Detection of a Ouabain-Induced Structural Change in the Sodium, Potassium-Adenosine Triphosphatase†

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ABSTRACT: The structural consequences of ouabain interaction with a highly purified Na^+,K^+-ATPase preparation, isolated from the outer medulla of porcine kidneys, were examined. The apparent heat capacity vs. temperature profile of the enzyme was obtained with a newly designed differential scanning calorimeter. The profile was characterized by a major endothermic transition at 55.3 °C. This transition appeared to correspond to irreversible protein denaturation since it was associated with loss of enzyme activity and the transition was not present in calorimetric profiles obtained after the initial scan of a sample. Interaction of ouabain with its receptor surface on the Na^+,K^+-ATPase shifted the endothermic transition from 55.3 to 59.5 °C and decreased the width of the transition. This indicated that the ouabain-Na^+,K^+-ATPase complex was more stable with respect to temperature and that the apparent cooperative nature of the transition was greater for the complex than for the untreated enzyme. The effects of the ouabain–enzyme interaction were examined with the fluorescence probe, 8-anilino-1-naphthalenesulfonic acid. The fluorescence of this dye in the presence of the enzyme was monitored as a function of temperature. These measurements also suggested that ouabain induces the formation of a more stable enzyme conformation. Incubation of the enzyme for 10 min at 53 °C with and without ouabain and measurement of remaining enzyme activity after the dissociation of bound ouabain confirmed the conclusions from the fluorescence and scanning calorimeter experiments.

Cardiac glycosides such as ouabain bind to and inhibit the sodium–potassium pump and its in vitro manifestation, the Na^+,K^+-ATPase (Schwartz et al., 1975). More recent studies have also suggested that this interaction alters calcium binding to phospholipids in the membrane bilayer adjacent to the Na^+,K^+-ATPase (Gervais et al., 1975, 1977). The latter consequence offers a possible explanation for the therapeutic and/or toxic effects of the digitalis-like glycosides (other possibilities also exist, e.g., see Langer, 1972), but such a mechanism requires that glycoside interaction with its receptor induce a significant perturbation in the Na^+,K^+-ATPase which is subsequently transmitted into the adjacent bilayer.

Structural changes in the Na^+,K^+-ATPase, subsequent to glycoside interaction, were predicted on the basis of kinetic and binding studies (Hoffman, 1966; Schwartz et al., 1968; Albers et al., 1968). Attempts to detect structural alterations in Na^+,K^+-ATPase preparations by spectroscopic techniques, however, have yielded varying results (Lindenmayer and Schwartz, 1970; Mayer and Avi-Dor, 1970; Nagai et al., 1970, 1972; Yoda and Hokin, 1972). Hart and Titus (1973a,b) did show that ouabain interaction with a partially purified preparation altered the reactivity of sulfhydryl groups in a 98 000-dalton polypeptide which is thought to be a major component of the Na^+,K^+-ATPase (see reviews: Dahl and Hokin, 1974; Schwartz et al., 1975). Also, Kuriki et al. (1976) more recently provided batch calorimetric data that support the proposition that the interaction of magnesium and inorganic phosphate with the Na^+,K^+-ATPase induced a conformational change in the Na^+,K^+-ATPase.

The present study was designed to test whether physically detectable macromolecular perturbations do or do not arise secondary to ouabain interaction with a highly purified Na^+,K^+-ATPase preparation (Lane et al., 1973). Differential scanning calorimetric profiles suggested the former in that ouabain appears to induce a conformation that is more stable than control to heat denaturation. This result was confirmed indirectly with fluorescence vs. temperature scans using the probe, 8-anilino-1-naphthalenesulfonic acid (ANS),[1] and directly by showing that ouabain interaction protects the preparation against the loss of Na^+,K^+-ATPase activity at 53 °C.

Experimental Procedure

Preparation of the Na^+,K^+-ATPase. The Na^+,K^+-ATPase was prepared from the outer medulla of porcine kidneys by the procedure of Lane et al. (1973) with slight modifications. The preparation obtained after the glycerol precipitation step in that procedure was used for the experiments described herein. Na^+,K^+-ATPase activities were obtained by the colorimetric determination of inorganic phosphate produced (Martin and Doty, 1949) during a 3-min assay at 37 °C in the presence of 1 mM Tris-EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 25 mM imidazole, pH 7.2, 5 mM Na_2ATP, and 5 μg of protein. Protein was determined by the method of Lowry et al. (1951). The calorimetric experiments were performed with two different enzyme preparations having specific activities of 900 and 500 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ h^{-1} and

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[1] Abbreviations used: ANS, 8-anilino-1-naphthalenesulfonic acid; T_m, transition midpoint.

protein concentrations of 2.3 and 5.2 mg/ml, respectively. For the other experiments, the specific enzyme activity of the preparation employed was 650 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ h^{-1}. The phospholipid concentration in the preparations was 1 μmol/mg of protein as determined by the method of Bartlett (1959).

For the calorimetric experiments the Na^+,K^+-ATPase preparation was suspended in a buffer consisting of 3.9 mM $MgCl_2$, 10 mM imidazole, 0.77 mM Tris, and 0.77 mM EDTA, pH 7.4. For the calorimeter scans the enzyme was divided into two aliquots and, to one, 0.01 M ouabain in water was added (final concentration, 7.7×10^{-4} M) and, to the other, an identical volume of water was added. The samples were incubated at room temperature for 30 min to allow ouabain to interact with the Na^+,K^+-ATPase (Inagaki et al., 1974) and were subsequently chilled and maintained at 3 °C until placed in the calorimeter.

Calorimetry. A newly designed heat-conduction-type differential scanning calorimeter (Suurkuusk et al., 1976) was used to measure the apparent heat capacities of the enzyme preparations as a function of temperature. The accuracy in terms of baseline reproducibility is better than ±100 μcal/deg and the sensitivity is ±25 μcal/deg. Absolute sample temperature determination in these experiments is better than ±0.01 °C. The calorimeter consists of two rectangular aluminum blocks each with a hole for a sample or reference ampule having a volume of 1.0 ml. Each aluminum block is sandwiched between a pair of thermoelectric modules (Cambion, Inc.) in thermal contact with the copper heat sink. The heat sink is positioned inside a steel cylindrical adiabatic shield which is insulated from the environment by a 2-in. layer of polyurethane foam surrounded by a steel outer cylinder. The measured voltage due to the differential heat flow is amplified, digitalized, and recorded on punched tape along with the time and heat sink temperature every 13.1 s. The slight mismatch of the thermoelectric modules, the temperature dependence of the calorimetric constants, the instrument response time, and the slight variability in the scan rate make extensive computations necessary to obtain heat capacity as a function of temperature.

In these experiments, the reference ampule was loaded with 0.7 g of water, and the sample ampule was loaded and scanned with water, buffer, enzyme, enzyme + ouabain, and buffer + ouabain. The samples were all loaded at 30 °C and cooled to about 4 °C, and then the calorimeter was heated at the rate of 15 °C/h to 72 °C. Each enzyme sample was then cooled to about 4 °C and rescanned without removing the sample from the calorimeter.

To assess the effect of the calorimetric scan on activity of the enzyme, ATPase activity vs. temperature was examined in a separate experiment by heating the enzyme preparation (3 mg/ml) at 15 °C per h. At various times aliquots were removed for the determination of Na^+,K^+-ATPase activity. The samples were cooled to ice-bath temperature until assayed.

Fluorometry. An Aminco-Bowman spectrophotofluorometer equipped with a jacketed cuvette housing and magnetic arc stabilizer was used for the fluorescence experiments reported. The probe employed for these studies was 8-anilino-1-naphthalenesulfonic acid (ANS). The ANS (Sigma Chemical Co.) was recrystallized prior to use and a 5.1 mM methanol solution was used. Excitation and emission wavelengths were 390 and 495 nm, respectively. The control and ouabain-pretreated preparations, prepared in the manner described for the calorimeter experiments, were cooled in separate experiments to 8 °C and 25 μl of ANS was added. After a 30-min incubation at 8 °C, the temperature of the solution was scanned from 8 to 70 °C, and the relative fluorescence was recorded continuously with a strip chart recorder. The temperature of the solutions in the cuvette was increased at a rate of 1.16 °C per min with the use of a Neslab temperature programmer connected to a Lauda K2/R refrigerated circulating bath. In the fluorescence data reported here, a correction was made for the large heat-induced increase in fluorescence quenching. No corrections were made for inner filter effects.

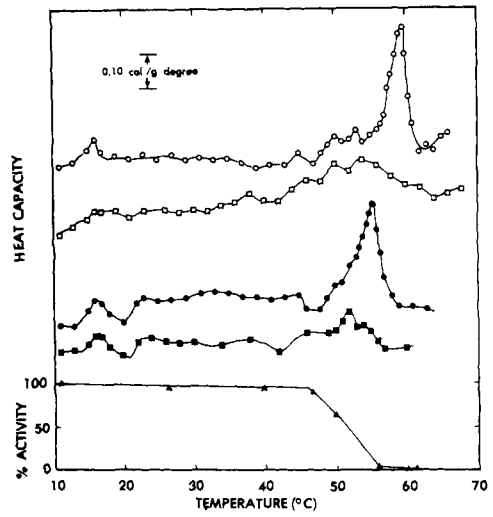

FIGURE 1: Apparent heat capacity and activity of the Na^+,K^+-ATPase (specific activity 500) vs. temperature. Heat capacities of the control (●) and ouabain-pretreated (○) Na^+,K^+-ATPase membrane preparations were measured with a differential scanning calorimeter. After the initial scan, the samples were chilled to about 4 °C and rescanned (■, □). In a separate experiment, the Na^+,K^+-ATPase preparation was heated at a similar rate (15 °C per h) over the same temperature range as used for the calorimetric scans, aliquots were taken at the points indicated (▲), and Na^+,K^+-ATPase activities were determined as described in Experimental Procedure.

Ouabain Protection at 53 °C. The enzyme (1 mg/ml in 0.02 M imidazole, 0.004 M $MgCl_2$, 0.001 M Tris-EDTA (pH 7.15)) was incubated for 30 min at 37 °C in the presence or absence of 5.0×10^{-6} M ouabain (Inagaki et al., 1974). The two samples were then incubated at 53 °C for 10 min, centrifuged at 180 000*g* for 40 min, resuspended at 0.67 mg/ml in 0.001 M Tris-EDTA (pH 7.4), and subsequently reincubated at 37 °C to allow the bound ouabain to dissociate. At intervals during the latter incubation, aliquots were removed, and the ATPase activity was measured.

Results and Discussion

The calorimetric scans of the Na^+,K^+-ATPase (5.2 mg/ml) were characterized by two transitions (Figure 1). A small transition was apparent in the range of 14–18 °C and may reflect a phase transition in the lipid bilayer of the preparation (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1974). A large endothermic transition was also present and was characterized by a peak at 55.3 °C. An enthalpy change of 1.3 cal/g was estimated by integrating the apparent heat capacity from 48 to 60 °C using the average value of the heat capacity at the two temperature extremes as the baseline. The transition as detected by calorimetry was correlated with the loss of Na^+,K^+-ATPase activity. Pretreatment of the Na^+,K^+-ATPase preparation with ouabain caused a shift in the peak of this transition to 59.5 °C and the enthalpy change for the transition in the latter case was estimated to be 1.1 cal/g by

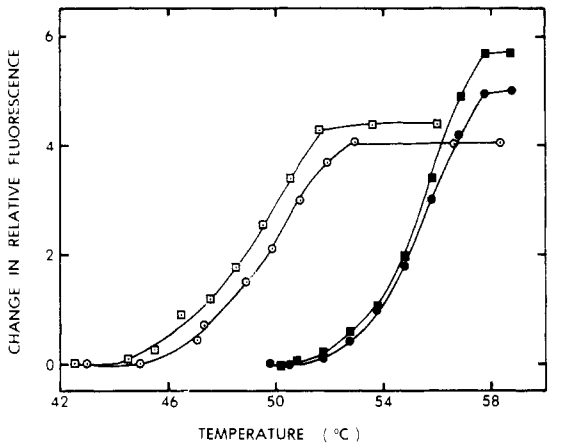

FIGURE 2: Effect of ouabain pretreatment on the change in relative fluorescence of ANS vs. temperature. The cuvette contained 0.37 mg/ml of the Na^+,K^+-ATPase preparation in 0.02 M imidazole, 0.004 M $MgCl_2$, 0.001 M Tris-EDTA (pH 7.15) and, where indicated, 5×10^{-4} M ouabain. The final ANS concentration was 104 μM. Following a 30-min preincubation to allow ouabain binding, the samples were heated at a rate of 1.16 °C per min with the simultaneous recording of relative fluorescence. (□ and ○) Control preparation with no ouabain; (■ and ●) ouabain-pretreated preparation. (See Experimental Procedure for details.)

TABLE I: Summary of Calorimetry and Fluorescence Scans.

Method	Enzyme	T_m (°C)	Transition Width[a] (°C)
Differential scanning calorimetry	Control	55.3	3.8
	Ouabain	59.5	2.5
ANS fluorescence	Control	49.4	2.9
	Ouabain	55.4	1.9

[a] Transition width for differential scanning calorimetry experiments is the temperature interval at half-peak height. Transition width for fluorescence experiment is the interval for ¼-¾ completion of reaction.

integrating the heat capacity from 55 to 62 °C. It should be noted that the observed values for the two transition enthalpies are not considered to be significantly different because of problems in selecting a baseline.

The absolute value of the excess heat capacity was noted by Jackson et al. (1973) to be very sensitive to small uncertainties in the amount of material in the calorimeter. For this reason no significance can be attributed to the absolute values of the heat capacity in the two scans and only relative apparent heat capacity differences are discussed. The width of the structural transition was markedly changed by the binding of ouabain. In the presence of the cardiac glycoside, the width of the large transition occurred over a 2.5 °C interval whereas, in the absence of drug, the transition width was 3.8 °C (see Table I).

The reversibility of these transitions was examined by rescanning the samples without removing them from the ampule. The high-temperature transitions for the enzyme with and without ouabain appeared to be largely irreversible. This and the loss of enzyme activity with the initial scan are consistent with the conclusion that the large endothermic transition reflects irreversible denaturation of the Na^+,K^+-ATPase.

When the buffer containing 7.7×10^{-4} M ouabain but no protein was scanned over this temperature range, no transitions were seen. The lipids in the ATPase preparation were extracted with chloroform–methanol, resuspended in an aqueous buffer after removal of the organic solvents, and scanned in the presence and absence of ouabain. No significant differences were observed between these two scans (data not shown). The results of scans of the enzyme with specific activity of 900 were similar to the scans in Figure 1 (specific activity 500).

The calorimetric results demonstrate that the binding of ouabain results in a more stable conformer of the Na^+,K^+-ATPase membrane system. It is possible that the increase in transition temperature results from a stabilization of the native form of the enzyme. However, the decreased transition width in the presence of the drug suggests that a significant drug-induced change in structure occurs. Since the protein transitions are largely irreversible, this system is not in equilibrium through this temperature range. Therefore, the change in transition width may also reflect differences in the kinetics of the unfolding reaction. The irreversibility of the protein transitions means that the calorimeter results are not measures of true excess heat capacity, a thermodynamic property.

The fluorescent probe ANS was chosen to confirm the calorimeter results since this dye is presumed to be able to interact with both proteins and lipids. The dye has a low quantum yield in water and a much larger quantum yield in nonpolar environments (Turner and Brand, 1968). The fluorescence of this dye in the presence of the ATPase with and without ouabain was monitored as the temperature was scanned from 8 to 70 °C. A monotonic decrease in fluorescence occurred as the temperature was increased up to about 46 °C for the control and to 53 °C for the ouabain-treated enzyme. In Figure 2, portions of four representative scans are shown, two with ouabain and two without. The observed fluorescence of ANS undergoes an increase with a transition midpoint (T_m) of 49.4 °C in the control enzyme and a T_m of 55.4 °C in the ouabain-treated enzyme. The change in ANS fluorescence indicates a change in the structure of the ATPase-lipid system, although it is not known what this change is in macromolecular terms. An increase in fluorescence might result from a change in the microenvironment of the bound probe or from a change in the amount of dye bound. Furthermore, it should be noted that the change in fluorescence could reflect alterations in both dye–lipid and dye–protein interactions.

These fluorescence data support the conclusion made from the calorimetry results that ouabain stabilizes the enzyme structure. As shown in Table I, the ouabain–enzyme was observed by both techniques to be more stable to thermal denaturation by about 5 °C. The T_m's for the thermally induced changes in ANS fluorescence occur at a lower temperature than the T_m's from the calorimetric scans. Presumably the calorimeter monitors the heat absorption changes for the overall denaturation process, whereas the fluorescent probe measures a more thermally labile structural rearrangement. Since the faster scan rates used in the fluorescence experiments would tend to shift the transition to a higher temperature, the differences in the calorimetric and fluorescence detected T_m's might in fact be greater than 4–6 °C if both experiments were performed at 15 °C per h. Even if the T_m's obtained using the two different techniques were to coincide, this would not constitute proof that the same event was being monitored. The fluorescence scans also indicate that the ouabain–enzyme is a more cooperative structure since the transition occurs over a smaller temperature interval (Table I).

The above two physical techniques indicate that ouabain stabilizes the enzyme to thermal denaturation. To confirm

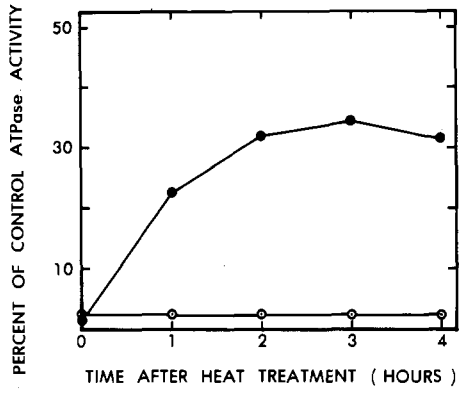

FIGURE 3: Protection of Na^+,K^+-ATPase activity by pretreatment with ouabain. The preparations (control and ouabain-pretreated) were heated for 10 min at 53 °C. The preparations were subsequently centrifuged, resuspended in fresh buffer (without drug), and incubated at 37 °C. At the times indicated, aliquots were removed and Na^+,K^+-ATPase activity of the preparations was determined as described in Experimental Procedures. (⊙) Control preparation; and (●) ouabain-treated preparation.

these findings, the ability of ouabain to protect the ATPase activity of the enzyme was investigated (Figure 3). The control and ouabain-pretreated preparations were incubated at 53 °C for 10 min. Subsequently, the two preparations were washed (to remove free ouabain from the pretreated preparation) and incubated at 37 °C (to allow bound drug to dissociate from the pretreated preparation; Allen et al., 1971; Akera and Brody, 1971; Lane et al., 1973). Under such conditions, the preparation pretreated with drug regains ATPase activity whereas the nontreated preparation remained essentially inactive. About 35% of the original enzyme activity was regained in the former after 3 h of incubation at 37 °C in fresh buffer. The protection of ATPase activity by ouabain was actually greater than 35% since, after 3 h of incubation, a second wash followed by 2 h of incubation resulted in another 10% increase in activity.

The Na^+,K^+-ATPase is an integral membrane protein that appears to span the bilayer of the plasma membrane (Schwartz et al., 1975). Cardiac glycosides bind to a site on the external surface of the membrane and inhibit the hydrolysis of ATP which occurs on the internal surface. Because this requires the transfer of information from the drug-binding site to the catalytic site, it has generally been assumed that the drug induces a fairly large conformational change in the enzyme rather than a simple perturbation localized in the binding site region. The present study confirms this possibility to the extent that thermodynamic stabilization was detected by the physical and spectroscopic techniques employed.

The enthalpy change for the major transition of the calorimetric profile (~1.2 cal/g) is large when calculated on a mole basis (Brandts, 1969). If the molecular weight of the Na^+,K^+-ATPase is assumed to be 300 000 g/mol (e.g., Lane et al., 1973), the enthalpy change for the transition would be 360 000 cal/mol. It is possible that the conformations of membrane phospholipids in the bilayer are functionally linked to the conformation of the enzyme and therefore also undergo a structural transition upon protein denaturation. If so, such linkage would contribute to the large enthalpy change and further suggests that perturbations of the Na^+,K^+-ATPase are transmitted to adjacent phospholipids (Gervais et al., 1975, 1977; also see Biltonen, 1977).

Acknowledgments

The authors thank Ed Van Alstyne for excellent technical assistance.

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