Photoaffinity Labeling of the Digitalis Receptor in the (Sodium + Potassium)-Activated Adenosinetriphosphatase†

Terry B. Rogers‡ and Michel Lazdunski*

ABSTRACT: Two photoaffinity labels have been synthesized from ouabain and strophanthidin. The photosensitive derivatives were formed through the reductive amination of *N*-(2-nitro-4-azidophenyl)ethylenediamine to the periodate-oxidized rhamnose moiety of ouabain (NAP-ouabain) and the C-19 aldehyde side chain of strophanthidin (NAP-strophanthidin). The binding of these photoaffinity labels to the digitalis binding sites was followed in the dark by two methods: the ability of the NAP-ouabain and NAP-strophanthidin to inhibit the enzyme activity of the (sodium + potassium)-activated adenosinetriphosphatase purified from the tissue of the electric organ of *Electrophorus electricus*; the inhibition of [^{3}H]ouabain binding to a microsomal fraction of the same tissue. The results of photoaffinity labeling experiments with the purified (sodium + potassium)-activated adenosinetriphosphatase indicate that only the large molecular weight protein (M_r = 93 000) of the enzyme is labeled with either [^{3}H]NAP-ouabain or [^{3}H]NAP-strophanthidin. Therefore the large chain contains both the sugar and steroid binding sites of the digitalis binding center of this enzyme. When either photoaffinity label was incubated with a membrane preparation from the electric organ and the solution irradiated with ultraviolet light, sodium dodecyl sulfate gel electrophoresis of the membrane proteins indicated that the large chain of the (sodium + potassium)-activated adenosinetriphosphatase was the major protein labeled. About 40% of the total radioactivity incorporated was found in this band in the gel. As with the purified enzyme preparation, the small chain (M_r = 47 000) was not labeled significantly. There were two other proteins in the gels which were labeled in these experiments with molecular weights of approximately 45 000 and 50 000. Protection experiments with 1.0 mM ouabain added to the solution indicated that this labeling was a specific affinity process. These results suggest there may be several proteins involved in the binding of digitalis in one or more specific sites on the membrane.

Digitalis preparations have been of immense clinical value in the treatment of chronic congestive heart failure as well as in other disorders associated with the cardiovascular system (Marks & Weissler, 1972). Besides its clinical value, this family of molecules has attracted the interest of biochemists, physiologists, and biophysicists because of its rather unique action on membrane function affecting the transport of sodium and potassium.

It is well known that the sodium pump in the plasma membrane of eukaryotic cells is inhibited by low concentrations of the digitalis glycosides (Schatzman, 1953; Wilson et al., 1970; Glynn, 1964). Studies on the nature of the interactions of the (Na^+-K^+)ATPase[1] with digitalis, ouabain, and other compounds of this class indicate that the inhibition of the sodium pump activity is due to the direct binding of these drugs to the enzyme (for reviews, see Lee & Klaus, 1971; Schwartz et al., 1975).

Although it is very generally accepted that molecules of the ouabain family are excellent (Na^+-K^+)ATPase inhibitors, there are many conflicting reports in the literature on whether the inhibition of the cardiac (Na^+-K^+)ATPase by digitalis is responsible for the inotropic effect of these drugs (Akera, 1977; Peters et al., 1974; Okita, 1977). There is a suggestion that other receptors exist which are responsible for the inotropic effect (Dutta et al., 1968). Affinity labels derived from digitalis type molecules are clearly needed: (1) to have a better knowledge of the receptor site of the ouabain type molecules in the (Na^+-K^+)ATPase; (2) to try to determine with a direct technique if the (Na^+-K^+)ATPase is the only ouabain receptor or, more important, if it is the receptor of pharmacological interest.

Several covalent labels of the digitalis binding site have already been synthesized. A photoaffinity label has been prepared by Ruoho & Kyte (1974) which labeled detergent-solubilized renal medulla (Na^+-K^+)ATPase on the large molecular weight subunit (M_r = 100 000). However, this derivative of cymarin does not seem to be generally useful for three reasons: (i) the photoaffinity label had a low specific radioactivity (5 mCi/mmol) and, as a consequence, the amount of radioactivity incorporated into the (Na^+-K^+)ATPase was low (on the order of 100 cpm); (ii) the photoreactive side chain, a diazomalonyl moiety, is prone to intramolecular rearrangements and reaction with water upon photolysis (Hexter & Westheimer, 1971); (iii) irradiation conditions required for covalent labeling of the enzyme with this derivative resulted in cross-linking of the high molecular weight subunit as well. Another photolabile inhibitor of the (Na^+-K^+)ATPase, 3-azidoacetylstrophanthidin has been reported by Tobin et al. (1976). However, the authors report a low yield of irreversible inactivation of the enzyme after irradiation (only 10%). In a brief report, a photoactivatable derivative of ouabain appears to be a useful affinity label of the (Na^+-K^+)ATPase enzyme system (Forbush et al., 1978). The authors report radiolabeling of the large peptide chain of the enzyme system. Periodate-oxidized ouabain has also been used as an affinity label of the digitalis receptor in (Na^+-K^+)ATPase (Hegyvary, 1975). When this compound was incubated with renal plasma

† From the Centre de Biochimie, Unité d'Enseignement et de Recherche Scientifique et Technique, Université de Nice, 06034 Nice, France. *Received June 22, 1978.* This work was supported by the Centre National de la Recherche Scientifique (LA 201), the Commissariat à l'Energie Atomique, and the Institut National de la Recherche Médicale (ATP 78-95).

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[1] Abbreviations used: (Na^+-K^+)ATPase, (sodium + potassium)-activated adenosinetriphosphatase; FNAP, 4-fluoro-3-nitrophenyl azide; NAP-ethylenediamine, *N*-(2-nitro-4-azidophenyl)ethylenediamine; NAP-strophanthidin, *N*-(strophanthidin)-*N'*-(2-nitro-4-azidophenyl)-ethylenediamine; NAP-ouabain, *N*-(ouabain)-*N'*-(2-nitro-4-azidophenyl)ethylenediamine; PNPP, *p*-nitrophenyl phosphate; PNPPase, *p*-nitrophenylphosphatase; TEA, triethanolamine; $NaDodSO_4$, sodium dodecyl sulfate; UV, ultraviolet; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; IR, infrared.

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membrane and sodium [^{3}H]borohydride, the only peptide chain labeled was the large subunit of the (Na^+-K^+)ATPase.

The present report describes the synthesis and characterization of photoreactive derivatives of ouabain and strophanthidin which have a large specific radioactivity and high chemical reactivity. Studies with a purified (Na^+-K^+)ATPase from the electric organ of *Electrophorus electricus* as well as with a microsomal preparation from the same tissue indicate that these compounds are useful affinity labels for the cardiac glycoside receptor.

Experimental Procedures

Materials

Ouabain was purchased from Sigma Chemical Co. and strophanthidin from E. Merck, Darmstadt. [^{3}H]Ouabain (14 Ci/mmol) was obtained from New England Nuclear Co. Sodium [^{3}H]borohydride was available from the Commissariat à l'Energie Atomique (Paris) at 25 Ci/mmol. A live electric eel, *Electrophorus electricus*, was purchased from World Wide Scientific Animals, Ardsley, N.Y. The electric tissue was dissected and frozen at −70 °C and was used in enzyme and membrane preparations. 4-Fluoro-3-nitroaniline was obtained from Fluka.

Methods

Synthesis of NAP-ethylenediamine. FNAP was prepared from 4-fluoro-3-nitroaniline by the method of Fleet et al. (1972). FNAP (1 mmol) was incubated with ethylenediamine (6 mmol) in dimethyl sulfoxide at 50 °C for 30 min. Solvent was removed by lyophilization and the product was recrystallized in a minimum of dimethyl sulfoxide (yield > 90%). Free amino reactivity was tested with trinitrobenzenesulfonic acid according to Inman & Dintzis (1969). UV-visible: maxima at 256 and 445 nm (in methanol). Infrared: strong absorption 2120 cm^{-1} (azide).

Synthesis of NAP-strophanthidin. Typically, 28 mM strophanthidin and 14 mM NAP-ethylenediamine were incubated with 17 mM sodium cyanoborohydride in methanol at 50 °C for 18 h in the dark. The orange-colored reduced product (R_f = 0.47) was purified from the orange-colored Schiff base (R_f = 0.77) and NAP-ethylenediamine (R_f = 0.09) using preparative thin-layer silica gel plates with chloroform–methanol (2:1) as the developing solvent. For a radiolabeled derivative, sodium [^{3}H]borohydride was used as the reductant. In this case, strophanthidin and NAP-ethylenediamine were incubated as before, but without sodium cyanoborohydride, until approximately 90% of the amine has been coupled to strophanthidin as followed by TLC analysis. At this point, sodium [^{3}H]borohydride, dissolved in isopropyl alcohol–water (90:10), 100 mM at 0 °C, was added to the reaction solution. A mole of sodium borohydride was added for each mole of NAP-ethylenediamine present. The reduction was stopped after 1 min by the addition of acid, the solution was applied to TLC plates, and the product isolated as before. A specific radioactivity of 4–5 Ci/mmol was obtained (yield = 20%). The product migrated as a single radioactive peak in chloroform–methanol (2:1) and ethanol–acetic acid (96:4) solvent systems on TLC silica gel plates.

Synthesis of NAP-ouabain. Ouabain (10 mM) and sodium periodate (11 mM) were incubated in an aqueous solution for 1.5 h at room temperature. There was a quantitative conversion of ouabain to the oxidized product as determined by: (1) the decrease in optical density at 220 nm due to the destruction of periodate (ϵ_{220} (IO_4^-) = 9500; ϵ_{220} (IO_3^-) = 1100); (2) TLC analysis of the reaction using [^{3}H]ouabain as a tracer in the reaction with chloroform–methanol (2:1) as the developing solvent on silica gel plates (R_f (ouabain) = 0.27, R_f (oxidized ouabain) = 0.57). At the end of the reaction, lead(II) acetate was added to 5 mM and the precipitate formed was removed by centrifugation. The supernatant was lyophilized and the product dissolved in methanol. The oxidized ouabain (47 mM) was incubated with NAP-ethylenediamine (47 mM) and 65 mM sodium cyanoborohydride at room temperature for 4 h. The orange product, NAP-ouabain (R_f = 0.45), was isolated on a TLC plate using ethanol–acetic acid (96:4) as the developing solvent. A radiolabeled [^{3}H]-NAP-ouabain was prepared in a similar manner from [^{3}H]ouabain. The reactants were less concentrated, 1.0 mM [^{3}H]ouabain, so that longer reaction times were necessary: 8 h for the oxidation, 7 h for the reductive amination (yield 20%). The product (approximately 1 Ci/mmol) migrated as a single radioactive peak in three separate TLC solvent systems: ethanol–acetic acid (96:4), chloroform–methanol (2:1), and butanol–acetic acid–water (4:1:1). Generally 60–70% of the specific radioactivity was retained during the oxidation of [^{3}H]ouabain. The following extinction coefficients were used: ouabain and strophanthidin, ϵ_{220} = 14 500; NAP-ouabain, ϵ_{460} = 5000; NAP-strophanthidin, ϵ_{445} = 5000 in methanol.

(Na^+-K^+)ATPase Binding Studies. A microsomal fraction from the electric organ of *Electrophorus electricus* was prepared according to the method of Albers et al. (1963). A Lubrol solubilized and purified (Na^+-K^+)ATPase was obtained from the same tissue using the method of Dixon & Hokin (1978).

The activity of the (Na^+-K^+)ATPase in both the purified and microsomal preparations was followed by two methods. The ATPase activity was assayed at 340 nm with a pyruvate kinase–lactate dehydrogenase linked system using conditions previously described (Gache et al., 1976). The K^+-stimulated *p*-nitrophenylphosphatase (PNPPase) activity was determined spectrophotometrically by following the increase in optical density at 410 nm due to the liberation of *p*-nitrophenol (Gache et al., 1977).

The binding of ouabain, strophanthidin, and their derivatives to the (Na^+-K^+)ATPase was determined by the inhibition of the PNPPase activity. The solubilized (Na^+-K^+)ATPase (usually 10 μg of protein) was preincubated with various concentrations of the NAP derivatives or parent compounds in 250 μL of an incubation buffer, 50 mM TEA, 5 mM Mg^{2+}, 5 mM P_i-TEA, pH 7.4, for 30 min at 37 °C. The solution was then diluted to 1.0 mL of 50 mM TEA, 20 mM Mg^{2+}, 20 mM K^+, 20 mM PNPP, 2 mM dithiothreitol for the determination of the enzyme activity at 25 °C. Each assay was performed in duplicate.

The binding of [^{3}H]ouabain to the membrane preparation was determined by filtration techniques. Generally, 25-μg protein aliquots of the microsomal preparation were incubated with various concentrations of the ligand in 1.0 mL of the same incubation buffer for 30 min at 37 °C. The membranes were then collected on Millipore HAWP filters and rinsed twice with 5-mL aliquots of 50 mM TEA, pH 7.4, at 4 °C. The filters were then added to vials for radioactivity determinations by scintillation techniques. In all cases the "specific binding" was determined from the difference between this value and from the radioactivity incorporated in a sample prepared in the same manner except for a 30-min preincubation with 1 mM ouabain (unlabeled) prior to the addition of [^{3}H]ouabain.

Irreversible inactivation of (Na^+-K^+)ATPase by NAP derivatives was determined using a purified enzyme preparation. The enzyme was incubated in the presence of 4 μM NAP-strophanthidin or NAP-ouabain in 5 mM Mg^{2+}, 5 mM

P_i buffer at 37 °C. The solution was irradiated with UV light for 3 min and the NAP ligands were dissociated at 37 °C by diluting an aliquot of the solution 100-fold in 50 mM TEA, pH 7.4. Aliquots were removed over time for PNPPAse activity determinations and the enzyme was reconcentrated by centrifugation. Then this process of reincubating with fresh photoaffinity label, irradiating, and diluting was repeated for as many as 4 to 5 cycles. UV light was found to inactivate the enzyme; therefore, the inactivation due to the NAP derivative binding irreversibly was obtained by correcting for the UV photoinactivation obtained in a control experiment where the enzyme was irradiated in the absence of NAP-ouabain or NAP-strophanthidin.

Irreversible binding of [^{3}H]NAP-strophanthidin and [^{3}H]NAP-ouabain after irradiation with UV light was studied using both the solubilized (Na^+-K^+)ATPase and the *Electrophorus electricus* microsomal preparation. An aliquot of either preparation (60–100 μg of protein) was incubated in 100 μL of the Mg^{2+}–P_i buffer system with either [^{3}H]NAP derivative for 30 min at 30 °C. The solutions were then irradiated for various times with UV light (Mineralight, Model UVSL13) 7 cm from the light source. The irradiated solutions were then diluted 10-fold with buffer and centrifuged for 20 min at 50 000*g*. The pellets were dissolved in 50 mM Tris, 1% $NaDodSO_4$, 2.5% mercaptoethanol and heated at 37 °C for 30 min before separation by $NaDodSO_4$ gel electrophoresis.

Sodium Dodecyl Sulfate Electrophoresis. A discontinuous $NaDodSO_4$ gel electrophoresis system was used to separate the membrane proteins as previously described by Laemmli (1970) with one modification. *N,N'*-Diallyltartardiamide was used to cross-link the acrylamide instead of the normal methylenebisacrylamide (Anker, 1970). A slab gel apparatus was used analogous to that described by Reid & Bieleski (1968). The gels were stained with Coomassie blue according to Fairbanks et al. (1971) before slicing into 2-mm segments. The slices were dissolved in 5% periodic acid before the addition of scintillation liquid for counting of the radioactivity.

Results

Synthesis and Characterization of NAP-ouabain and NAP-strophanthidin. The products obtained from the reductive coupling of NAP-ethylenediamine with strophanthidin or periodate-oxidized [^{3}H]ouabain were purified with a yield of approximately 20% (mole of product/moles of radioactive reactant). When 25 Ci/mmol [^{3}H]$NaBH_4$ was used to prepare NAP-strophanthidin, a product of 4.7 Ci/mmol was obtained. It should be possible to prepare [^{3}H]NAP-ouabain at a level of 9–10 Ci/mmol starting from 14 Ci/mmol of [^{3}H]ouabain commercially available, since only 30% of the radioactivity is lost during the oxidation and coupling steps. Usually a product with a specific radioactivity of 1–2 Ci/mmol was prepared after isotopic dilution of the [^{3}H]ouabain.

NAP-ouabain and NAP-strophanthidin were characterized by IR, NMR, and UV–visible spectroscopy. The salient spectral features for both the aryl azide and α,β-unsaturated lactone (of the steroid portion) ring systems were observed. Table I summarizes these data for NAP-strophanthidin, and similar results were obtained for NAP-ouabain. UV difference spectroscopy at 220 nm was used to estimate the stoichiometry of the coupling reaction, i.e., the number of moles of 2-nitrophenyl azide per mole of steroid. Using an equivalent amount of NAP-ethylenediamine in the reference cell in methanol, values of 0.90 mol of NAP-ethylenediamine/mol and 0.80 mol of NAP-ethylenediamine/mol were obtained for NAP-strophanthidin and NAP-ouabain, respectively. The stoichiometry of the NAP-ouabain reaction was calculated using ^{3}H-labeled, oxidized ouabain of known specific radioactivity in the reaction and, in this case, a ratio of 0.90 was estimated.

Table I: Summary of Spectroscopic Properties of NAP-strophanthidin

technique	absorption maxima	functional group
IR	2120 cm^{-1}	azide
	1745 cm^{-1}	α,β-unsaturated lactone
NMR[a]	δ 5.92	C-22 vinylic proton of lactone ring[b]
	δ 7.25, 7.45	phenyl azide
UV–vis	220 nm	α,β-unsaturated lactone
	258, 465 nm	phenyl azide

[a] Samples dissolved in dimethyl-d_6 sulfoxide. [b] Kyte (1972).

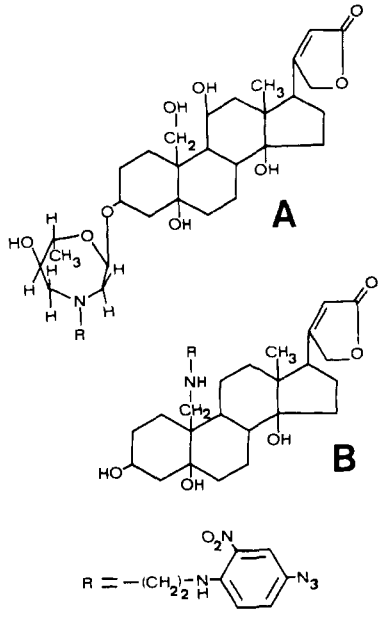

FIGURE 1: The proposed structures of the photoaffinity labels. The products of the reductive amination of NAP-ethylenediamine with ouabain and strophanthidin are shown: NAP-ouabain, structure A; NAP-strophanthidin, structure B.

The most likely structures for the products are shown in Figure 1. NAP-ouabain has the NAP-ethylenediamine coupled to the sugar portion of the molecule. Structure A can be obtained through a mechanism analogous to that previously published (Khym, 1963). NAP-strophanthidin is formed through the reductive amination of the C-19 aldehyde side chain on the steroid portion of the molecule, as shown in structure B.

The photoreactivity of NAP-ouabain or NAP-strophanthidin was followed by changes in the UV–visible spectrum upon irradiation with UV light. Figure 2 shows the changes that were observed for NAP-strophanthidin. The decreases in the optical densities at 258 and 445 nm are characteristic of the photoreaction of the aryl azide moiety. The absorption spectra before and after irradiation were identical if the reaction was carried out with sodium borohydride or with sodium cyanoborohydride. Therefore, under the reaction conditions used, no significant borohydride-mediated reduction of the aryl azide occurred. NAP-ouabain was also found to be photosensitive by the same methods.

Binding of the Photoaffinity Labels to the Digitalis Receptors in the Dark. The inhibition of the (Na^+-K^+)ATPase activity by the NAP derivatives in the dark is evidence that these products are able to bind reversibly to the enzyme, a

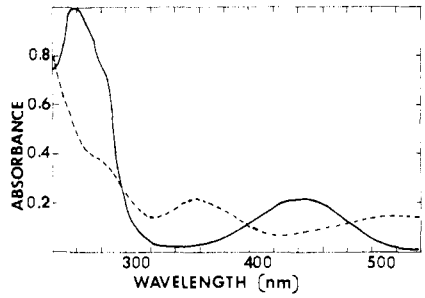

FIGURE 2: Absorbance spectrum of NAP-strophanthidin. The spectrum for NAP-strophanthidin, 44 μM in methanol, was made before (solid line) and after (broken line) irradiation for 5 min with UV light.

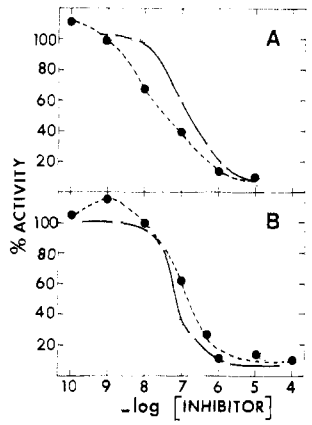

FIGURE 3: The effect of cardiac glycosides on the (Na^+-K^+)ATPase enzyme activity. A solubilized, purified (Na^+-K^+)ATPase preparation was incubated with the inhibitors or their NAP derivatives in 5 mM Mg^{2+}, 5 mM P_i, TEA buffer as described in Methods, and the K^+-stimulated PNPPase activity of the enzyme was determined. The specific ATPase activity of the enzyme was 13 μmol of P_i $(min)^{-1}$ (mg of protein)$^{-1}$ at 37 °C. The log–dose curves are displayed in panel A, strophanthidin (●---●) and NAP-strophanthidin (○—○), and panel B, ouabain (●---●) and NAP-ouabain (○—○).

property typical for active digitalis type compounds. When the purified (Na^+-K^+)ATPase was incubated with various concentrations of either NAP-strophanthidin or NAP-ouabain, log–dose inhibition curves were obtained as shown in Figure 3. The concentrations of the half-maximal effect (ID_{50}) for all of the derivatives are in the region of 0.1 μM. These results indicate that the addition of a NAP-ethylenediamine side chain to strophanthidin or ouabain has not significantly affected the ability of these compounds to interact directly with the digitalis binding site on the enzyme since the ID_{50}'s for the NAP derivatives and their parent compounds are very similar. Ouabain and strophanthidin appeared to slightly stimulate the enzyme activity at low concentrations, 1–0.1 nM, an observation that has previously been reported (Peters et al., 1974; Cohen et al., 1976). However, NAP-ouabain and NAP-strophanthidin did not display this effect.

The binding of [^{3}H]ouabain to the microsomal preparation of the electric organ of *Electrophorus electricus* was determined using a rapid filtration technique. The binding isotherm is shown in the inset of Figure 4. Half-maximal binding ($K_{0.5}$) occurred at 0.59 μM and the number of ouabain sites was found to be 196 pmol/mg of protein.

NAP-ouabain was able to compete with [^{3}H]ouabain for the binding sites on the membrane as shown in Figure 4. NAP-ouabain was preincubated with the membranes and the binding of [^{3}H]ouabain was followed over a 50-min period. The half-maximal protection given by NAP-ouabain against [^{3}H]ouabain binding is observed at 0.5 μM NAP-ouabain.

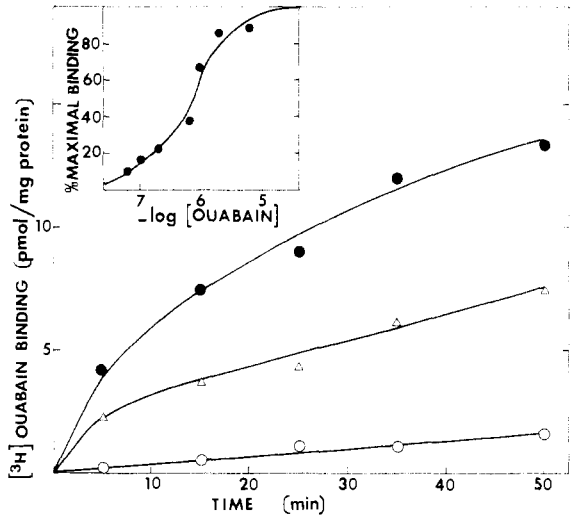

FIGURE 4: The binding of [^{3}H]ouabain to a microsomal preparation of *Electrophorus electricus* tissue. NAP-ouabain, at various concentrations, was preincubated for 30 min at 37 °C with membranes in 5 mM Mg^{2+}, 5 mM P_i, 50 mM TEA, pH 7.4. [^{3}H]Ouabain, 0.2 μM, was added and the rate of binding was followed at 30 °C by rapid filtration techniques (see Methods) in the presence of 5 μM NAP-ouabain (○); 0.5 μM NAP-ouabain (△); control (no NAP-ouabain present) (●). The inset shows the binding curve of [^{3}H]-ouabain to the microsomal preparation using the same buffer as in Figure 4. See Methods for experimental details. The curve drawn is the best fit of the data to the Hill equation with a Wang 2200 calculator by methods previously described (Gache et al., 1976).

These data confirm that ouabain and NAP-ouabain bind to their receptor with a very similar affinity.

Irreversible Binding of the Affinity Labels. Irreversible inhibition of the PNPPase activity observed when the NAP derivatives are irradiated in the presence of the enzyme is evidence that the photoaffinity labels covalently bind to the (Na^+-K^+)ATPase. NAP-ouabain or NAP-strophanthidin were incubated with the purified (Na^+-K^+)ATPase and the complexes were irradiated with UV light. After irradiation, the photolyzed NAP derivatives were dissociated from the enzyme by dilution and the remaining PNPPase activity was measured. Either NAP-ouabain or NAP-strophanthidin promoted a 30–35% irreversible inactivation of the enzyme. The enzyme could be reconcentrated by centrifugation, reincubated with fresh NAP-strophanthidin, and irradiated again. With 5 cycles performed in this manner, the enzyme activity decreased to the following levels, 70%, 53%, 45%, 35%, 30%; that is, after 5 cycles of irradiation with fresh NAP-strophanthidin, as much as 70% of the activity was irreversibly inhibited. Similar experiments with NAP-ouabain were not practical due to its slow dissociation from the enzyme ($t_{1/2}$ = 60 min). There was some inactivation due to UV light so these data are corrected for this photoinactivation.

Photoaffinity Labeling of the Purified (Na^+-K^+)ATPase. The results of the irreversible binding of the [^{3}H]NAP derivatives to the enzyme after irradiation with UV light are shown in Figure 5. After the photolysis and electrophoresis, only one protein band is radiolabeled on the gel with either NAP derivative incubated in solution with the purified (Na^+-K^+)ATPase. This band was found to correspond to the large molecular weight subunit of the enzyme (M_r = 93 000). Approximately 30% of the labeling was nonspecific, that is, not protectable by 1 mM ouabain for either NAP-ouabain or NAP-strophanthidin. Greater than 95% of the total radioactivity incorporated into the gel is found in this region and the low molecular weight band (M_r = 47 000) was not labeled to any extent, specifically or nonspecifically.

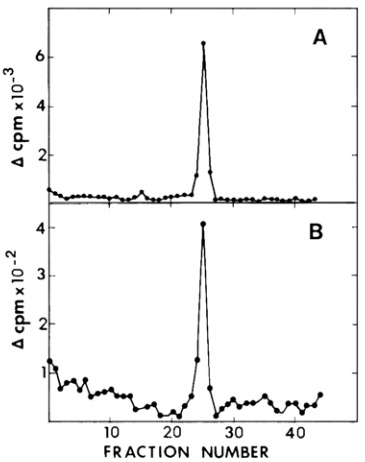

FIGURE 5: $NaDodSO_4$ gel electrophoresis of radiolabeled purified $(Na^+\text{-}K^+)$ATPase after photolysis. The experimental details are described in Methods. The proteins were separated on a gel with a 6–15% linear gradient in acrylamide. The gels were sliced into 2-mm segments. Panel A shows the radioactivity incorporated when the enzyme was irradiated in the presence of 2 μM [^{3}H]NAP-strophanthidin; panel B shows the results when approximately 0.2 μM [^{3}H]NAP-ouabain was used. Both panels A and B represent specific labeling which is the difference in radioactivity incorporated in experiments conducted in the presence and absence of 1 mM ouabain.

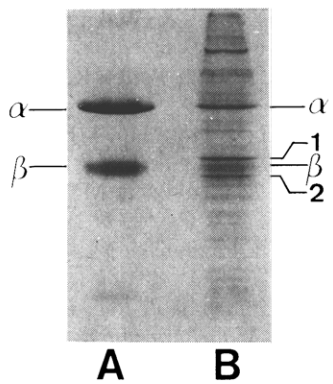

FIGURE 6: $NaDodSO_4$ electrophoresis of the $(Na^+\text{-}K^+)$ATPase and microsomal preparations. Gel A is the solubilized purified enzyme, and gel B shows the results for the microsomal preparation from the electric organ of *Electrophorus electricus*. The bands labeled α and β correspond to the large and small chain, respectively, of the $(Na^+\text{-}K^+)$ATPase. The bands labeled 1 and 2 are discussed in the text.

Several controls were used in order to indicate that this labeling was a photoaffinity process. First, the radioactive incorporation was protected when the enzyme was preincubated with 1 mM ouabain. There was no labeling in the absence of UV light. The radioactivity incorporated was proportional to the time of irradiation for the first 2 min at which time the maximum incorporation was obtained.

Photoaffinity Labeling of Electrophorus electricus Electric Organ Membranes. The microsomal preparation from the electric organ was also used in labeling studies to characterize the ouabain receptors in a membrane system. The position of the characteristic ATPase bands in the membrane samples on $NaDodSO_4$ gels was determined by an electrophoretic comparison with the solubilized purified $(Na^+\text{-}K^+)$ATPase from the same tissue as shown in Figure 6 (bands labeled α and β). The results of the photolabeling studies are displayed in Figure 7. The precise positions of the proteins of the $(Na^+\text{-}K^+)$ATPase in the gels were determined since the gels were stained with Coomassie blue before slicing and are labeled α and β in the figure. This procedure also had the advantage of lowering the background radioactivity. It is clear that the

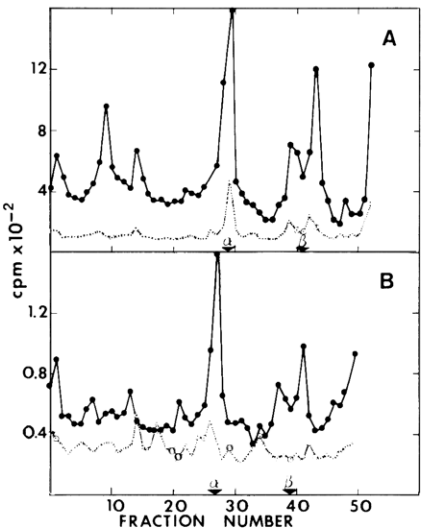

FIGURE 7: Electrophoresis of membrane proteins after photolabeling. After irradiation, the proteins were separated on a $NaDodSO_4$ gel with a linear gradient of 6–15% in acrylamide. Gels were sliced into 2-mm segments after staining with Coomassie blue. (Panel A) Membranes were incubated with 2 μM [^{3}H]NAP-strophanthidin (●—●) or with 2 μM [^{3}H]NAP-strophanthidin plus 1 mM ouabain (○···○). (Panel B) Incubation and irradiation of the membranes was carried out in the presence of approximately 0.2 μM [^{3}H]NAP-ouabain (●—●) or 0.2 μM [^{3}H]NAP-ouabain plus 1 mM ouabain (○···○). The arrows in both panels labeled α and β indicate the exact position of the bands observed for the large and small chains of the $(Na^+\text{-}K^+)$ATPase.

major band labeled is the large chain of the $(Na^+\text{-}K^+)$ATPase in both NAP-ouabain and NAP-strophanthidin labeling experiments. In contrast to the solubilized enzyme experiments, the nonspecific labeling of this protein is very low with less than 10% of the radioactivity not protected by 1.0 mM ouabain. Approximately 33% and 40% of the total radioactivity irreversibly bound to the membrane was incorporated into this protein with 0.2 μM [^{3}H]NAP-ouabain and 2 μM [^{3}H]NAP-strophanthidin, respectively. Of the two proteins of the $(Na^+\text{-}K^+)$ATPase enzyme, α and β, only the α band was labeled by these two photoderivatives. This was seen in the purified enzyme photolabeling experiments as well.

It is also evident that there are two other protein bands significantly labeled in the gels. They are found on either side of the $(Na^+\text{-}K^+)$ATPase β protein and correspond to the bands designated 1 and 2 in Figure 6 with molecular weights of approximately 50 000 and 45 000, respectively. The distinction between the positions of these three bands was possible only since the gels were stained prior to slicing. The labeling of these bands is specific since these protein chains are protected with 1 mM ouabain. The same controls were performed with the membranes as with the purified enzyme to verify that this labeling was due to a photoaffinity process.

Discussion

Two photoaffinity labels have been synthesized by methods which give products in practical yields with a specific radioactivity of at least 5–6 Ci/mmol. The products obtained have the photoreactive side chain coupled to different portions of the cardiac glycoside molecule: NAP-strophanthidin to the steroid ring system and NAP-ouabain to the oxidized sugar.

The NAP derivatives were found to have the properties necessary for effective photolabels. First, the products contained a photoactivatable phenyl azide moiety as determined spectrally. In addition, NAP-strophanthidin and NAP-ouabain were specifically bound to the digitalis receptor sites in the presence of Mg^{2+} and inorganic phosphate. The binding

in the dark was studied by two methods: (1) inhibition of the enzymatic activity of the (Na^+-K^+)ATPase where the NAP derivatives have a half-maximal effect similar to the parent compounds; (ii) the ability of NAP-ouabain to compete with [^{3}H]ouabain binding to the *Electrophorus* membrane preparation.

Finally, both NAP-ouabain and NAP-strophanthidin were found to irreversibly bind to the ouabain receptor since they irreversibly inhibited the activity of the purified (Na^+-K^+)-ATPase after UV irradiation. As much as 70% of the activity was irreversibly lost in the NAP-strophanthidin experiments. The yields are much higher than previously reported where a 10% inhibition was observed when 3-azidoacetylstrophanthidin was used (Tobin et al., 1976).

There are two subunits in the (Na^+-K^+)ATPase system: a large chain with a molecular weight in the region of 100 000 and a small chain glycoprotein (M_r = 50 000). The functions of these two subunits, which have been isolated from a number of sources, are not well defined. It is known that the large chain is phosphorylated by ATP (Dixon & Hokin, 1974; Schwartz et al., 1975). It has also been established that digitalis binds to the phosphorylated form of the enzyme. When the purified (Na^+-K^+)ATPase was irradiated with UV light in the presence of either [^{3}H]NAP-ouabain or [^{3}H]-NAP-strophanthidin, only the high molecular weight protein was labeled. There was no labeling of the smaller protein, either specifically or nonspecifically. These results indicate that the larger protein, which is phosphorylated by ATP, contains the digitalis binding site and support the conclusions of earlier studies (Hegyvary, 1975; Ruoho & Kyte, 1974). In a brief communication, it was also reported that a small proteolipid (M_r = 12 000) was labeled in addition to the large chain of the kidney (Na^+-K^+)ATPase by a photoactivatable derivative of ouabain (Forbush et al., 1978). The functional significance of this small protein chain remains to be understood.

It has been reported that there are separate binding sites for the sugar and steroid portion of the cardiac glycosides (Yoda, 1973). Since both of the earlier affinity labeling studies utilized derivatives containing the reactive chemical group in the sugar region of the molecule, little could be said with respect to the steroid binding site. In the present report, the large polypeptide chain is labeled exclusively no matter whether the NAP-ethylenediamine side chain is attached to the sugar or steroid portion of the molecule. Due to the large amount of radioactivity incorporated in the NAP-strophanthidin experiments, even a small 5% labeling of the smaller chain would be easily detectable in these experiments. These results strongly support the notion that the digitalis binding center containing both the steroid and sugar binding sites is located on the larger protein of the (Na^+-K^+)ATPase and that the smaller protein is not involved directly in the binding.

The labeling experiments with the *Electrophorus electricus* membrane preparation indicate that the large chain of the (Na^+-K^+)ATPase is the major protein radiolabeled. As in the solubilized enzyme preparation, the small subunit does not irreversibly bind the NAP derivatives. However, there are two other protein bands which do incorporate significant amounts of radioactivity when either NAP product is used. The nature of these proteins has not been determined; yet they appear to be involved in a specific, high affinity process since labeling occurs when 0.2 μM [^{3}H]NAP-ouabain and this labeling is ouabain protectable. It is not clear whether these proteins represent other receptors for ouabain or whether they are proteins found in the immediate vicinity of the (Na^+-K^+)-ATPase receptor site. It is also possible that these bands represent proteolytic fragments of the large polypeptide chain which have been labeled as well, although this theory was not tested here in this work. In any case, these proteins may have a role in the specific binding of digitalis to these membranes in one or more receptor sites.

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