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Anthroylouabain: A Specific Fluorescent Probe for the Cardiac Glycoside Receptor of the Na-K ATPase†

P. A. George Fortes

ABSTRACT: Anthroylouabain (AO) was synthesized by reaction of anthracene-9-carboxylic chloride with ouabain. Nuclear magnetic resonance spectroscopy of AO suggests that the anthracene is esterified to the rhamnose in the glycoside. AO inhibits Na-K ATPase from human red cells, eel electroplax and rabbit and dog kidney with a $K_I < 1$ μM. AO bound to rabbit or dog kidney Na-K ATPase shows enhanced fluorescence and characteristic spectral shifts. AO binding requires Mg and is optimum in the presence of Mg + P_i or MgATP + Na; ouabain prevents AO binding and fluorescence enhancement if added before AO or reverses it if added after AO is bound. Na inhibits AO binding in the presence of Mg + P_i and K inhibits it in the presence of MgATP + Na. AO binding and dissociation rate constants measured by fluorescence agree qualitatively with reported measurements for ouabain, using other methods, although AO shows faster kinetics than ouabain. Dissociation constants obtained from kinetic measurements are 1.5×10^{-7} and 1.8×10^{-7} M for the MgATP + Na complex and Mg + P_i complex, respectively. K_D from fluorescence titrations is 2.3×10^{-7} M for the latter. The enzyme has 2-2.5 nmol of AO binding sites/mg of protein. No differences in the fluorescence parameters of the Mg + P_i or MgATP + Na complexes were observed, suggesting that the same enzyme conformation binds AO under both ligand conditions. Comparison of the AO fluorescence parameters in the enzyme with those of model systems suggests that the binding site is hydrophobic and/or viscous and shielded from H_2O. The results indicate that AO is a specific fluorescent probe of the cardiac glycoside receptor of the Na-K ATPase. Possible applications are discussed.

The mechanism of the sodium-potassium adenosine triphosphatase (Na-K ATPase)[1], which is the primary component of the pump responsible for the active transport of Na and K across plasma membranes, is not known. Under physiological conditions the enzyme catalyzes the transport of 3 Na ions out of the cell, 2 K ions into the cell, and the hydrolysis of 1 ATP molecule in a single cycle (Sen and Post, 1964; Garrahan and Glynn, 1967). The ion movements are thought to be obligatorily coupled to ATP hydrolysis in a series of intermediate steps which involve the formation of a phosphoenzyme, changes in conformation, and cleavage of the phosphoenzyme (for recent reviews, see Dahl and Hokin, 1974; Skou, 1974; Glynn and Karlish, 1975; Schwartz et al., 1975). The Na-K ATPase has been purified from a variety of sources (Kyte, 1971a; Jørgensen, 1974a; Hokin et al., 1973). Two polypeptides are found in all the purified preparations (Jørgensen, 1974b): a large chain of about 90 000 daltons containing the phosphorylation site (Kyte, 1971b) and a smaller glycopeptide.

Although conformation changes of the enzyme are probably related to the actual transport of Na and K, at present they can only be studied indirectly. Fluorescence spectroscopy is a possible approach to obtain information on the structure and molecular dynamics of the enzyme during different functional states owing to the sensitivity of the fluorescence parameters

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[1] Abbreviations used: Na-K ATPase, sodium-potassium adenosine triphosphatase; ATP and ADP, adenosine triphosphate and adenosine diphosphate, respectively; AO, anthroylouabain; ANS, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Cl_3AcOH, trichloroacetic acid; P_i, inorganic phosphate; NMR, nuclear magnetic resonance.

of certain probes to the properties of their immediate environment (Stryer, 1968; Azzi, 1975; Radda, 1975). Using 8-anilino-1-naphthalenesulfonate (ANS) in microsomal Na-K ATPase preparations, Nagai et al. (1970) observed changes in fluorescence induced by different ligands, which they interpreted as due to conformational changes of the enzyme. However, although ANS inhibits active Na and K transport and Na-K ATPase in human red cells, suggesting that it does interact with the pump (Fortes and Ellory, 1975), the fluorescence changes with ANS are difficult to interpret since the probe is nonspecific and sensitive to electrostatic forces that depend on interactions between the probe, the membrane and the ionic composition of the solution (Fortes and Hoffman, 1971; Fortes, 1976a). Site-specific fluorescent probes, such as suramin, which resembles ANS and inhibits the Na-K ATPase, probably at the ATP site (Fortes et al., 1973), offer the advantage that their interaction with the enzyme can be localized and defined independently of the fluorescence studies. A good candidate for directing site-specific fluorescent probes is the cardiac glycoside receptor since cardiac glycosides are potent and specific inhibitors of the Na-K ATPase (Glynn, 1964). Their site of action is located on the extracellular aspect of the pump (Hoffman, 1966) on the large chain (Ruoho and Kyte, 1974). In previous attempts to obtain specific probes of the cardiac glycoside receptor, naphthalenesulfonate derivatives of hellebrigenin (Yoda and Hokin, 1972) and strophanthidin (Azzi, 1975) were synthesized and fluorescence changes induced by ligands in the presence of Na-K ATPase were observed. However, as in the case of ANS, a significant portion of the fluorescence resulted from nonspecific interactions of the probes (Yoda and Hokin, 1972), probably due to electrostatic factors affecting the negatively charged derivatives.

Thus, the main limitation in using fluorescence spectroscopy to study the Na-K ATPase is the availability of adequate probes. Attempting to obtain a specific probe for the cardiac glycoside receptor that would have minimal nonspecific interactions, I have synthesized a fluorescent derivative of ouabain using anthracene as the fluorophore. Ouabain was chosen in order to attach the fluorophore to the sugar, hoping to minimize perturbation of the high affinity and inhibitory activity which depend mainly on the steroid part of the glycoside. Anthracene was chosen since it is uncharged, its fluorescence parameters are sensitive to the environment, and certain anthroyl esters have been shown to be useful fluorescent probes (Waggoner and Stryer, 1970).

This paper describes the synthesis and purification of anthroylouabain (AO), experiments designed to determine its usefulness as a specific fluorescent probe of the Na-K ATPase, and some properties of the cardiac glycoside receptor derived from the spectroscopic parameters of the AO–Na-K ATPase complex. A preliminary report of these experiments has been previously presented (Fortes, 1976b).

Materials and Methods

Enzyme Preparations. Rabbit kidney Na-K ATPase was prepared by incubation of microsomes with sodium dodecyl sulfate and ATP, followed by centrifugation in an angle rotor (865 Ti, Sorvall OTD-2 centrifuge) in a discontinuous sucrose gradient, exactly as described by Jørgensen (1974a), except that 1 mM Tris–EDTA was included in the 0.25 M sucrose, 30 mM histidine, pH 7.5 buffer for homogenization and during isolation of the microsomes. If EDTA was not included, low specific activities (1–2 units/mg) and low yields were obtained. The preparations had, typically, a specific activity of 12–14 μmol of ATP hydrolyzed min^{-1} mg^{-1} of protein at 37 °C and less than 5% of the activity was insensitive to 1 mM ouabain.

Dog kidney Na-K ATPase (Lane et al., 1973) was a generous gift of Dr. Lois Lane of the Department of Cell Biophysics, Baylor College of Medicine. The preparation had a specific activity of 8 units/mg. Electroplax microsomes from *Electrophorus* were prepared by Dr. A. Jesaitis in our laboratory according to the method of Dixon and Hokin (1974). The Na-K ATPase activity was 1.4 units/mg.

Frozen–thawed human red cell ghosts were prepared from freshly drawn heparinized blood or from recently outdated bank blood by the method of Heinz and Hoffman (1965) and stored frozen at −20 °C. After thawing, the ghosts were washed twice in the medium to be used for assay.

Protein content of all the preparations was measured by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Phosphatidylcholine liposomes were prepared by transferring 20 mg of egg lecithin dissolved in hexane to a polycarbonate tube and evaporating the hexane under a stream of nitrogen. Twenty milliliters of 30 mM histidine (pH 7.5) was added and the tube was shaken by hand to make a turbid suspension. The suspension was sonicated 10 min with a Branson sonifier set at power 4. The resulting suspension was centrifuged 30 min at 35 000*g*, and the supernatant was used for the fluorescence measurements.

ATPase Activity Determination. The purified Na-K ATPase preparations (1–2 μg/ml) were incubated 2–5 min at 37 °C in a medium containing 3 mM Na_3ATP, 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, and 30 mM histidine (pH 7.5). The reaction was started by adding the enzyme to the prewarmed medium and stopped with trichloroacetic acid to give a final concentration of 8% (w/v). The tubes were immediately transferred to an ice bath and inorganic phosphate was determined by the method of Sanui (1974). Ouabain sensitivity and nonenzymatic ATP hydrolysis were determined in tubes containing 1 mM ouabain or no enzyme, respectively. Each determination was done in duplicate or triplicate. ATP hydrolysis was linear with time under these conditions. Since ouabain and AO binding to the enzyme is relatively slow, particularly at low concentrations, and K^+ decreases the affinity for the glycosides, the dose–response curves (Figure 2) were measured by incubating the enzyme in 50 μl of the above medium, at pH 6.6 without K and with the glycosides at the indicated concentrations, for 30 min at 37 °C. The reaction was started by adding 0.45 ml of medium containing K and no glycoside, and stopped with Cl_3AcOH, after 4 min. Since some dissociation of AO occurs with the addition of K and dilution, the actual affinity for AO is probably higher than that implied by Figure 2. The activity during the 4 min of incubation with K was determined by subtracting the activity measured in the presence of 1 mM ouabain. Similar results were obtained at pH 7.5.

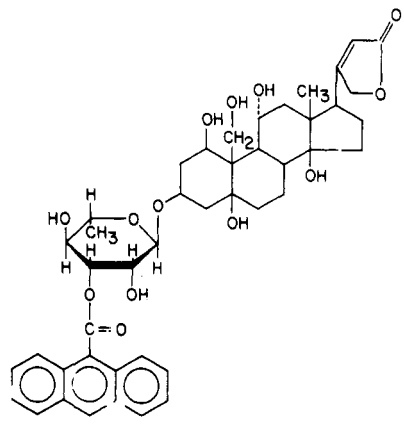
For the experiments with red cell ghosts 2–3 mg/ml of ghost protein was incubated in 5 mM $MgCl_2$, 5 mM Tris-P_i, 20 mM Tris-Cl, pH 7.5, and the indicated ouabain or AO concentrations for 1 h at 37 °C. After the incubation the ghosts were washed six times in 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 20 mM Tris-Cl (pH 7.5), resuspended in this medium at a concentration of about 3 mg of protein/ml and warmed to 37 °C. The reaction was started by adding 3 mM ATP and stopped after 1 h with 8% Cl_3AcOH. The denatured protein was sedimented by centrifugation and inorganic phosphate in the supernatant was determined as described above. The Na-K ATPase activity was determined as the dif-

ference in ATP hydrolyzed in the presence and absence of 0.1 mM ouabain.

Fluorescence Measurements. A Hitachi Perkin-Elmer MPF-4 spectrofluorimeter equipped with a thermostated cell compartment containing a magnetic stirrer was used in the ratio mode. Corrected spectra (200–600 nm) were obtained with a corrected spectra computer (Perkin-Elmer) using rhodamine B in ethylene glycol as a quantum counter. Spectra were recorded using a 2–5-nm band-pass. To obtain the AO spectra in buffer and in the presence of Na-K ATPase (Figure 3), "blank" spectra representing stray and scattered light were recorded in the absence of the fluorescent probe and subtracted from the recorded spectra. The intensity of the "blank" spectrum was 20–40% of the intensity recorded in the presence of 1 mM ouabain. The spectra in solvents were measured at 23 °C in air-equilibrated solutions containing 1–3 μM AO. Kinetic measurements and titrations were recorded with a 10-nm band-pass. In some experiments a Corning CS 7-60 filter and a 430-nm cutoff filter were used in the excitation and emission sides, respectively, to decrease stray and scattered light. AO was added from concentrated (0.1–1 mM) solutions in ethanol to achieve the final concentrations noted in the figure legends. The final ethanol concentration was less than 1.5% (v/v). Controls adding ethanol alone had no effect on either enzyme activity or fluorescence. A 1-cm path length cuvette containing 1.5–2 ml of suspension and a magnetic stirring bar was used in all measurements. The mixing time was about 1 s. Binding curves (cf. Figure 6) were plotted according to Scatchard (1949). The amount of AO bound to the cardiac glycoside receptor was obtained from the difference in fluorescence in the absence and presence of 1 mM ouabain. The equivalence between fluorescence units and nanomoles of AO bound was obtained by measuring the fluorescence of a fixed (0.5–1 μM) AO concentration as a function of the Na-K ATPase concentration. It was found that the fluorescence reached a maximum value at Na-K ATPase concentrations around 1.2 mg/ml, above which no further fluorescence increase with enzyme was observed. The magnitude of the maximum fluorescence at high enzyme concentrations was taken as the fluorescence when all the AO is bound. Absorbance measurements were done with either a Cary Model 15 or a Perkin-Elmer Model 356 spectrophotometer.

Synthesis of Anthroylouabain. Ouabain (2 mmol) and anthracene-9-carboxylic chloride (2 mmol) were dissolved in 20 ml of dry pyridine and incubated 14 days at 23 °C. The pyridine was removed by evaporation under vacuum and the yellow product dissolved in chloroform. Thin-layer chromatography on silica gel G in chloroform–methanol–water (10:5:1) showed a strong blue fluorescent spot (R_f 0.72) and seven weak fluorescent spots (R_f 0.38–0.93). The material with R_f 0.72 represented about 70% of the total product. Purification of this band was accomplished by loading a silica gel column with the chloroform extract and eluting with 5% methanol in chloroform (v/v). Fractions of the eluate exhibiting high absorbance at 362 nm were chromatographed as above and those showing a single spot at R_f 0.72 were pooled and evaporated to dryness in vacuo. No detectable contamination with unreacted ouabain exists in the purified fraction as shown by the absence of a ouabain spot (R_f 0.35) and a single spot at R_f 0.72.

To characterize the purified material, 220-MHz NMR spectra were measured in a Varian spectrometer. The solvent was deuterated pyridine and the standard tetramethylsilane. AO spectra were compared with those of ouabain, strophanthidin, and rhamnose. The main findings from the NMR



ANTHROYL OUABAIN

FIGURE 1: Structure of Anthroylouabain.

studies are: (a) the new compound contains 1 mol of anthracene/mol of ouabain; (b) no significant differences between proton resonances due to the steroid in ouabain or anthroylouabain are observed; (c) a multiplet present in both ouabain and rhamnose spectra at δ 4.30–4.36 is absent in the anthroylouabain spectrum, which instead shows a broadened singlet at δ 4.497; (d) both rhamnose and ouabain show a broad resonance (~3 H) centered at δ 6.303 which is substituted by a doublet (~2 H) at δ 6.50–6.53 in anthroylouabain. These findings suggest that the anthroyl group is attached to the sugar, not the steroid in ouabain. A tentative structure of anthroylouabain consistent with the NMR data is shown in Figure 1. It should be noted that it is possible that the anthroyl group is on carbons 2 or 4 of the rhamnose, instead of C_3, and/or that a mixture of isomers is present. Further work is in progress to establish a definite structure.

The calculated molecular weight of AO ($C_{44}H_{52}O_{13}$) is 788.9. Absorption spectra of AO solutions in ethanol show that AO has identical maxima as other anthroyl esters (Waggoner and Stryer, 1970). The extinction coefficients of AO in ethanol at the absorption maxima in the near ultraviolet were estimated assuming that AO was 100% pure and unhydrated. The values of ϵ_{max} (M^{-1} cm^{-1}) are: $\epsilon_{329-331}$ (2353), ϵ_{345} (4589), ϵ_{362} (6738), and ϵ_{381} (5976).

The AO concentrations in the experiments presented here were determined using the above extinction coefficients. These values are 18% lower than those reported by Waggoner and Stryer (1970) for other anthroyl esters. If the values of Waggoner and Stryer are used, a molecular weight of 976, corresponding to ~10 mol of H_2O/mol of AO, is obtained, and the AO concentrations in the figure legends should be 18% lower.

Reagents. Ouabain, ATP disodium salt, egg lecithin, and bovine serum albumin were obtained from Sigma. Anthracene-9-carboxylic chloride was obtained from Aldrich. All solvents used were either spectroscopic or fluorimetric grade. Water was deionized and distilled in glass. All other chemicals were reagent grade.

Results

Inhibition of Na-K ATPase by Anthroylouabain. In order to see if the fluorescent derivative retained the high affinity and inhibitory activity of ouabain, the effect of AO on the activity of Na-K ATPases from different sources was studied. Figure 2 shows the effect of increasing AO concentration on the Na-K ATPase activity of rabbit and dog kidney, electric organ, and

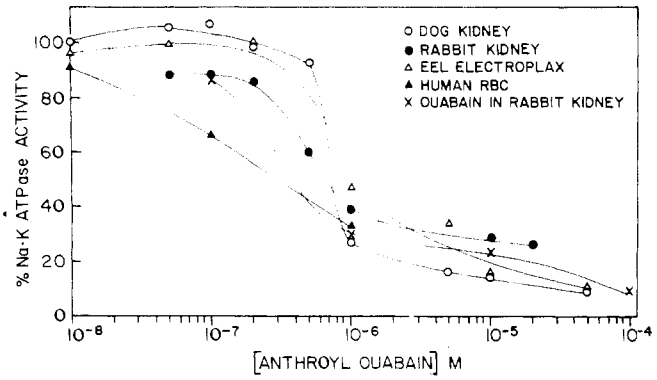


FIGURE 2: Inhibition of Na-K ATPase by anthroylouabain. See Materials and Methods for details of the experimental procedure and composition of the incubation media. For comparison, the effect of ouabain on rabbit kidney (x) is also shown.

TABLE I: Effect of Preincubation with ATP on Inhibition of Red Cell Na-K ATPase by AO and Ouabain.[a]

Additions to Preincubation Medium[b]			
AO (μM)	Ouabain	ATP (mM)	ATPase Act.[c] (nmol of P_i mg^{-1} h^{-1})
0	0	0	105
0.12	0	0	126.5
0.12	0	2	64.5
0.12	0.1 mM	2	65
0	0.1 μM	0	97.2
0	0.1 μM	2	51.5

[a] Ghosts (2 mg/ml) were incubated 30 min at 37 °C in the indicated media and washed twice in ice cold medium and ATPase activity was assayed in 40 mM NaCl, 20 mM KCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 2 mM ATP, and 10 mM Tris-Cl (pH 7.4). [b] Contains 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM Na_2EDTA, 10 mM Tris-Cl (pH 7.5). [c] Means of duplicate determinations.

human red cell membranes. In all these preparations, 1 μM AO causes more than 50% inhibition, indicating that the presence of the anthroyl group does not alter the inhibitory activity nor the high affinity of ouabain for its binding site on the enzyme. This supports the conclusion that the anthroyl group is attached to the rhamnose since the high affinity and inhibitory activity of ouabain depend on the structure of the steroid part of the molecule. It is unlikely that the inhibition with AO is due to contamination with ouabain. In fact, the degrees of inhibition of the kidney and electroplax enzymes shown in Figure 2 are lower limits since dilution and the addition of K necessary to study the reaction causes dissociation of the enzyme–AO complex (see below). With red cell ghosts, inhibition is observed even at 10^{-8} M AO. AO binding and inhibition of the red cell Na-K ATPase appears to behave similarly to that of ouabain and other glycosides since it is essentially irreversible in the presence of appropriate ligands. Incubation at 37 °C of ghosts with AO in the presence of Mg^{2+} or Co^{2+} with or without inorganic phosphate, or Mg, Na, and ATP, followed by extensive washing results in inhibition of Na-K ATPase activity as shown in Figure 2. However, incubation with AO or ouabain in the presence of Mg and Na, followed by washing, causes no inhibition unless ATP is present (Table I). These observations indicate that AO is similar to ouabain in its ability to inhibit Na-K ATPase and its requirements for high affinity binding to the enzyme: promotion by Mg + P_i or Mg + Na + ATP and inhibition of binding by Na in the absence of ATP.

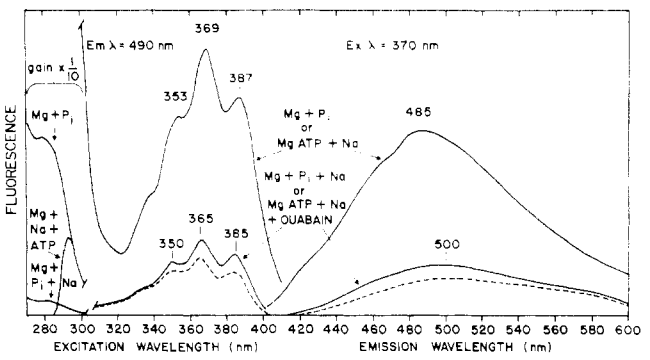


FIGURE 3: Corrected excitation and emission spectra of AO in rabbit kidney Na-K ATPase. Incubation conditions: for all spectra the medium had 30 mM histidine, pH 7.5, and 0.66 μM AO. The dashed spectra are in the absence of Na-K ATPase. For the top spectra the media had either 5 mM $MgCl_2$ + 7.5 mM Tris-phosphate or 5 mM $MgCl_2$ + 5 mM Na_3ATP + 80 mM NaCl. For the spectra in the middle, 80 mM NaCl was added to the Mg + P_i media or 1 mM ouabain to the ATP media. Na-K ATPase: 130 μg/ml, volume 1.5 ml, temperature 37 °C. Note that the gain was reduced to one-tenth below 300 nm. Blank spectra, in the absence of AO were subtracted from these curves, as in Figure 9.

Fluorescence of Anthroylouabain in the Presence of Na-K ATPase. In order to see if the interaction of AO with Na-K ATPase could be detected spectroscopically, fluorescence measurements were done using purified rabbit kidney Na-K ATPase incubated with Na, Mg, and ATP or Mg and inorganic phosphate, conditions that promote optimal ouabain binding (Schwartz et al., 1968). To determine whether or not AO interacts specifically with the ouabain binding site of the Na-K ATPase, controls included 0.1–1 mM ouabain to saturate all the binding sites.

Figure 3 shows corrected AO excitation and emission spectra, under the conditions mentioned above. AO fluorescence in buffer is low with a maximum emission at 500 nm and excitation maxima at 254, 350, 365, and 385 nm. When Na-K ATPase is added in the presence of either Mg + P_i or Mg + Na + ATP there is a large increase in fluorescence accompanied by a shift in the emission maximum to 485 nm and in the excitation peaks to 259, 353, 369, and 387 nm. In addition, a new excitation peak at 280 nm appears in the presence of Mg + P_i. This new peak is seen at 293 nm with ATP, due to a filter artifact caused by the nucleotide absorbance below 290 nm (Figure 3). In contrast, without either Mg or ATP in the presence of P_i or Mg + Na, respectively, or if ouabain is added to the medium, the changes in AO fluorescence with Na-K ATPase are strongly inhibited: the intensity upon addition of Na-K ATPase increases only about 30%, the emission maximum shifts to 495 nm, and the excitation maxima remain the same as in buffer alone, except the 365 nm peak shifts to 367 nm.

The fluorescence spectra with Mg + P_i are identical with those with Mg + Na + ATP, and the spectra in the presence of ouabain are identical with those without Mg or ATP. These results indicate that the fluorescence enhancement and the spectral shifts reflect a specific interaction of AO with the cardiac glycoside binding site of the Na-K ATPase, and that this site has an environment different from water. Furthermore, the new excitation band in the 280–300-nm region is due to energy transfer from tryptophan to AO. This is shown by the decrease in tryptophan fluorescence at 333 nm at the expense of increased AO fluorescence above 400 nm when excitation is in the tryptophan absorption band (Figure 4). The

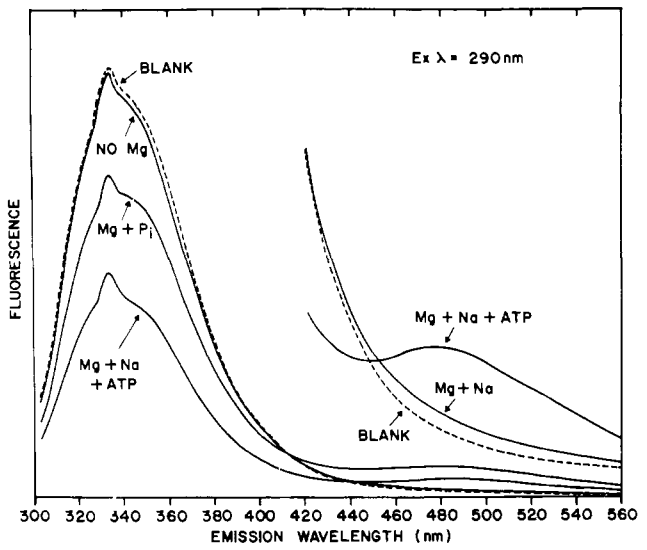


FIGURE 4: Energy transfer between tryptophan and AO. Conditions as in Figure 3 except Na-K ATPase was 1.3 mg/ml and [AO] was 2 μM. The dashed spectra were in the absence of AO. Since ATP absorbs some of the excitation light, part of the decrease in tryptophan fluorescence is due to a filter effect. The inset above 400 nm shows spectra amplified tenfold.

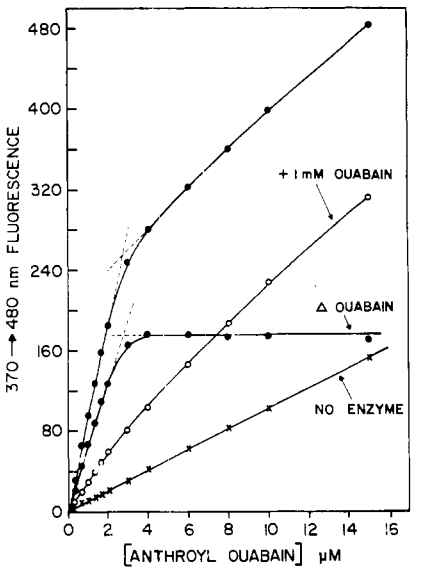


FIGURE 5: Titration of Na-K ATPase with AO. Experimental conditions: 30 mM histidine, 5 mM $MgCl_2$, 7.5 mM Tris-phosphate, pH 7.5, and the indicated AO concentrations. Where indicated, 1.3 mg/ml Na-K ATPase and 1 mM ouabain were also present. Temperature = 37 °C; volume = 1.5 ml. Excitation and emission wavelengths were 370 and 480 nm, respectively.

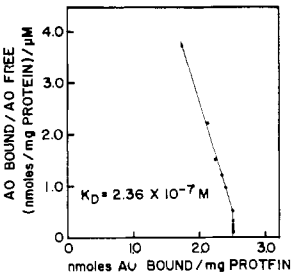


FIGURE 6: Scatchard plot of AO binding to the Na-K ATPase. Conditions as in Figure 5, except Na-K ATPase was 18.6 μg/ml. [AO] was varied from 0.5 to 17.5 μM.

absence of energy transfer with ouabain or without Mg or ATP (Figures 3 and 4) indicates that no binding of AO occurs at other sites in the protein. Nevertheless, there appears to be some nonspecific binding of AO, probably to membrane lipid, since there is some ouabain-insensitive fluorescence enhancement, which can be as much as 100% at higher Na-K ATPase concentrations (1-2 mg/ml). Although these nonspecific sites also shift the emission maximum to 485 nm, their environment is different from the specific site since the excitation peaks are 254, 350, 367, and 385 nm for the former, and 259, 280-290, 353, 369, and 387 nm for the latter.

In order to see if the specific and nonspecific AO binding sites could be distinguished by their affinities for AO, fluorescence titrations were done in the absence and presence of 1 mM ouabain. Figure 5 shows that AO fluorescence in buffer is linear with AO concentration up to the highest concentration tested (15 μM). In the presence of Mg + P_i and Na-K ATPase at high concentration (1.3 mg/ml), which binds essentially all the added AO below 1 μM since increasing the enzyme concentration does not increase fluorescence further, fluorescence is much higher and increases linearly with AO concentration up to about 2 μM, above which the fluorescence increases with a smaller slope. In the presence of ouabain, fluorescence is only about twice as high as in buffer and the increase in intensity with AO becomes essentially parallel to the curve without ouabain above 4 μM AO. The difference in fluorescence in the presence and absence of ouabain, also plotted in Figure 5, shows that the ouabain-sensitive fluorescence enhancement is saturated above 4 μM AO. AO titrations in the presence of Mg ATP and Na are similar to those of Figure 5, except that ATP must be added in large excess (>25 mM) since it is hydrolyzed by the uninhibited enzyme during the titration.

Extrapolations of the linear rise and the maximum fluorescence of the ouabain-sensitive curve intercept at 2.7 μM AO. Since the enzyme concentration is 1.31 mg/ml, maximum AO binding is 2.06 nmol/mg of protein. The dissociation constant of the ouabain-sensitive sites cannot be estimated accurately from the plot in Figure 5 since both the enzyme and the ligand are at high concentrations and the binding is stoichiometric. Using about a hundred times lower enzyme concentrations results in a similar curve. A Scatchard plot (Figure 6) under the latter conditions is linear and the derived parameters are n = 2.49 nmol/mg of protein and $K_D = 2.36 \times 10^{-7}$ M. The difference in the maximum number of AO sites determined by direct titration (Figure 5) or by the Scatchard plot (Figure 6) is probably due to the use, for the latter, of the maximum AO fluorescence in the presence of excess enzyme concentrations to estimate the amount of AO bound from fluorescence (see Methods). Since this method includes the fluorescence of the nonspecific sites, the values are less accurate and probably overestimated. Therefore, the correct value is probably closer to 2 nmol/mg of protein. This value is lower than the reported number of ouabain binding sites (3-4 nmol/mg of protein), measured with [^{3}H]ouabain binding (Jørgensen, 1974b) and kinetic titrations (Kyte, 1972), in highly purified Na-K ATPase preparations. Since the specific activities of our enzyme preparations are about 50% lower than those used for the [^{3}H]ouabain binding studies (Jørgensen, 1974a,b), a lower number of ouabain binding sites is expected.

In contrast, the nonspecific AO binding sites, represented by the fluorescence enhancement in the presence of ouabain (Figure 5), have a much lower affinity than the specific sites, since the former are far from saturation at 15 μM AO. Scatchard plots of the nonspecific sites (not shown) are nonlinear

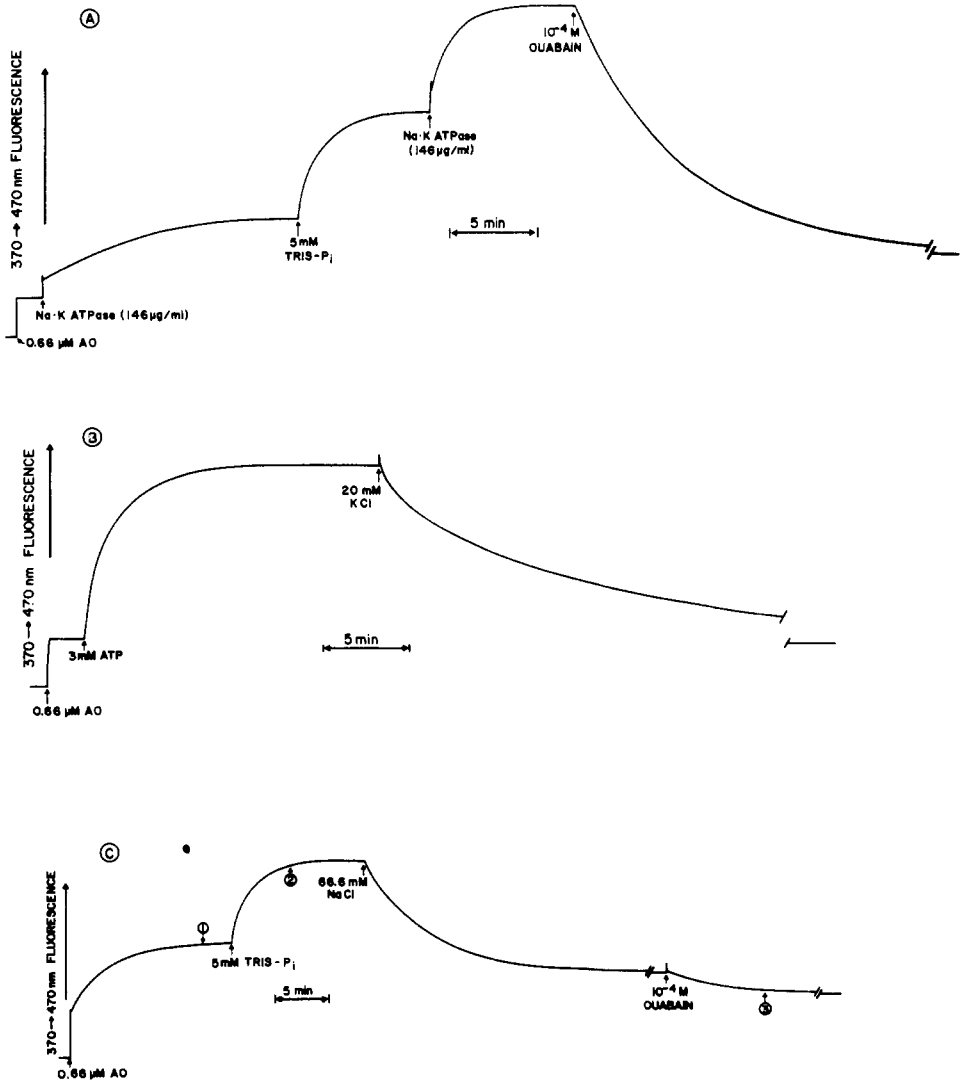


FIGURE 7: Kinetics of AO fluorescence changes induced by ligands. (A) The cuvette had 5 mM $MgCl_2$, 30 mM histidine, pH 7.5; (B) the cuvette had 3.3 mM $MgCl_2$, 66.6 mM NaCl, 30 mM histidine, pH 7.5, and 0.29 mg/ml Na-K ATPase; (C) the cuvette had 5 mM $MgCl_2$, 30 mM histidine, and 0.29 mg/ml Na-K ATPase. All additions were at the indicated final concentrations. Volume = 1.5 ml; temperature = 37 °C. For the experiment in C, excitation and emission spectra were recorded at the points indicated by the numbered arrows. The spectra are shown in Figures 8 and 9.

indicating heterogeneity of these sites. The lowest dissociation constant of the nonspecific sites is 15–20 μM.

Kinetics of the Interaction of Anthroylouabain with the Na-K ATPase. Figure 7 shows the time course of the AO fluorescence changes induced by various ligands. Mg alone causes a slow fluorescence increase and inorganic phosphate causes a further increase at a faster rate (Figure 7A). Na prevents the fluorescence increase due to Mg but increases the rate in the presence of Mg and ATP (Figure 7B). ADP does not promote enhanced fluorescence in the presence of Mg and Na since the fluorescence decreases when the ATP is hydrolyzed by the residual activity of the enzyme. In the absence of Mg no increase in fluorescence is observed upon addition of ATP or P_i. Thus maximum rate of fluorescence enhancement is observed when the enzyme is incubated with either Mg + Na + ATP or Mg + P_i. The ligand requirements and time course suggest that the fluorescence enhancement represents AO binding to the cardiac glycoside receptor. Ouabain inhibits the fluorescence enhancement if present before the addition of the ligands or reverses the increase with slow kinetics if added after the maximum fluorescence has been reached (Figure 7A). The increased fluorescence upon AO binding to the Na-K ATPase is also reversed by addition of EDTA (not shown), Na to the Mg + P_i complex (Figure 7C), and K to the Mg + Na + ATP complex (Figure 7B). These observations suggest that the fluorescence decrease represents dissociation of the AO–enzyme complex, caused by occupation of sites by ouabain, and decreased affinity for AO in the presence of Na or K, or absence of Mg. This conclusion is supported by excitation and emission spectra taken after the fluorescence decrease induced by Na or K reached steady state, which show the same intensity and peak wavelengths as those in the presence of saturating concentrations of ouabain (Figures 8 and 9).

If the fluorescence changes represent the binding and dissociation of AO to the cardiac glycoside binding site of the ATPase, the association and dissociation rate constants can be obtained from the fluorescence measurements. The half-time of the fluorescence increase upon addition of ATP in the presence of Mg and Na is inversely proportional to the AO concentration. Figure 10 shows that the apparent association rate constant calculated from the half-times of fluorescence increase induced by ATP, assuming pseudo-first-order kinetics ($k = \ln 2/t_{1/2}$), is a linear function of the AO concentration

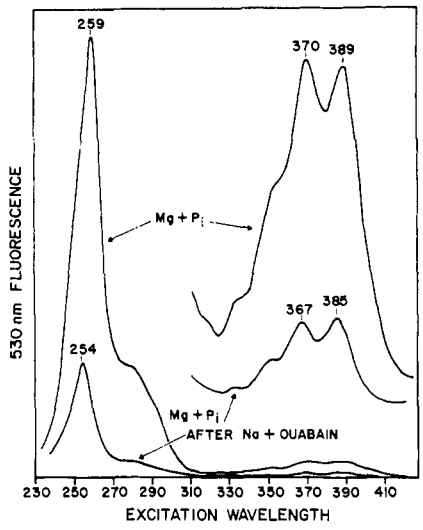


FIGURE 8: Uncorrected excitation spectra of AO in Na-K ATPase. Conditions as in Figure 7C. The region above 300 nm is shown amplified 30-fold.

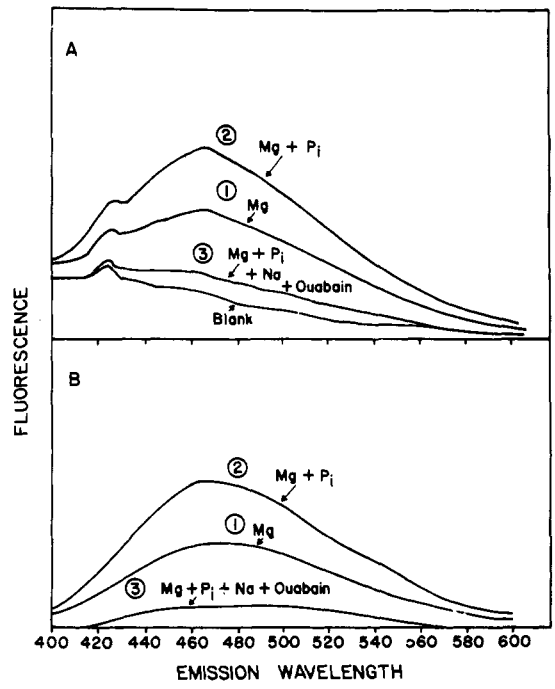


FIGURE 9: Uncorrected emission spectra of AO in Na-K ATPase. Conditions as in Figure 7C. (A) Spectra as recorded; (B) fluorescence measured in the absence of AO was subtracted. Numbers correspond to those in Figure 7C.

and the line intercepts at the origin. The second-order rate constant, k_{on}, obtained from the slope of the curve is 1.5×10^4 M^{-1} s^{-1}. Similarly, k_{on} in the presence of Mg + P_i is also 1.5×10^4 M^{-1} s^{-1}.

The fluorescence decrease induced by ouabain and Na follows first-order kinetics as shown by the semilog plots of the fractional fluorescence enhancement vs. time after ouabain addition, which are linear and independent of the AO concentration (Figure 11). The derived first-order dissociation rate constants, k_{off}, for the ouabain-induced AO dissociation are 2.3×10^{-3} and 2.8×10^{-3} s^{-1} for the Mg + Na + ATP and the Mg + P_i complexes, respectively. Assuming that ouabain at this concentration binds much faster than AO, and its only effect is to prevent AO association by occupation of the binding sites, the above AO dissociation rate constants should be the same during the binding reaction. If so, the equilibrium dissociation constant for AO bindings, K_D, can be obtained from the ratio of the association and dissociation rate constants: $K_D = k_{off}/k_{on}$. The dissociation constants derived from the rate constants are 1.5×10^{-7} and 1.8×10^{-7} M for the Mg + ATP + Na and Mg + P_i complexes, respectively. The latter value is considered in good agreement with $K_D = 2.3 \times 10^{-7}$ M for the Mg + P_i complex obtained from the fluorescence titrations mentioned above.

The AO dissociation caused by Na also follows first-order kinetics and the rate constant is lower than that in the presence of ouabain (Figure 11). However, Na must decrease the AO association rate much more than the dissociation rate in order to cause the observed AO dissociation, which indicates a large decrease in the affinity of the enzyme for AO. The K-induced AO dissociation is also slower than that induced by ouabain (Figure 11), although the kinetics is complex since the semilog plot is nonlinear in the first minutes and becomes linear at longer times. This nonlinearity is probably due to hydrolysis of the remaining ATP, stimulated by K addition, since the curvature in the plot depends on the initial ATP concentration and the time of incubation.

Fluorescence Parameters of AO in Model Systems. Since the fluorescence of AO bound to the Na-K ATPase results in characteristic excitation and emission spectra, it was of interest to study these parameters in known environments to see which resembles the AO binding site.

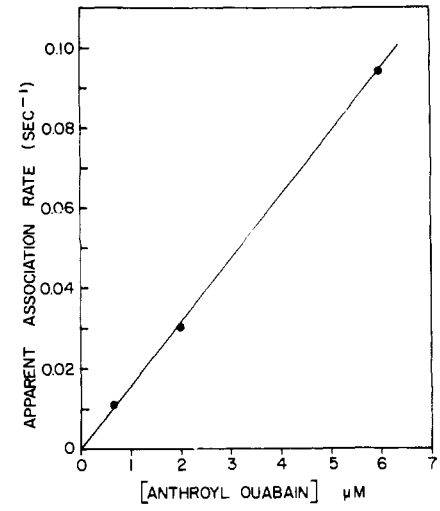


FIGURE 10: Pseudo-first-order association rate constants vs. AO concentration. The rate constant was estimated from the half-time of fluorescence increase induced by ATP as in Figure 7B assuming pseudo-first-order kinetics: $k_{app} = \ln 2/t_{1/2}$. The slope of the curve gives the second-order rate constant, $k_{on} = 1.5 \times 10^4$ M^{-1} s^{-1}.

Table II shows the excitation and emission maxima, obtained from corrected spectra, of AO in a variety of solvents and in sonicated egg lecithin liposomes. As with other fluorescent probes, there is a bathochromic shift of the emission maximum and increased quantum yield in solvents of low polarity (Figure 12). The fluorescence increase in apolar solvents is mainly due to variations in quantum yield. Absorbance measurements show that the extinction coefficient of AO in ethanol is only about 12% higher than in water, while fluorescence in ethanol is more than 20-fold higher than in water (Figure 12). However, polarity is not the only factor since polar but viscous solvents, such as glycerol, also cause a blue-shift in the emission spectrum and increased quantum yield. The

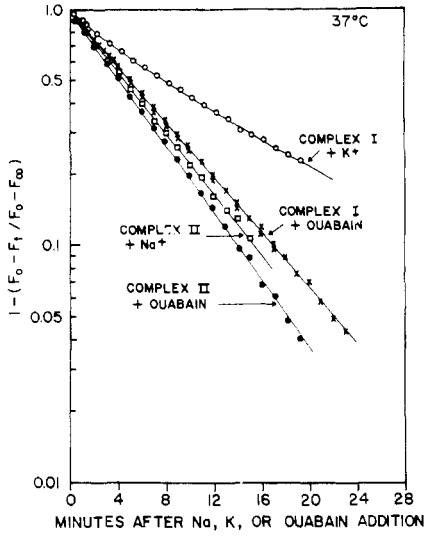


FIGURE 11: Dissociation of AO complexes I and II by ouabain, Na, and K. Conditions as in Figure 7. Complexes I and II refer to AO binding promoted by Mg + ATP + Na and Mg + P_i, respectively. In the ordinate, F_0 is the fluorescence before the addition of the ligand that induces the fluorescence decrease, F_∞ is the fluorescence at equilibrium, and F_t the fluorescence at time t. The crosses show two different experiments with [AO] = 0.66 and 2 μM.

TABLE II: Corrected AO Fluorescence Parameters in Solvents.

Solvent	Emission Maximum (nm)	Excitation Maxima 1	2	3	4
H_2O	500	253	349	365	385
Methanol	485	253	347	363	382
Ethanol	483	254	345	362	381
Butanol	480	255	347	363	383
Glycerol	481	257	350	367	386
Toluene	482		349	365	385
Dioxane	465		348	365	385
Dimethylformamide	464		349	366	385
Hexane	463		350	369	389
Lecithin liposomes	480		349	367	385
Na-K ATPase	485	259	353	369	387
Na-K ATPase + ouabain	485	254	350	367	385

excitation spectrum is less sensitive to the environment, although small changes are observed. Alcohols cause a blue shift of 2–3 nm, whereas dioxane, toluene, and dimethylformamide, which increase the fluorescence intensity 30–80-fold over that in H_2O, show the same excitation maxima as in H_2O. Hexane shifts the excitation spectrum to the red. Sonicated lecithin liposomes increase the intensity and also shift the emission maximum to the blue but have little effect on the excitation spectrum.

Discussion

The observations presented in this paper indicate that anthroylouabain is a sensitive fluorescent probe and highly specific for the cardiac glycoside receptor of the Na-K ATPase. AO resembles ouabain in its ability to inhibit Na-K ATPases from eel electroplax, rabbit and dog kidney, and human erythrocytes with a $K_I < 1$ μM. AO and ouabain binding to the red cell Na-K ATPase, as measured by inhibition of the enzyme, are not reversed by extensive washing and both require certain ligands—i.e., Mg + P_i or Mg ATP + Na—for optimum binding.

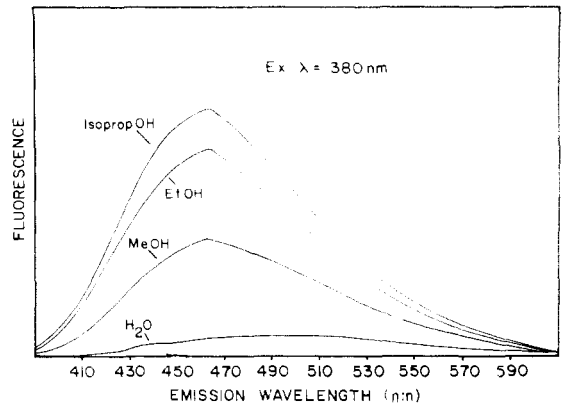


FIGURE 12: Effect of alcohols on AO fluorescence. [AO] was 1 μM, except in H_2O where [AO] = 2 μM. Spectra are uncorrected. Corrected values are shown in Table II. Temperature = 23 °C.

Enhanced fluorescence and shifts in the excitation and emission spectra are observed when AO interacts with the Na-K ATPase. A variety of observations indicates that these fluorescence changes arise specifically from AO molecules bound to the cardiac glycoside receptor: (a) the fluorescence changes are prevented or reversed when ouabain is added in a 100–1000-fold excess over AO; (b) they occur only in the presence of ligands that promote ouabain binding and are prevented by ligands known to inhibit ouabain binding, i.e., K in the presence of Mg ATP + Na, Na in the absence of ATP, or no Mg; (c) the maximum number of specific AO binding sites as determined from fluorescence titrations is similar to that determined from [^{3}H]ouabain binding (Jørgensen, 1974a,b), if the differences in specific activity between preparations are taken into account; (d) the time course of the fluorescence changes is consistent with association and dissociation kinetics; the dissociation constants derived from the kinetic measurements are similar to those derived from equilibrium binding.

Since AO fluorescence is sensitive to the environment, the spectroscopic parameters of AO bound to the Na-K ATPase provide information on the properties of the cardiac glycoside receptor.

It must be pointed out that, since AO is a relatively large molecule and the fluorophore is not attached to the steroid, the environment sensed by this probe is in the immediate vicinity of the steroid site, rather than in the site itself. Since the synthesis of AO yields a variety of products, presumably isomers with the anthroyl group attached to the different hydroxyls in ouabain, it will be interesting to compare the properties of these isomers, to map possible environmental differences and steric restriction in the receptor site.

The increased fluorescence intensity and blue shift in the emission spectrum upon AO binding to Na-K ATPase indicate that the environment of its binding site is more hydrophobic and/or viscous than water since similar changes are observed with AO in alcohols, glycerol, aprotic solvents, and liposomes (Table II). The red shift in the excitation peaks of the AO–enzyme complex is different from that produced by the alcohols, which cause a blue shift, and the aprotic solvents and liposomes, which show little change from the excitation spectrum in water. However, hexane and, to a lesser extent, glycerol, cause a similar red shift in the excitation spectrum, again suggesting a hydrophobic and/or viscous environment of the binding site in the enzyme. These results indicate that, although the cardiac glycoside receptor is accessible only on the external membrane surface (Hoffman, 1966), it must be

shielded from quenching processes that would occur if it were exposed to the solvent.

The appearance of an excitation peak due to energy transfer from tryptophan indicates that one or more tryptophan residues are close to the binding site. A definite distance between AO and tryptophan cannot be calculated at the present, because of the large number of tryptophan residues in the molecule, which would make an average distance estimate meaningless. More precise localization of this site may be possible by measurements of energy transfer between AO and probes located at other sites, and through the sensitivity of AO fluorescence to quenchers or perturbations of the solvent in which the membranes are suspended. In this respect, we have found that cobalt does not quench AO fluorescence by energy transfer when Co^{2+} is bound to the Mg^{2+} binding site that promotes AO binding, but occupation of a second Co^{2+} binding site of lower affinity results in energy transfer from AO to Co^{2+} with ~80% efficiency. The estimated distances between AO and these Co^{2+} binding sites will be published elsewhere (Moczydlowski and Fortes, in preparation).

Although most of the AO fluorescence arises from AO bound to the cardiac glycoside receptor site, there is some nonspecific binding as shown by the fluorescence enhancement and spectral shifts observed in the presence of saturating concentrations of ouabain. The fluorescence arising from AO bound to nonspecific sites varies from less than 10% to about 50% of the total fluorescence with excitation at 330–390 nm, depending on the AO and Na-K ATPase concentrations. With excitation in the tryptophan absorption band the fluorescence of the nonspecific sites is negligible. These nonspecific sites are probably in the lipid since little or no energy transfer from tryptophan is observed in the presence of ouabain, which would be expected if the nonspecific sites were on the protein. Although the nonspecific binding sites have a similar emission spectrum, they have a lower affinity and a different excitation spectrum than the specific sites, indicating a different environment. The environment of the nonspecific sites is mimicked by glycerol and lecithin liposomes, suggesting further that the bilayer is responsible. This is supported by nanosecond fluorescence measurements which show that the specific site has a lifetime of about 10 ns while the nonspecific sites show a longer lifetime, 13–14 ns, similar to AO in lecithin liposomes or glycerol (Fortes, in preparation).

The long lifetime of the nonspecific sites indicates a high quantum yield. Since the fluorescence arising from the nonspecific sites is quite low, very few AO molecules must be bound nonspecifically at the concentrations that saturate the specific sites, in accordance with the low affinity of the nonspecific sites indicated by AO titrations in the presence of ouabain (Figure 5).

The steady-state fluorescence intensities and spectra of the bound AO are the same for the Mg ATP + Na and the Mg + P_i complexes (Figure 3). This suggests that the same enzyme conformation ($E_2 - P$?) is produced by the two sets of ligands. A similar suggestion has been made on the basis of the activation energy for [^{3}H]ouabain binding which is the same in the presence of either Mg + P_i or Mg ATP + Na (Wallick and Schwartz, 1974). However, the AO–Na-K ATPase complex in the presence of Mg alone also shows the same excitation and emission maxima, although a 30–50% lower intensity. Since under these conditions the enzyme is not phosphorylated, at least two conformations bind AO and give rise to similar spectra. Thus, it is possible that the differences between these conformations are simply not detectable by changes in excitation or emission spectra.

A slow fluorescence decrease is observed when Na or K is added in the presence of Mg + P_i or Mg ATP + Na, respectively. These changes reflect the dissociation of AO due to a decreased affinity for the probe and, therefore, they reflect the conformation changes induced by the ions. Since it is unlikely that the actual conformation changes induced by Na and K have such slow kinetics, it follows that AO fluorescence can only detect these conformation changes through variations in the rate-limiting steps for AO interaction with the enzyme.

Although the fluorescence experiments presented in this paper were all done with the rabbit kidney enzyme, we have observed that dog kidney and eel electroplax enzymes interact with AO in a similar way and cause identical excitation and emission spectra as the rabbit enzyme. Thus, the environment of the cardiac glycoside receptor is similar, within the sensitivity of this probe, in these different species.

The present results suggest that AO and similar probes may be useful in studying the properties and conformational dynamics of the Na-K ATPase. Thus, it may be possible to measure the distances between active sites by energy transfer, their microviscosity and orientation by fluorescence polarization, and the nature and kinetics of conformational transitions induced by ligands and temperature. The use of fluorescence spectroscopy has the advantage that kinetic studies are monitored continuously in the same sample and can be carried out down to the millisecond or microsecond time scales. AO may also be useful in studies with intact cells and in reconstitution studies, to study the localization, orientation, mobility, and dynamics of the Na-K ATPase. In addition, fluorimetric assays of binding constants for various ligands and other cardiac glycosides may be devised, based on their ability to promote AO binding or competition with AO for their receptor site, respectively.

It must be noted that the effects of Na and K on ouabain binding depend on the side of the membrane which is exposed to the ions (Bodemann and Hoffman, 1976). Since the preparations used in the present work are freely permeable to ions, it is not possible to study their sidedness. AO fluorescence studies in red cell ghosts may be feasible despite their low content of Na-K ATPase (200 molecules/cell (Hoffman, 1969)) since we have been able to measure specific AO fluorescence signals with 10^{-8} M binding sites of the rabbit enzyme.

Of course, an important disadvantage of AO is that the probe itself inhibits the enzyme and it is not possible to study the Na-K ATPase during turnover. Other specific probes, located at noninhibitory sites may be useful in this respect.

Acknowledgments

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