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Circular dichroism of the two major conformational states of mammalian ($Na^+ + K^+$)-ATPase

David F. Hastings, Jacqueline A. Reynolds and Charles Tanford

Department of Physiology, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

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No alteration in the circular dichroic spectrum of fully active, membrane-bound ($Na^+ + K^+$) – ATPase is observed when the protein is cycled between the two major conformational states, E_1 and E_2. This finding is in agreement with the infrared study by Chetverin and Brazhnikov (J. Biol. Chem. 260 (1985) 7817) and demonstrates that any difference in secondary structure between the two conformers must be less than 2%.

Introduction

Transmembrane proteins engaged in active transport must have a minimum of two conformational states [1] in which the uptake and discharge sites for the transported ions have access to opposing surfaces of the membrane. Two such states of the ($Na^+ + K^+$)-ATPase have been identified directly by a variety of techniques and are usually referred to as E_1 and E_2, the former being the uptake conformer for intracellular Na^+ and the latter the uptake conformer for extracellular K^+. Jørgensen and co-workers [2] observed differences in the pattern of trypsin cleavage of the α chain that are dependent upon the monovalent cation present, and Karlish and co-workers [3] demonstrated a change in intrinsic fluorescence as the distributions between E_1 and E_2 states is altered.

The question arises as to what extent the two conformers differ at the secondary structural level. Recently, Gresalfi and Wallace [4] have reported alterations in the circular dichroic spectra of the ($Na^+ + K^+$)-ATPase that are dependent on the specific monovalent cation present and thus are presumed to reflect changes in secondary structure between E_1 and E_2. The authors interpret their data as consistent with a 7% decrease in α helix and a 10% increase in β sheet in the E_2 conformation as compared to E_1. Chetverin and Brazhnikov [5,6], however, observed essentially no difference in the magnitude of infrared absorbance in the amide region of the spectra when E_1 and E_2 were compared. They found no changes in the peaks associated with antiparallel β structure (1631, 1688 cm^{-1}) and less than 2% decrease in the 1654 cm^{-1} band (α helix) when E_1 was compared to E_2. These authors consider the latter change marginally significant and suggest it may reflect a minor distortion of the α helices in the E_1 form.

Nakamoto and Inesi [7] have reported no change in the circular dichroic spectrum of the Ca^{2+}-ATPase from sarcoplasmic reticulum when this transport protein is alternated between the two major conformational states by addition and removal of the transported cation. This protein appears to be closely related functionally to the ($Na^+ + K^+$)-ATPase, and, in fact, Andersen et al.

Correspondence address: Dr. J.A. Reynolds, Department of Physiology, Duke University Medical Center, Durham, NC 27710, USA.

Abbreviations: ($Na^+ + K^+$)-ATPase, Na^+ and K^+ transporting adenosinetriphosphatase (EC 3.6.1.3); Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid.

[8] have demonstrated analogous differences in tryptic cleavage products between the Ca^{2+} uptake and discharge states.

We have examined the far-ultraviolet circular dichroic specta of the E_1 and E_2 conformers of the $(Na^+ + K^+)$-ATPase under carefully controlled conditions in which protein concentration and light scattering intensity do not differ. We observe no significant differences, in accord with the findings of Chetverin and Braznikov [5,6].

Materials and Methods

Membrane-bound $(Na^+ + K^+)$-ATPase was purified from the outer medulla of fresh porcine kidney [9,10]. Final sodium dodecyl sulfate concentration for differential detergent extraction was calculated using the relationship of Jørgensen [11]. Isopycnic centrifugations on 24–32% continuous sucrose gradients were performed using an SW-27 rotor, 27000 rpm, $\omega^2 t = 2 \cdot 10^{11}$, 4°C. Sucrose density gradient solutions were prepared by the addition of sucrose to storage buffer: 250 mM sucrose/0.1 mM H_4EDTA/12 mM K^+/25 mM Tes (pH 7.4), 23°C. K^+ was included in the gradient buffer to lower the ATP affinity and assist in the removal of the bound nucleotide. The high specific activity fractions were pooled and washed free of K^+ by three successive centrifugations (50.2 Ti rotor, $150000 \times g$, 30 min, 4°C) and resuspended in Na^+- and K^+-free buffer as indicated in the text and figure legends. Final alkali cation concentrations assayed by atomic absorption were less than 10 μM. Specific activities ranged from 30 to 38 IU at 37°C.

Protein concentrations were determined by the method of Lowry et al. as modified by Bensdoun and Weinstein [12]. ATPase activities were measured by the method of Ottolenghi [13] except that bovine serum albumin was omitted from the assay media.

Na^+ and K^+ concentrations were determined by carbon rod atomizer atomic absorption using a Varian Techtron Model 61. Fluorescence measurements were carried out on a Perkin-Elmer MPF-44A. Excitation and emission wavelengths were 295 and 325 nm, respectively, and 10 nm slit widths were used.

Circular dichroic spectra were obtained using a Jobin-Yvon Dichrograph V calibrated with isoandrosterone. The spectral bandpass was 1 nm, the cell path-length 0.1 cm, and the photomultiplier acceptance angle approx. 26°. Data were collected at 0.2 nm intervals with an instrument time constant of 2 s. The cuvette was maintained at 20.6°C. No systematic differences were observed in replicate spectra. Spectra have not been smoothed in order to assure an unbiased comparison.

Turbidity was measured on a Cary 17D spectrophotometer at 350 nm in a 0.1 cm path-length cell.

Specially pure sodium dodecyl sulfate was obtained from BDH Chemicals, Poole. Vanadium free ATP was purchased from P-L Biochemicals, Milwaukee, WI. Imidazole free of far-ultraviolet-absorbing material was obtained from Boehringer-Mannheim. All other chemicals were standard reagent grade.

Cyclical experimental protocols were followed which placed the enzyme successively in the states $(E_1 \rightarrow E_2 \rightarrow E_1)$ or $(E_2 \rightarrow E_1 \rightarrow E_2)$. Transitions were induced by the sequential addition of aliquots of concentrated Na^+ or K^+ stock solutions, and protein concentrations were determined for initial and/or final sample conditions. The protein concentration of the intermediate state was determined from the dilution factor. The maximum change in protein concentration for any given cyclical protocol was 2%.

Results

Karlish and Yates [3] initially described the intrinsic fluorescence changes associated with the E_1 to E_2 transition using protein in 100 mM Tris-HCl, buffer which places the enzyme predominantly in the E_1 conformation [14]. The transition to E_2 was then induced by addition of KCl at increasing concentrations. The transition was complete at 0.5 mM KCl. We have duplicated these experimental conditions and examined both the circular dichroic spectra and the fluorescence emission intensity. We have also measured the turbidity of the sample at 350 nm using the same 0.1 cm path-length cuvette used for circular dichroic measurements. In 100 mM Tris-HCl the turbidity was 8.36 AU $mg \cdot protein^{-1} \cdot cm^2$ and

was unaltered within the 1.5% detection limit by the addition of up to 10 mM KCl or 49 mM NaCl.

Fig. 1 shows the circular dichroic spectra of the $(Na^+ + K^+)$-ATPase in 100 mM Tris-HCl in the E_1 and E_2 conformations. As detailed in the figure legend, the protein was first examined in the absence of either Na^+ or K^+ but in the presence of Tris-HCl, which induces the E_1 state. The solution was then brought to 1 mM KCl (E_2 state) and returned to E_1 with 49 mM NaCl. The spectra in Tris buffer and in 49 mM NaCl were identical and these data are plotted in Fig. 1. The spectrum of E_2 is also given in the figure and is superimposable on that of E_1 as is evident from the difference spectrum (D) which is zero over the wavelength range investigated. Gresalfi and Wallace observed a 10% difference in $[\theta]$ at 222 nm when comparing E_1 and E_2. The coefficient of variance in the signal at 222 nm in Fig. 1 is 3.2% and the minimal detectable difference in this experiment is 2.6%.

The intrinsic fluorescence measured under conditions identical to those in Fig. 1 showed a 2% increase in emission intensity at 325 nm when E_2 was compared to E_1. The protein in 100 mM Tris-HCl alone was deduced to be 85% E_1 by comparison of the fluorescence emission intensity with that in the presence of either Na^+ or K^+.

The turbidity of the membrane sample was lowered by 40% at 350 nm when the the buffer concentration was reduced to 4.2 mM as compared to 100 mM. However, under these conditions marked and variable increases in turbidity ranging from 17 to 33% at 350 nm occurred on the addition of either Na^+ or K^+ at sufficient concentrations to induce the conformational transitions between E_1 and E_2. We observed that addition of 0.29 mM sodium dodecyl sulfate to this buffer system reduced the magnitude of the turbidity changes to 5 to 7% on addition of Na^+ or K^+. The specific activity of the enzyme was unaffected by this concentration of sodium dodecyl sulfate (32 IU at 37°C), but the difference in fluorescence emission intensity at 325 nm between E_1 and E_2 was reduced from 2.0 to 0.7%.

Fig. 2 shows the circular dichroic spectra for the $(Na^+ + K^+)$-ATPase in the E_1 and E_2 con-

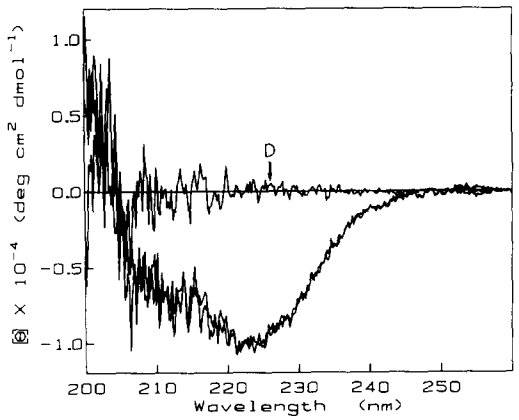

Fig. 1. Circular dichroic spectra of the E_1 and E_2 conformational states of the $(Na^+ + K^+)$-ATPase in high-ionic-strength buffer (100 mM Tris-HCl (pH 7.1)/0.1 mM H_4EDTA). 10 μl of the protein in 2 M glycerol/0.1 mM EDTA/10 mM imidazole/10 mM Tes (pH 7.4) were added to 240 μl of buffer solution to give a final protein concentration of 0.039 mg·ml^{-1}. 1 μl of 250 mM KCl was added to bring the solution concentration to 1 mM KCl (E_2 conformer). 5 μl of 2.5 M NaCl were then added to yield a final concentration of 49 mM NaCl (E_1 conformer). Data are collected and plotted at 0.2 nm intervals. E_1 and E_2 replicate spectra showed no systematic differences. Averages of these replicates are plotted here and are not labeled separately because they are indistinguishable. D is the difference curve between E_1 and E_2. To assure unbiased comparisons the curves have not been smoothed.

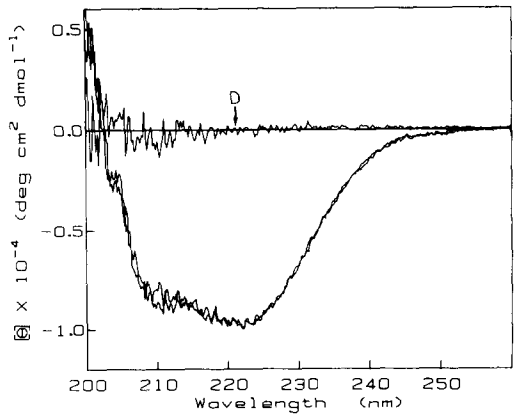

Fig. 2. Circular dichroic spectra of the E_1 and E_2 conformational states of the $(Na^+ + K)$-ATPase in low-ionic-strength Tes buffer (4.2 mM Tes (pH 7.4)/0.16 mM H_4EDTA/1.63 mM K^+/0.29 mM sodium dodecyl sulfate). Protein concentration was 0.137 mg·ml^{-1} and the enzyme was initially in the E_2 state. 10 μl of 2.5 M NaCl were added to raise the Na^+ concentration to 49 mM (E_1 conformer). Data are collected and plotted at 0.2 nm intervals. The curves are not labeled separately because they are indistinguishable. D is the difference curve between E_2 and E_1. The curves have not been smoothed.

formations in the low ionic strength buffer containing 0.29 mM sodium dodecyl sulfate. The E_2 state was induced by bringing the KCl concentration to 1.63 mM and the E_1 state by subsequent adjustment to 49 mM NaCl as described in the figure legend. No difference in the spectra of the two conformations is observed. The coefficient of variance in the value of $[\theta]$ at 222 nm is 1.5% and the detection limit for a significant difference is 1.5%.

The results presented in figs. 1 and 2 are at variance with the report from Gresalfi and Wallace [4], who used a modified Cary 60 spectropolarimeter equipped with a Model 6001 CD attachment. Their data are collected by means of an unspecified computer interface at 1 nm intervals and are presented as smoothed curves that give no indication of the signal-to-noise ratio. Thus, it is not possible to evaluate the significance of their reported differences in ellipticities because the smoothing operation destroys signal variance information. Additionally, it is not clear whether the calculated spectra are normalized to a measured protein concentration or to the magnitude of the signal at 208 nm since both E_1 and E_2 forms of the enzyme are reported to have identical ellipticities at this wavelength but to differ in the 222 nm region. Gresalfi and Wallace state that differences in absorption flattening should not be present in their experiments, since the same preparation was used to examine both the E_1 and E_2 states. However, we have reported here a marked and variable increase in turbidity on the addition of either Na^+ or K^+ to membrane fragments suspended in low-ionic-strength media – conditions used in Ref. 4. Changes in turbidity reflect alterations in the state of the membrane suspension that are in turn reflected in absorption flattening.

Discussion

We have demonstrated that any alteration in the circular dichroism of porcine medulla ($Na^+ + K^+$)-ATPase as it cycles between the E_1 and E_2 state is less than 2%. The experiments were carried out under conditions where light scattering from the sample was constant and the protein concentration (measured directly) changed a maximum of 2% as the result of dilution. These data are in agreement with the recently published work of Chetverin and Brazhnikov [6], who observed no significant change in α helix or β sheet for this protein by the criterion of amide infrared absorption intensities.

We conclude that there are minimal alterations in secondary structure associated with the alternating access states of this ion transport protein, a result that is consistent with the active ion transport model proposed by Tanford [15] in which this conformational change is not obliged to be major. The same result has been obtained by Nakamoto and Inesi [7] with respect to the closely related Ca^{2+}-ATPase from sarcoplasmic reticulum.

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