration on Whatmann GF/A filters previously soaked in 0.3% polyethyleneimine in H$_2$O. The filters were then washed with 2 × 5 ml of ice-cold 140 mM KCl, 0.1 mM EGTA, avoiding filter drying between rinses. In both protocols, blanks were obtained by replacing KCl by NaCl in the $^{45}\text{Ca}^{2+}$ uptake medium. All solutions were buffered with 10 mM MOPS/Tris, pH 7.4.

**Infrared Attenuated Total Reflection Spectroscopy**

**Sample Preparation—**20 to 50 μl of sample was deposited on one side of the ATR germanium plate and was slowly evaporated, under a stream of nitrogen, yielding a dry thin film of reconstituted protein sample. The internal reflection element (ATR plate) is a germanium plate (50 × 20 × 2 mm, Harrick EJ 2121) with an aperture angle of 45°, yielding 25 internal reflections (37). The ATR plate was then sealed in a universal sample holder (Perkin-Elmer 186–0354).

**Secondary Structure Analysis—**The sample on the ATR plate was deuterated by flushing with a $^2\text{H}_2\text{O}$-saturated nitrogen stream for at least 2 h. Deuterium/hydrogen ($^2\text{H}/\text{H}$) exchange allows differentiation of $\alpha$-helical secondary structures from a random one (36) by shift of the absorption band of the former from 1,655 to approximately 1,642 cm$^{-1}$, yielding 25 internal reflections (37). The ATR plate was then sealed in a universal sample holder (Perkin-Elmer 186–0354).

**Orientation of the Secondary Structure—**Determination of peptide orientation by infrared ATR spectroscopy was performed as described previously (42, 40). Briefly, spectra were recorded with a parallel and perpendicular polarization with respect to the incidence plane. Then, the dichroism spectrum was obtained by subtracting the perpendicular polarized spectrum from the parallel one. A larger absorbance of the parallel polarization indicates a dipole oriented preferentially parallel to the normal to the ATR plate, whereas, a larger absorbance of the perpendicular polarization indicates a dipole orientation close to the plane of the ATR plate. It has been shown that in $\alpha$-helices, amide I dipole orientation makes a 27° angle with respect to the helix axis and that the $\beta$-sheet long axis is mainly perpendicular to the amide C = O axis (43).

**Deuteration Kinetics—**Films containing 10–20 μg of protein were prepared on a germanium plate as described above. Nitrogen gas was saturated with $^3\text{H}_2\text{O}$ (by bubbling through a series of three $^3\text{H}_2\text{O}$-containing vials) at a flow rate of 90 ml/min (controlled by a Brooks flow-meter). Bubbling was started at least 1 h before starting the experiment, i.e. before connecting the tubing carrying the $^3\text{H}_2\text{O}$-saturated nitrogen flux to the sealed microchamber containing the sample film. Before starting $^3\text{H}/\text{H}$ exchange measurements, 10 spectra of non-deuterated dried sample on ATR plates were recorded to verify measurement stability and reproducibility of band area computation. At $t = 0$, the tubing was connected to the sealed microchamber containing the studied sample. For each kinetic point, 12 spectra, with a 4 cm$^{-1}$ resolution, were recorded and averaged. Spectra were recorded every 15 s for the first 2 min. After which, the recording time interval was exponentially increased. After 16 min, the time interval between scans was large enough to allow the insertion of another $^3\text{H}/\text{H}$ exchange kinetics. Thus, a second sample, prepared on another ATR setup of the

**Structure of the Na$^+/\text{Ca}^{2+}$ Exchanger 70-kDa Polypeptide**

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**Fig. 1.** Schematic representation of the Na$^+/\text{Ca}^{2+}$ exchanger reaction scheme based on a consecutive countertransport mechanism in the forward mode. The exchanger exposes its ion-binding site to the extracellular side (conformation $E_1$) and releases Ca$^{2+}$ with concomitant change in conformation to $E_2$, which binds 3 Na$^+$. The binding site is then exposed to the cytoplasmic side, Na$^+$ ions are released, and the exchanger returns to an $E_1$ conformation, that binds Ca$^{2+}$, completing the transport cycle (25).

1. **Na$^+/\text{Ca}^{2+}$ Exchanger Protein**
2. **Sample Preparation**
3. **Secondary Structure Analysis**
4. **Orientation of the Secondary Structure**
5. **Deuteration Kinetics**
Perkin-Elmer shuttle was analyzed with the same time sampling (with a 16-min offset) by connecting the 2H2O-saturated nitrogen flux in series with the first sample. Accordingly, our software changed the shuttle position to follow both kinetics (43, 44). In our case, the first sample was the reconstituted Na\(^+/\)Ca\(^{2+}\) exchanger in the absence of ligands (see reconstitution protocol), and the second sample had 0.2 mM CaCl\(_2\) added.

The spectrophotometer chamber was flushed with dry air for 20 min before starting the experiment. However, further removal of traces of water vapor occurred as the \(^2\)H/H exchange kinetics proceeded, superimposing water vapor’s sharp bands onto the protein spectra (45). At this stage, the water contribution was subtracted as described (45). Areas of the amide I, II, and III bands were obtained by integration between 1,702 and 1,596, 1,596 and 1,502, and 1,492 and 1,412 cm\(^{-1}\), respectively. The amide II area was divided by the corresponding lipid \(\nu(C = O)\) area which allowed us to take into account small, but significant, variations in the overall spectra intensity due to sample layer swelling caused by the presence of \(^2\)H2O. Indeed, ATR spectrum intensity highly depends on the distance between sample layer and germanium plate surface (46); and, as this distance increases by sample swelling, the spectrum intensity decreases by a few percent for all measured bands.

The 0% deuterated sample spectra were recorded before starting the \(^2\)H/H exchange kinetics as described above, and the 100% deuterated sample value was extrapolated by assuming a zero value for the amide II band area. Accordingly, throughout the kinetics, the amide II band area (normalized to the lipid \(\nu(C = O)\) area) was expressed between 0 and 100% deuteration for each kinetic point.

**Statistics and Calculations**

All data are presented as mean ± S.E.

**RESULTS**

**Purification and Reconstitution of the Na\(^+/\)Ca\(^{2+}\) Exchanger**—The bovine heart exchanger was purified using a combination of ion-exchange and affinity chromatography, and reconstituted into asolectin vesicles. The purification resulted in a 70-kDa protein showing Na\(^+/\)Ca\(^{2+}\) exchange activity, that was inserted inside-out in the lipid vesicles, i.e. with the large cytoplasmic loop facing the extravesicular milieu. The Na\(^+/\)Ca\(^{2+}\) exchange activity in control reconstituted sarcolemmal vesicles was 18 ± 1 nmol of Ca\(^{2+}\)/mg protein/s at 40 \(\mu\)M Ca\(^{2+}\) and 1155 ± 81 nmol of Ca\(^{2+}\)/mg of protein/s at the same Ca\(^{2+}\) concentration for the reconstituted Na\(^+/\)Ca\(^{2+}\) exchanger, the purification factor being 64. The amount of protein in the control sample was ~1850 \(\mu\)g, while that in the purified sample was ~15 \(\mu\)g (30). The protein content of control reconstituted vesicles comprises all membrane proteins, including the exchanger; whereas, the purified samples contain only the Na\(^+/\)Ca\(^{2+}\) exchanger protein. The purified 70-kDa polypeptide was characterized in a previous publication (30).

**Secondary Structure Analysis of the Reconstituted Exchanger**—Fig. 2 shows spectra of purified reconstituted exchanger in the absence of ligands (Fig. 2A) and in the presence of 0.2 mM Ca\(^{2+}\).
The lipid C = O band maximal absorption was near 1,737 cm\(^{-1}\) for ligand-free (sample A) and Ca\(^{2+}\)-containing (sample B) reconstituted sample. The amide I band had a maximal absorption near 1,650 cm\(^{-1}\) for sample A and sample B (Fig. 2, A and B, respectively). A quantitative evaluation of the protein secondary structure was obtained by Fourier deconvolution and curve-fitting analysis of the amide I region for spectra recorded in the absence and presence of Ca\(^{2+}\) (42). The curve-fitting of amide I band showing different structural components (α-helices, β-sheets, β-turns, and random) for sample A and sample B is shown in Fig. 3, A and B, respectively. The confirmation of these assignments is discussed by Goormaghtigh et al. (47). Analysis of the shape of the amide I absorption band of the reconstituted Na\(^{+}/Ca^{2+}\) exchanger has been carried out as described previously (37). Typical absorbance regions of different peptide chains secondary structures are the following: 1,646–1,661 cm\(^{-1}\) (α-helice), 1,615–1,637 cm\(^{-1}\) and 1,682–1,698 cm\(^{-1}\) (β-sheet), 1,661–1,681 cm\(^{-1}\) (β-turn), and 1,637–1,645 cm\(^{-1}\) (random).

**Table I**

Summary of the secondary structure analysis results of the purified exchanger

| Structure of the Na\(^{+}/Ca^{2+}\) Exchanger 70-kDa Polypeptide |
|---------------------------------------------------------------|
| Assignment of percentage for α-helices, β-sheets, β-turns, and random structures was obtained by self-deconvoluted amide I band from spectrum of purified and reconstituted Na/Ca exchanger in the absence and presence of 0.2 mM Ca\(^{2+}\). The number of amino acids (aa) per secondary structure group calculated on the basis that the 70-kDa polypeptide is about 620 amino acids, assuming 110 Da per amino acid. |
| Na\(^{+}/Ca^{2+}\) exchanger (no Ca\(^{2+}\) added) | 44% | 25% | 16% | 15% |
| (273 aa) | (155 aa) | (99 aa) | (93 aa) |
| Na\(^{+}/Ca^{2+}\) exchanger (0.2 mM Ca\(^{2+}\) added) | 42% | 26% | 17% | 15% |
| (260 aa) | (161 aa) | (105 aa) | (93 aa) |

Orientation of the Na\(^{+}/Ca^{2+}\) Secondary Structure with Respect to the Lipid Membrane—The orientation of the reconstituted exchanger secondary structure was determined from FTIR-ATR spectra recorded with parallel and perpendicular polarized incident light, respectively (see “Experimental Procedures” for details). The dichroism spectrum (Fig. 4) shows a
maximal absorption of about 1,656 cm\(^{-1}\) in the amide I region, characterizing an \(\alpha\)-helices axis having a preferential orientation near perpendicular to the lipid bilayer. No dichroism could be detected for the \(\beta\)-sheet component in the spectrum.

**Deuterium/Hydrogen Exchange**—In the absence of secondary structure changes upon ligand addition, the rate of \(^2\text{H}/\text{H}\) exchange reveals tertiary structure modifications which follows interaction with \(\text{Ca}^{2+}\) (at constant pH and temperature). Kinetics of \(^2\text{H}/\text{H}\) exchange of the reconstituted exchanger was recorded in the absence and presence of \(\text{Ca}^{2+}\) to check whether any tertiary structure modifications occurred upon ligand addition.

Peptide N-H group hydrogen exchange was followed by monitoring the amide II absorption peak decrease (N-H) maximal absorption at 1,544 cm\(^{-1}\) as a function of \(^2\text{H}_2\text{O}\)-saturated \(\text{N}_2\) flow exposure time, as illustrated in Figs. 5 and 6. Spectra were recorded during the same experiment for protein samples with no ligands and with 0.2 mM \(\text{Ca}^{2+}\) added (Fig. 5 and 6, respectively). The amide II area evolution, between 0 and 100% \(^2\text{H}/\text{H}\) exchange, was computed as described under “Experimental Procedures” and is reported in Fig. 7. It immediately appears in Fig. 7 that \(^2\text{H}/\text{H}\) exchange is faster for protein sample prepared in the absence of ligands than that for sample with 0.2 mM \(\text{Ca}^{2+}\) added. Indeed, after 2 h of deuteration, about 60% of the peptide N-H is exchanged for the former sample, compared with around 45% for the latter one.

Hydrogen atoms with different exchange rates are involved in the exchange process. Considering that the \(^2\text{H}/\text{H}\) exchange rate is a first-order reaction, the exchange curve can be represented by a multiexponential decay function taking into account different amide proton groups \((\alpha_i)\), each characterized by a time period \((T_i)\),

\[
F(t) = \sum \alpha_i \exp(-t/T_i)
\]  
(Eq. 1)

Because of the large number of protons, it is impossible to compute individual rate constants. Three exponentials were chosen characterized by a period, \(T_i\) \((i = 3)\), and a group of amide hydrogens, \(\alpha_i\). Decomposing of the experimental curve into three families of exchanging protons (slow, intermediate, and fast exchanging) allows to fit the experimental data within the reproducibility of the experiment. A nonlinear fitting of all experimental curves without constraints on \(T_i\) and \(\alpha_i\) yields three similar periods, \(T_1, T_2,\) and \(T_3\) for analyzed kinetics curves (corresponding to \(^2\text{H}/\text{H}\) exchange kinetics in the absence of ligands, and that in the presence of \(\text{Ca}^{2+}\)). A second fitting
was then carried out for each curve, setting $T_i$ to its average value: $T_1 = 2.5$ s, $T_2 = 40$ s, and $T_3 = 10^4$ s, to compare the $a_i$ proportion of each amide group for different experimental conditions used (44). Constraining the $T_i$ to its average value did not modify the proportion of amide protons in each class. Table II summarizes the results of that analysis. The $T_1$ period corresponds to the rapidly exchanging amide protons which represents 32% of the whole protein amide groups (198 amino acids). Upon Ca$^{2+}$ addition, the number of rapidly exchanging amino acids is decreased by about 50%, which were essentially converted to slowly ($T_3 = 10^4$ s) and intermediate ($T_2 = 40$ s) exchanging species. This increased protection of 93 amino acids confirms a direct interaction between Ca$^{2+}$ and the exchanger, and demonstrates that Ca$^{2+}$ induces a major conformational change of the protein.

**DISCUSSION**

The aim of this work was to study the secondary structure of the purified and reconstituted 70-kDa bovine heart Na$^+$/Ca$^{2+}$ exchanger, the orientation of protein secondary structure in the lipid vesicle membrane, and possible secondary/tertiary structure changes in the presence of Ca$^{2+}$ ion. Whereas the protein secondary structure is not significantly modified upon specific ligand addition, amide $^2$H/H exchange experiments provided information as to solvent accessibility to protein amide group, allowing detection of conformational changes and quantification of the number of amino acid residues involved. Comparison of FTIR-ATR experiments carried out on film with data obtained in solution by FTIR or NMR studies demonstrate the validity of this approach (48).

Recently, we have purified and characterized a unique 70-kDa polypeptide that retained Na$^+$/Ca$^{2+}$ exchange activity when reconstituted into asolectin vesicles and that corresponded to the NH$_2$ terminus portion of the exchanger (30). In the reconstitution protocol, we used a lipid:protein ratio of $\approx$ 7:1 to obtain a homogeneous population of reconstituted protein (inserted inside-out), which is essential for such structural studies (30, 40, 41).

Analysis of ATR-FTIR results provides information on the secondary and tertiary structure of the reconstituted active exchanger. The estimated secondary structure of the protein was evaluated to be 44% $\alpha$-helices, 25% $\beta$-sheets, 16% $\beta$-turns, and 15% random structures (Table I).

Because the 70-kDa polypeptide, studied herein, has only the 5 NH$_{2}$-terminal membrane-spanning segments compared with the entire exchanger (30), and assuming that those 5 segments are $\alpha$-helical, the 70-kDa protein should be constituted of at least 18% $\alpha$-helices. The proportion of $\alpha$-helices determined in this study (44%) is markedly higher. This discrepancy between hydrophyo plot estimations and experimental secondary structure assignment has been previously observed for other ion transporting membrane proteins (40, 41, 51, 52). A possible explanation for this difference could be that protein topology models based on hydrophathy profile analysis underestimate the proportion of transmembrane domains (assuming those domains as exclusively $\alpha$-helical) and/or that ordered $\alpha$-helical structures are present outside the lipid bilayer. Table III com-

![Infrared spectra of reconstituted Na$^+$/Ca$^{2+}$ exchanger in the absence of ligands](https://example.com/fig5.png)
pares the secondary structure proportions of the Na\(^+/Ca^{2+}\) exchanger with those of other proteins such as the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (53), Na\(^+/K^{+}\)-ATPase (54), P-glycoprotein (41), H\(^+\)-ATPase (44), and H\(^+/K^{+}\)-ATPase (52) as determined by ATR-FTIR. A very high similarity between the secondary structure of the 70-kDa polypeptide Na\(^+/Ca^{2+}\) exchanger and that of PMCA can be observed (92% of similarity between structures, 96% for \(\beta\)-sheet structures, 81% for \(\beta\)-turn structures, and 93% for random structures). This high similarity may reflect a homology between the Na\(^+/Ca^{2+}\) exchanger and the PMCA secondary structure (55), especially at the level of secondary structure organization of their intracytoplasmic domains. The 70-kDa polypeptide also shows high similarity with secondary structure proportions of P-type ion-transporting ATPases. Interestingly, addition of Ca\(^{2+}\) (0.2 mM) does not alter the Na\(^+/Ca^{2+}\) exchanger secondary structure (Table I) which is an observation common to P-glycoprotein (41) and P-type H\(^+\)-ATPase upon addition of ligands (44).

Infrared dichroism brings evidence for the presence of \(\alpha\)-helices with a transmembrane orientation perpendicular to the lipid bilayer, in agreement with the currently accepted model of the Na\(^+/Ca^{2+}\) exchanger protein (13, 14). The other secondary structures did not show any significant orientation.

\(^2\)H/H exchange measurements, in the absence of ligands (0.05 mM MOPS), showed that a considerable proportion of the Na\(^+/Ca^{2+}\) exchanger (about 40% of the total amino acid content), characterized by a slow exchange rate (half-decay \(T_{1/2} = 10^4\) s), was inaccessible to the aqueous phase (Fig. 7 and Table II). This lack of accessibility could be partly due to the lipid bilayer shielding effect. Inaccessibility of solvent to membrane-spanning regions of proteins was previously demonstrated for many membrane proteins such as glycoporin, bacteriorhodopsin, P-glycoprotein, and some P-type ATPases (41, 44, 52, 56). In our case, theoretical predictions of transmembrane regions of the 70-kDa polypeptide account for only 18% of total amino acids. Accordingly, two explanations are possible for this difference: more membrane-spanning segments are present than theoretically predicted and/or a considerable proportion of the extramembrane region is organized in a highly structured manner accounting for solvent inaccessibility. Such highly organized domains could represent membrane-associated and/or membrane-embedded regions at the level of the large cytoplasmic loop.

Upon Ca\(^{2+}\) addition (0.2 mM), \(^2\)H/H exchange measurements showed a major conformational change characterized by a 15% reduction in solvent accessibility of the 70-kDa polypeptide total amino acids compared with solvent accessibility in the absence of ligands (Fig. 7 and Table II). Accordingly, at least 93 amino acid residues become shielded from the solvent after addition of Ca\(^{2+}\). Interestingly, adding Ca\(^{2+}\) does not alter the secondary structure of the protein; thus, the recorded conformational change is most probably due to tertiary structure changes induced by Ca\(^{2+}\) binding to its regulatory site (amino acids 445–455). Detailed analysis of \(^2\)H/H exchange curves indicated that Ca\(^{2+}\) binding to the Na\(^+/Ca^{2+}\) exchanger leads to an increase of about 99 amino acid residues essentially of the slow and, to a smaller extent, intermediate exchanging population at the expense of the fast one (Table II).
induced increase of protection of a relatively large number of amino acids can be interpreted in terms of a transient protein folding or "membrane penetration" of a protein portion, resulting in a decreased solvent accessibility. A similar phenomenon of tertiary structure changes upon addition of ligands was observed in a recent study of the purified and reconstituted P-glycoprotein where addition of MgATP (which is hydrolyzed by the protein) induced an increased accessibility to at least 76 amino acid residues. But, addition of MgATP-verapamil lead to the protection of 106 amino acid residues (41). Verapamil is known to be a stimulator of P-glycoprotein MgATP hydrolysis activity (57–60). Similar important tertiary structure modifi-

**TABLE II**

Summary of 2H/H exchange results

| Substrate     | a₁ (%) | a₂ (%) | a₃ (%) |
|---------------|--------|--------|--------|
| No ligands    | 32%    | 25%    | 43%    |
| Ca²⁺         | 16%    | 29%    | 56%    |

* aa, amino acids.

**TABLE III**

Comparison of secondary structure proportions between Na⁺/Ca₂⁺ 70-kDa exchanger and other ion-transporting membrane proteins

| Protein                | α-Helices | β-Sheets | β-Turns | Random structures |
|------------------------|-----------|----------|---------|-------------------|
| Na⁺/Ca₂⁺ exchanger     | 44%       | 25%      | 16%     | 15%               |
| PMCA                   | 48%       | 24%      | 13%     | 14%               |
| P-glycoprotein          | 32%       | 26%      | 29%     | 13%               |
| H⁺-ATPase (44)          | 39%       | 30%      | 16%     | 14%               |
| H⁺/K⁺-ATPase (52)       | 32%       | 31%      | 16%     | 20%               |
| Na⁺/K⁺-ATPase (54)      | 30–35%    | 37–40%   | 13–15%  |                   |

* The value represents the percentage of β-sheets and β-turns taken together.
flections were observed for a P-type ATPase upon ligand addition. Indeed, 2H/H exchange measurements on the H+-ATPase show that it undergoes a major conformational change involving the additional shielding of about 175 amino acid residues (out of 920) when adding MgADP or MgATP-venadate (vanadate is known to stimulate the H+-ATPase activity) (44, 61).

Ca2+-besides being counter-transported with Na+-is a regulatory ion having its binding site located between amino acids 445 and 455. As our 2H/H exchange measurements indicate, Ca2+-binding is a significant shielding of about 150 amino acid residues which involves a major conformational change (Fig. 1). Such Ca2+-binding induction of structural changes has been observed in the case of the PMCA (62). Outstandingly, the missing COOH-terminal region in the 70-kDa polypeptide does not prevent protein-structure modifications upon Ca2+-binding. This is not surprising as the 70-kDa protein includes a large part of the cytoplasmic loop (about 400 amino acids) and retains Na+/Ca2+-exchange activity (30). Further investigations need to be carried out to elucidate the role of the COOH portion of the Na+/Ca2+-exchanger with respect to regulation.

In conclusion, the present study indicates that the secondary structure of the 70-kDa exchanger is composed of 44% α-helices, 25% β-sheets, 16% β-turns, and 15% random. Moreover, the 70-kDa polypeptide secondary structure showed high similarity to that of the PMCA. Ca2+-binding induced a major conformational change in the protein, with no apparent secondary structure modifications. The Na+/Ca2+-exchanger most probably has a consecutive ion-transport mechanism. Such transport mechanisms are generally characterized by important conformational changes. Tertiary structure modifications observed upon Ca2+-binding may reflect protein folding throughout the Na+/Ca2+-exchange catalytic cycle.

REFERENCES
1. Hilgemann, D. W. (1986) J. Gen. Physiol. 87, 675–706
2. Bers, D. M., and Bridge, J. H. B. (1989) Circ. Res. 65, 334–342
3. Reeves, J. P. (1990) in Intracellular Calcium Regulation (Brunner, F., ed) pp. 305–347, A. R. Liss, New York
4. Miyamoto, H., and Racker, E. (1980) J. Biol. Chem. 255, 139–145
5. Philipson, K. D., McDonough, A., Frank, J. S., and Ward, R. (1987) J. Gen. Physiol. 89, 305–347
6. Goormaghtigh, E., Cabiaux, V., De Meuter, J., Rosseneu, M., and Ruysschaert, J.-M. (1992) Biochim. Biophys. Acta 1148, 1–19
7. Philipson, K. D., and Reeves, J. P. (1993) Biochim. Biophys. Acta 1180, 271, 262–270
8. Goormaghtigh, E., Vigneron, L., Scarborough, G., and Ruysschaert, J.-M. (1994) J. Biol. Chem. 269, 27409–27413
9. Goormaghtigh, E., Vigneron, L., Scarborough, G., and Ruysschaert, J.-M. (1994) J. Biol. Chem. 269, 2713–2714
10. Goormaghtigh, E., Cabiapis, V., and Ruysschaert, J.-M. (1995) Biochim. Biophys. Acta 1228, 258–268
11. Goormaghtigh, E., Vigneron, L., Scarborough, G., and Ruysschaert, J.-M. (1994) J. Biol. Chem. 269, 2713–2714