Secondary Structural Composition of the Na/K-ATPase E₁ and E₂ Conformers*

Thomas J. Gresalfi† and B. A. Wallace‡

From the Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York, New York 10032

The existence of conformers of the sodium- and potassium-dependent adenosine triphosphatase has been known for some time, yet their structures remain poorly characterized. In this study, circular dichroism spectroscopy was utilized to assess the secondary structural composition of the enzyme, particularly with regard to the E₁ and E₂ states that are associated with the presence of Na⁺ and K⁺, respectively. Parallel experiments were performed in which highly purified Na/K-ATPase from guinea pig kidney outer medulla was incubated with various cations and then examined by CD. The spectra were corrected for optical effects which arise due to the particulate nature of the membrane-bound protein, and then fit to reference data derived from a set of proteins with known secondary structures. In the peptide backbone region of the spectrum (190–240 nm), significant differences between the E₁ and E₂ conformers were detected and quantified in terms of the proportions of secondary structures present. An extensive conformational change rather than a small local perturbation must be responsible for the differences observed.

The Na/K-ATPase is a ubiquitous intrinsic membrane protein with vital physiological function that has been implicated in numerous pathological states including hypertension, hypothyroidism, obesity, and manic-depressive psychosis. Despite the enzyme’s biological significance and the availability of pure preparations for some time, there is a dearth of information concerning the enzyme’s structure and its relationship to function.

The Na/K-ATPase utilizes ATP hydrolysis as an energy source for the coupled active transport of Na⁺ and K⁺ across a wide variety of cell membranes. Following identification of the Na/K-ATPase as the “sodium pump,” it was reasonable to postulate that this translocation involved at least two conformers. Evidence for the existence of distinct Na/K-ATPase conformations has been provided from a number of investigations. Proteolytic digestion experiments by Jørgensen (1975), Giotta (1975), and Castro and Farley (1979) have demonstrated that in the presence of K⁺, trypsin cleaves the α-subunit of the enzyme into two polypeptides of 58,000 and 48,000, while in Na⁺ solutions, trypsin yields a 78,000 fragment. These experiments have been carried out in the presence of many combinations of Na⁺/K⁺-ATPase ligands, and in each case, the tryptic digestion patterns were similar to either those obtained in the presence of Na⁺ alone (E₁) or those obtained with K⁺ alone (E₂). Labeling studies have also detected differences in accessibility of hydrophobic probes to membrane-embedded portions of the protein under similar conditions (Farley et al., 1980).

Intrinsic and extrinsic fluorescence experiments have provided additional evidence for the existence of distinct conformers. Karlish and Yates (1978) reported that the E₂ form of the enzyme has a 2–3% higher tryptophan fluorescence than the E₁ form. Experiments with a number of extrinsic fluorescence probes have also demonstrated the existence of two distinct conformations (Karlish, et al., 1978; Karlish, 1980; Skou and Esman, 1981; Moczydlowski and Fortes, 1981). The results of the tryptic digestion, labeling, and fluorescence experiments strongly favor the existence of at least two conformations of the Na/K-ATPase, but do not necessarily rule out the existence of conformational subclasses not detected by these techniques, nor do they quantitate the structural differences between the E₁ and E₂ forms. The results could be explained by either an overall refolding of the molecule or simply motion at a “hinge” region where very minimal secondary structural alterations could protect or expose proteolytic cleavage sites, or significantly alter fluorescence properties.

In addition to the Na⁺- and K⁺-induced conformations, it has been reported that there are ionic strength effects (Skou and Esman, 1980) and pH influences on the equilibrium between the two states (Skou, 1982).

Circular dichroism is a measure of the optical activity exhibited by biomolecules as a consequence of their asymmetry. In the 190–240 nm region, the CD spectra of proteins are dominated by the π → π* and σ → σ* electronic transitions of the peptide backbone which are sensitive to the molecular geometry. CD spectroscopy may be used to determine protein secondary structure and is valuable in monitoring conformational changes. To date, the major difficulty with CD studies of membrane proteins has been in assessing the extent of the optical artifacts introduced by the particulate nature of the lipid-protein complexes. Differential light-scattering and absorption flattening effects produce distortions of the spectral shapes and magnitudes, particularly in the low wavelength region of the spectrum. These technical problems have been addressed using instrumental modification (Schneider and Harmatz, 1976) and calculational correction methods (Mao et al., 1982)* so that accurate studies of membrane proteins are now feasible.

In this study, CD has been used to characterize the E₁ and E₂ conformations of mammalian Na/K-ATPase induced by

* This work was supported in part by National Institute of Health Grants AM 31089 and GM 27292. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement,” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a National Institutes of Health Institutional National Research Service Award Fellowship.
‡ Recipient of a Hirschl Career Scientist Award. To whom correspondence should be addressed.

1 D. Mao and B. A. Wallace, manuscript in preparation.
Na" and K", respectively. The effects of the ionic strength of choline chloride, and of NaCl and KCl, are examined. Estimates of the secondary structural compositions are presented in each case, as well as estimates of the magnitudes of the structural alterations involved.

MATERIALS AND METHODS

The Na/K-ATPase utilized in these experiments was a highly purified derivative of fresh guinea pig kidney outer medulla (Pel-Freeze Biologicals) prepared by slight modification to the zonal rotor method of Jorgensen (1974a), including addition of dithiothreitol to the buffers (Hayashi and Post, 1981). The purified enzyme was washed twice in 3 mM imidazole, 1 mM HEDTA, adjusted to pH 7.4 with -7 mM Tris base and stored at 0 °C. The Na/K-ATPase suspension was analyzed for protein content by the method of Lowry et al. (1951). Ouabain-sensitive specific ATPase activity was determined (Jorgensen, 1974b). Polyacrylamide gel electrophoresis using 7.5% gels with 5.0% stacking gels was performed (Laemmli, 1970). Selected fractions of the purified enzyme were combined to give a single high quality homogeneous preparation of sufficient quantity to complete this series of experiments.

Electron microscopy was performed by diluting the Na/K-ATPase to -0.1 mg/ml with imidazole/EDTA buffer with or without 100 mM NaCl or KCl present. The samples were placed on Formvar-coated copper grids and stained with 0.5 or 1% aqueous uranyl acetate and examined in a Phillips Model 300 transmission electron microscope.

For CD studies, parallel experiments were performed in which the buffered enzyme was incubated with 100 mM NaCl, KCl, LiCl, choline chloride, or without salt, added in each case as 4 µl of a 3 M solution to 117 µl of a 0.90 mg/ml suspension of enzyme. For estimates of light scattering and absorption flattening effects, samples were incubated with 1.2% (w/v) n-octylglucopyranoside for at least 24 h to allow adequate solubilization. Corresponding blanks for all samples were prepared by substituting the buffer alone for the enzyme suspension with or without n-octylglucopyranoside as appropriate.

CD spectra were recorded on a Cary 60 recording spectropolarimeter with a Model 6901 CD accessory and equipped with variable detector geometry (Schneider and Harmatz, 1976) which allowed variation of the acceptance half-angles from 1° to 45°. Most spectra were obtained with the photomultiplier tube placed directly adjacent to the sample cell, resulting in a total acceptance angle of -90°. The instrument was calibrated with (+)-d-10-camp-horsulfonic acid at 290 nm. Measurements were made at 25.9 ± 1.6 °C using a calibrated 0.10-mm path length cell. Spectra were generally run at a scanning speed of 6.3 nm/min and a time constant of 3 s over the wavelength range of 300-190 nm. Spectra used for computations usually represent an average of 4 of 5 spectra, selected on the basis of reproducibility. Three independent samples of either the Na" or K" plus enzyme samples were examined; several independent preparations of Na/K-ATPase were also used in preliminary experiments, although all the results reported herein are from a single very highly purified batch of enzyme. The UV absorption spectra from 400-190 nm were obtained with a Cary 15 recording spectrophotometer with the cell placed adjacent to the detector and scanning at a speed of 21 mm/min.

Uncorrected mean residue ellipticities were calculated based on a mean residue Mr = 116, as determined from amino acid analysis of the purified protein.2 The CD data was then corrected for optical artifacts by a method which assumes that the measured absorption of a particulate sample is the sum of the absorption of the chromophores modified by light scattering and absorption flattening effects (Mao et al., 1982).1 The magnitude of apparent absorption due to scattering, A", was measured in a region of the spectrum where the chromophore absorption is negligible (i.e. 400-310 nm) and was then extrapolated to lower wavelengths according to the relationship: A"(λ) = A"(λ°) [1 - exp (-k(λ° - λ))], where k and n are constants and λ is the wavelength (Leach and Scheraga, 1960). Absorption flattening, A", was estimated by comparison of the absorption of the particulate sample with the detergent-solubilized sample containing a homogenous chromophore distribution after correction for scattering artifacts. The effect of differential light scattering on the measured ellipticity was evaluated by varying the detector geometry (Schneider and Harmatz, 1976). The flattening corrections to the ellipticities were then computed, assuming that differential absorption flattening is identical for left and right circularly polarized light.

Following correction for optical artifacts, data points at 1-nm intervals between 194 and 240 nm were computer fitted to a reference data set by an unconstrained linear least square method similar to that of Magar (1968) which did not require the fractions to be positive nor the sum of the fractions to be equal to unity. The fractions generated were then normalized to 100% to obtain estimates of the relative proportions of secondary structures present in the samples (Mao et al., 1982). The reference data set used was derived from 15 water-soluble proteins (Chang et al., 1978). As the magnitude of the helix spectrum is a function of helical length, the fitting program permitted helical length as an input variable. The quality of each computer fit was evaluated by calculating a normalized standard deviation for each curve (Mao et al., 1982), as given by the equation:

\[ \text{NRMSE} = \frac{\sum (\theta_{\text{exp}} - \theta_{\text{obs}})^2}{\sum \theta_{\text{obs}}^2} \]

where \( \theta_{\text{exp}} \) and \( \theta_{\text{obs}} \) are the experimental and calculated mean residue ellipticities and \( n \) is the number of data points used.

RESULTS

Characterization of Samples—Based on an estimated specific activity of 2200 µmol of P<sub>i</sub>/mg of protein/h, the activity of this preparation (2000), would suggest that approximately 90% of the protein in these membrane patches is Na/K-ATPase. Analysis of the preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed only two bands, corresponding to the α- and β-subunits. Even on greatly overloaded gels (Fig. 1) no other significant bands are detected, consistent with the estimate of at least 90% purity.

While Lowry assays of Na/K-ATPase tend to overestimate the protein concentration by ~12% (Peterson et al., 1982), the normalization procedure in the secondary structural analysis will correct for this (Mao et al., 1982), as reflected in the values of 1/sum (Table II). They suggest that for the guinea pig enzyme, the concentration determined by the Lowry assay

2 T. J. Gresalfi and B. A. Wallace, unpublished data.

---

**FIG. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the highly purified Na/K-ATPase from guinea pig outer medulla which was used in these CD studies. The migration positions of molecular weight standards (ovalbumin, bovine serum albumin, phosphorylase a, β-galactosidase, and myosin, from bottom to top) are noted.
terions, a minimal concentration of the standard Na/K-
P(H) effect on the equilibrium between the species (Skou, 1982).
The will not distort the fractional calculations in the unconstrained analysis (Mao et al., 1982; Mao, 1983).

The Na/K-ATPase in these samples is localized in single membrane sheets of average diameter 0.3 μm, as estimated by negative stain electron microscopy (Fig. 2). The patches were reasonably uniform in size, ranging from 0.2 to 0.4 μm. The protein distribution within the membrane seems to be relatively random and similar in both Na⁺ and K⁺ samples. Since the membrane patch size is large relative to the wavelength of light used for the CD measurements, light scattering effects must be considered (see below). Because of excessive absorbance encountered with a number of buffers tested, as well as limitations due to ligand-like effects of several buffer counterions, a minimal concentration of the standard Na/K-ATPase buffer, imidizole, was used. Since it is possible that the E₁ to E₂ change involves a protonation, there may be a pH effect on the equilibrium between the species (Skou, 1982). Hence, the pH was monitored and maintained to within 0.1 unit for all the Na⁺ and K⁺ additions; these minor pH differences would tend to underestimate the structural alterations rather than overestimate them.

E₁ and E₂ Conformers—In parallel experiments, in which a single sample of membrane patches was divided into two portions and either KCl or NaCl added to form a final salt concentration of 100 mM, significantly different CD spectra were obtained (Fig. 3). The samples contained the same protein and lipid concentrations and were the same ionic strength; the only difference between them was the cation type. These results suggest that the conformational changes reflected by the differences in spectral magnitudes and shapes are due specifically to the presence of the different cations. The magnitudes of the differences between the Na and K spectra are significantly larger than the standard deviations between either two different Na⁺ or two different K⁺ data sets (Table I) and suggest a relatively large structural change. In order to quantitate the type of secondary structure involved in such a change, the spectra were fit by a least squares procedure to a reference data set of secondary structural types derived from soluble globular proteins. Although there must be some concern for the applicability of a reference data set from soluble proteins for estimating the structure of a membrane protein, this procedure has been shown to give a good correspondence to known structures of two hydrophobic membrane-embedded proteins (Mao et al., 1982). This may be because the hydrophobic interiors of large globular proteins may present an environment similar to that of transmembrane segments of membrane proteins. Furthermore, another assay of the appropriateness of the reference data set can be ascertained from the unconstrained analysis. If large negative coefficients were obtained for the secondary structural types, this would indicate the structural characteristics of the Na/K-ATPase were not well represented in the standard set of globular proteins. However, only positive values were obtained (Table II). Finally, the extent of agreement between the calculated structure and the measured CD spectrum is reflected in the NRMSD parameter. For soluble proteins, an NRMSD of ≤ 0.1 usually indicates that the calculated structure and x-ray structure are in excellent agreement. If 0.1 ≤ NRMSD ≤ 0.2, the calculated structure is characterized as having secondary structure similar to the actual structure. If NRMSD is ≥ 0.2, the calculated structure generally does not resemble the actual structure (Brahms and Brahms, 1980).

Fig. 2. Electron micrograph of membrane patches containing Na/K-ATPase used in these studies showing size and homogeneity of population. Magnification × 64,000.

![Electron micrograph of membrane patches containing Na/K-ATPase used in these studies showing size and homogeneity of population. Magnification × 64,000.](image_url)

**Fig. 3.** CD spectra of highly purified Na/K-ATPase in intact membrane patches to which either 100 mM NaCl (---) or 100 mM KCl (— — —) have been added, indicating the significant conformational change the molecule undergoes between the E₁ and E₂ states. The spectra are uncorrected for differential absorption flattening effects but have been obtained under conditions for which light scattering effects are minimal.

**Table I**

| Wavelength (nm) | 194 | 209 | 214 | 218 | 222 |
|-----------------|-----|-----|-----|-----|-----|
| Na⁺ – K⁺        | 53.9| 3.4 | 11.2| 10.9| 12.0|
| Na⁺ – Na⁺       | 8.7 | 4.3 | 0.6 | 0.2 | 0.5 |

* Standard deviation between data sets.

³ Wallace, B. A., Kohl, N., and Teeter, M. M. (1984) Proc. Natl. Acad. Sci. U. S. A., in press.
NRMSD values in this study ranged from 0.04 to 0.08, indicating particularly good fits. Small NRMSD values such as these are necessary but not sufficient for concluding that the calculated structures correspond well with the actual structures. However, given all the criteria discussed above, it is likely that the secondary structures calculated in this study reflect the features present in the molecule in intact membranes.

Since the CD spectral characteristics are somewhat dependent on helix length (Chang et al., 1978), and this is unknown for Na/K-ATPase, this was input as a variable in the structural analysis. One expects a better fit (lower NRMSD) with a more appropriate average helix length. As is evident from Table III, slightly better fits are obtained for Na/K-ATPase as one increases the helix length to ~20 amino acids. At larger values, both the structure and fit are independent of helix length suggesting that the helices present must be reasonably long and consistent with the length of helix necessary to span a bilayer. Although it is possible that some helical segments of Na/K-ATPase may be extramembranous, it seems likely that a significant portion of the transmembrane spans of the protein may have helical structure (Wallace, 1982). A helical length of 26 residues would be the approximate length of helix necessary to span the membrane, and therefore was used to calculate the secondary structures. However, variation of average helix length from 10 to 30 residues had little effect on the results (Table III).

The net structure calculated for the Na⁺ (E₁) form of the Na/K-ATPase is a mixture of helices, sheets, and random coils (Table II). The K⁺ (E₂) form appears to have a lower α-helix content but a higher β-sheet content than the Na⁺ form, although there appears to be no significant difference in turn or random coil structures in the presence of the two cation types. The 7% differences measured are well above the ±1% error level of the calculated data. The most simple interpretation of this result is that the major structural transition between the E₁ and E₂ states is an α-helix to β-sheet transition. However, since CD measures the net secondary structure present, it is possible that changes also involving random coils and turns in various parts of the molecule could occur but that compensating changes in these secondary structures result in no net change detected for them. In any case, the ~7% change from α to β secondary structure is the minimum alteration that must occur. This would involve at least 50 amino acids, a relatively massive structural transition. That the transition is between α and β is somewhat unexpected, as it would require breakage and formation of a large number of intramolecular hydrogen bonds as well as a topological refolding of the molecule, even though the net numbers of hydrogen bonds in the two conformers may be similar. The location of the amino acids undergoing this transition is indeterminate. They could either be in the membrane-spanning channel region or they could be primarily in the globular aqueous-soluble head group region; but in either case they entail a substantial conformational change.

It is important to demonstrate the reversibility of this transition in order to assure that the presence of neither the 100 mM Na⁺ nor K⁺ causes an irreversible structural change in the molecule. To do so, we took advantage of the ionic strength independence of the conformation (see below) and the estimated relative binding constants (Kaniike et al., 1976; Matsui et al., 1977) of the enzyme for Na⁺ and K⁺. In these studies, two series of experiments were done: first, membrane patches in 10 mM NaCl were examined and the resulting CD spectrum was shown to be E₂-like. KCl was added to this sample to a final potassium concentration of 90 mM. The resulting spectrum was E₁-like, thus demonstrating that the transition from E₂ to E₁ can be attained. In a second experiment, membrane patches which were examined in 10 mM NaCl were examined and the resulting CD spectrum was shown to be E₁-like. KCl was added to this sample to a final potassium concentration of 90 mM. This then reversed to the original E₂-like spectrum (Fig. 4). This experiment showed transitions from E₁ to E₂ and back to E₂, thus demonstrating the freely reversible nature of the conformational transition.

Ionic Strength Dependence—It has been suggested that the structural transitions associated with the E₁ and E₂ states might also be correlated with ionic strength (Skou and Esman, 1981). To control for this in the previous experiments, a constant ionic strength was used in Na⁺ and K⁺ samples of the enzyme (except for the reversibility experiments). In order to examine the ionic strength dependence of the conformations (independent of cation type), two types of experiments were done. The first were experiments where neither sodium nor potassium ions were present. The low ionic strength specimens had only 7 mM Tris necessary to buffer the system; the high ionic strength samples were made 100 mM with choline chloride. In both cases, Na⁺-type (E₁) CD spectra were obtained (Fig. 5) and the calculated secondary structures (Table II) compared very well with the Na⁺-type.
data, suggesting the absence of an ionic strength dependency. Although it is possible that small conformational differences exist as a function of ionic strength, they would have to be less than, or comparable to, the 1–2% error level of these experiments. The second type of experiment was done to examine if there was a specific ionic strength effect associated with either Na⁺ or K⁺. In these experiments the spectra of membrane patches in 10 and 100 mM Na⁺ were compared, as were the spectra in 10 and 100 mM K⁺. The Na⁺ spectra were indistinguishable from each other, as were the K⁺ spectra, again supporting the ionic strength independence of the conformations.

Light Scattering and Absorption Flattening Effects—When the size of a particle is large compared to the incident wave-length of light being used, non-Rayleigh scattering by the particle must be considered. This scattering is manifest in unpolarized absorption spectra as increased apparent absorption since light scattered in directions other than 180° from the incident will not be collected by the detector. The extent of the wavelength dependency of this scattering can be determined from absorption data in a region of the spectrum where absorbance due to peptide backbone chromophores is negligible, i.e. 310–400 nm, according to the equation $A_0(\lambda) = k \lambda^n$ (Leach and Scheraga, 1960). By fitting a plot of log $A_0$ versus log $\lambda$, the wavelength dependency ($n$) can be determined. For the Na/K-ATPase sheets, $n$ was calculated to be 3.4 as compared to a theoretical value of 4.0 for small spherical objects. This value then can be used to extrapolate the value of the scattering contribution to the UV spectrum at all wavelengths, including those wavelengths where there is appreciable scattering as well as absorbance due to the peptide chromophore, and will be used later to calculate absorption flattening effects.

In circular dichroism measurements, differential light scattering effects will be detected for left- and right-handed polarized light. These effects can be demonstrated and eliminated experimentally by differential detection geometries which result in different acceptance angles. That is, for large particles, at small angles (i.e. 2°), much of the scattered light is lost and is detected as apparent ellipticity. As the angle is increased, more of the scattered light is collected and the detected differential scatter is decreased. Using an acceptance angle of ~90° (attained by placing the sample cell directly adjacent to the detector), essentially all forward scattered light will be collected and thus not contribute significantly to the apparent ellipticity. For objects which act as spherical scatterers, the scattered light will be almost entirely in the forward direction and thus collected by this geometry. Since these membrane particles appear to scatter similarly to spheres, it is reasonable to expect that using this detector geometry, the CD spectra will exhibit very little apparent light scattering effects.

Unlike small sonicated vesicles which experience little light scattering and for which CD measurements at all acceptance angles are very similar, (Mao and Wallace, 1984) for Na/K-ATPase patches, the spectra detected at 2° and 90° are somewhat different. However, these differences from the 90° spectrum drop off quite rapidly with increasing angle, suggesting that most of the scatter is in the forward direction.

Despite this instrumental correction for scattering effects, the CD spectra obtained will still be distorted by absorption flattening effects. These arise from the non-random distribution of chromophores in the solution. Although proteins are more or less randomly distributed in the membranes and the membranes are randomly distributed in solution, the proteins are sequestered in the membrane patches which comprise a relatively small portion of the total volume of the solution, resulting in a breakdown of Beer's law, which is manifest as absorption flattening, an apparent decrease in absorption. Absorption flattening is dependent on the absorbance of the sample, and this is maximal at wavelengths corresponding to absorption peaks. It is also dependent on membrane concentration and size (Mao and Wallace, 1984). All the experiments herein described were done using the same membrane and protein concentrations and patch sizes (since they were parallel experiments done with divided samples of the same preparation), so their unpolarized absorbance spectra were also equivalent. Hence, the absorption flattening coefficients for all samples will be the same and so should have little effect on the differences between spectra from the two conformations and on the difference in secondary struc-
tured. However, it will alter the absolute amounts of secondary structure detected and so the magnitude of the effect must be estimated. This can be done using a flattening coefficient calculated from the unpolarized UV data. The difference in absorbance between the protein in intact membrane patches and in a solubilized sample with random distribution of chromophores will be due to three factors: light scattering, absorbance flattening, and any conformational change in the protein caused by the solubilization procedure. A_{\text{memb}} = A_{\text{sol}} + A_f - A_t + A_d, where A_{\text{memb}} is the apparent absorbance of the membrane patches, A_{\text{sol}} is the absorbance of the solubilized membranes, A_f is the scattering contribution, A_t is the absorption flattening, and A_d is the distortion in the structure upon solubilization. Since it is possible to estimate A_t (see above) and the effects of conformational changes on the unpolarized UV spectrum are small (Rosenheck and Doty, 1961), the magnitude of A_f can be determined. Since A_t = Q_f A_{\text{nus}}, where Q_f is the flattening coefficient, Q_f (which is assumed to be the same for left and right polarized light) can then be used to correct the measured CD spectrum for absorption flattening, i.e. \theta_{\text{corr}} = \theta_{\text{meas}}/Q_f, where \theta_{\text{corr}} is the corrected ellipticity and \theta_{\text{meas}} is the ellipticity measured with the acceptance angle = 90°. These procedures have been used with bacteriorhodopsin in purple membranes, the only hydrophobic protein whose secondary structure is known to any precision, and have been shown to give reasonable estimates of their structures (Mao et al., 1982). While the corrected spectra (Fig. 6) differ somewhat from the uncorrected (Fig. 3), especially at wavelengths associated with high absorbance (i.e. 195 nm), very little change in the calculated secondary structures is seen (Table II). Furthermore, the differences between the Na+ and K+ samples are virtually the same as in the uncorrected spectra, so the conclusions concerning secondary structural changes are not dependent upon, nor caused by the correction procedure.

Since identical scattering coefficients were obtained for both Na+ and K+ treated samples, this suggests any differences detected are not a consequence of optical artifacts. Furthermore, examination of the two types of samples by electron microscopy indicates that no apparent particle rearrangement or membrane aggregation accompanies the conformational change detected, so the differences measured are not a consequence of alterations in physical state of the samples, but rather in the structure of the Na/K-ATPase protein molecules.

**DISCUSSION**

The results shown here suggest that highly purified Na/K-ATPase in intact membranes contains a mixture of helical, sheet, and random structures. In less pure preparations (as assayed both by sodium dodecyl sulfate gels and ATPase activity), a higher total helical content was detected although similar trends in changes of secondary structures with Na⁺ and K⁺ were seen. This result suggests that other proteins which are copurified with Na/K-ATPase through much of the preparation must have a higher helical content than the Na/K-ATPase.

To our knowledge, the only previously reported CD study on preparations containing Na/K-ATPase was for a crude brain microsomal fraction (Long et al., 1973). With a maximal reported specific activity of 16.9 μmol of Pi/mg of protein/h, the protein content of that preparation was less than 1% Na/K-ATPase enzyme, so the reported structure did not in any way reflect the actual structure of the Na/K-ATPase. The apparent helix content of that sample was much higher than that calculated for the highly purified sample used in this study. This result corresponds to our observation that less pure preparations had a higher helix content.

Although some investigators have predicted negligible structural differences between the E₂ and E₃ states (e.g. Tanford, 1982), the results of this study suggest at least 80 amino acid residues are involved in the transition, a very large structural change. However, the n-octylglucopyranoside-solubilization procedure, which results in significant loss of ATPase activity, produces a sample which does not undergo the cation-induced conformational change seen in membranes (Table II). It will be of interest in the future to examine if other detergent-solubilized samples which retain more ATPase activity, are capable of undergoing this transition, or if the ATPase- and ion-associated effects are separable.

Unlike some other membrane proteins, such as bacteriorhodopsin or porin, which exhibit a vast predominance of either α-helix or β-sheet secondary structures (Wallace, 1982), the Na/K-ATPase contains a roughly equal mixture of all types of secondary structures. This may be because the molecule is comprised of three distinct domains, the aqueous-soluble globular cytoplasmic and exoplasmic regions and a hydrophobic membrane-spanning region (Fachence et al., 1983), rather than only a single predominantly membrane-embedded domain, as in bacteriorhodopsin and porin. Although there is presently no evidence that the membrane-spanning portions of this protein are helices, the helix seems to be a dominant structural motif in many membrane-embedded regions of proteins (Wallace, 1982). The secondary structures measured, even for the K⁺ conformer, contain a sufficient amount of helix to account for at least eight helices of ~26 amino acids, the length of a helix which would be required to span a bilayer of ~40 Å.

In the future, it may be possible to proteolytically dissect the molecule to determine the nature of the different domains by CD spectroscopy, as has been done for bacteriorhodopsin. 

---

Fig. 6. CD spectra of Na/K-ATPase membrane patches with 100 mM NaCl (---) or KCl (----) added, respectively, as in Fig. 3, which have been corrected for absorption flattening effects.

---

4 B. A. Wallace and N. Kohl, manuscript submitted for publication.
This would provide an experimental basis for model-building of the protein structure and more precisely localize the conformational change. Furthermore, the large differences which were detected and quantitated in this study would suggest that studies which examine the effects of phosphorylation and inhibitor binding, may provide further understanding of the intermediate states of the molecule and provide insight into its mechanism of action.

In summary, this study using highly purified and active enzyme in intact membrane patches, has demonstrated that distinctly different secondary structures are found for the E, and E2 conformers associated with 100 mM Na+ and K+, respectively, although no such changes were detected with ionic strength variation.

Acknowledgments—We thank I. S. Edelman for helpful discussions, D. Mao and C. Teeters for computer programs utilized in data processing and calculation of secondary structures, and J. Lustbader for the amino acid analysis.

REFERENCES
Brahms, S., and Brahms, J. (1980) J. Mol. Biol. 138, 149–178
Castro, J., and Farley, R. A. (1979) J. Biol. Chem. 254, 2221–2228
Chang, C. T., Wu, C.-S. C., and Yang, J. T. (1978) Anal. Biochem. 82, 4790–4792
Farley, R. A., Goldman, D. W., and Bayley, H. (1980) J. Biol. Chem. 255, 860–864
Giotta, G. J. (1975) J. Biol. Chem. 250, 5159–5164
Hayashi, Y., and Post, R.L. (1981) Fed. Proc. 40, 1330
Jergensen, P. L. (1974a) Biochim. Biophys. Acta 356, 36–52
Jergensen, P. L. (1974b) Methods Enzymol. 32, 277–290
Jergensen, P. L. (1975) Biochem Biophys. Acta 401, 399–415
Kaniike, K., Lindenmayer, G. E., Wallick, E. T., Lane, K. K., and Schwartz, A. (1976) J. Biol. Chem. 251, 4794–4795
Karlish, S. J. D., and Yates, D. W. (1978) Biochim. Biophys. Acta 527, 115–130
Karlish, S. J. D., Yates, D. W., and Glynn, I. M. (1978) Biochim. Biophys. Acta 525, 230–251
Karlish, S. J. D. (1980) J. Biol. Chem. 255, 111–136
Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
Leach, S., and Scheraga, H. A. (1960) J. Am. Chem. Soc. 82, 4790–4792
Long, M. M., Masotti, L., Sachs, G., and Urry, D. W. (1973) J. Supramol. Struct. 1, 259–268
Lowey, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
Magar, M. E. (1968) Biochemistry 7, 617–620
Mao, D. (1984) Ph.D. dissertation, Columbia University
Mao, D., Wachter, E., and Wallace, B. A. (1982) Biochemistry 21, 4960–4968
Mao, D., and Wallace, B. A. (1984) Biochemistry, in press
Matsui, H., Hayashi, Y., Homaredo, H., and Kimimura, M. (1977) Biochim. Biophys. Res. Commun. 75, 373–380
Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2357–2366
Puchence, J. M., Schoenborn, B. P., and Edelman, I. S. (1983) Biophys. J. 41, 370a
Peterson, G. L., Churchill, L., Fisher, J. A., and Hokin, L. E. (1982) Ann. N. Y. Acad. Sci. 402, 185–205
Rosenheck, K., and Doty, P. (1961) Proc. Natl. Acad. Sci. U. S. A. 47, 1775–1779
Schneider, A. S., and Harmatz, D. (1976) Biochemistry 15, 4158–4162
Skou, J. C., and Esman, M. (1980) Biochim. Biophys. Acta 601, 386–402
Skou, J. C., and Esman, M. (1981) Biochim. Biophys. Acta 647, 232–240
Skou, J. C. (1982) Ann. N. Y. Acad. Sci. 402, 169–184
Tanford, C. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2882–2884
Wallace, B. A. (1982) Methods Enzymol. 88, 447–462
Secondary structural composition of the Na/K-ATPase E1 and E2 conformers.

T J Gresalfi and B A Wallace

J. Biol. Chem. 1984, 259:2622-2628.

Access the most updated version of this article at http://www.jbc.org/content/259/4/2622

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/4/2622.full.html#ref-list-1