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Specific Photoaffinity Labeling of the Digitalis Binding Site of the Sodium and Potassium Ion Activated Adenosinetriphosphatase Induced by Energy Transfer†

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ABSTRACT: A ouabain *p*-aminobenzenediazonium derivative with a high specific radioactivity has been synthesized from ouabain and used as a photolabel for the (sodium plus potassium)-activated adenosinetriphosphatase from *Electrophorus electricus* electric organ and from dog kidney. In the dark it binds reversibly to the digitalis receptor site, with binding characteristics comparable to those of ouabain. The photoactivation of the ouabain derivative to produce covalent labeling of the receptor was obtained by energy transfer from a tryptophan residue in the (Na^+,K^+)ATPase to the ouabain *p*-aminobenzenediazonium molecule bound at the active site. The great advantage of this procedure compared to previous methods is that free molecules of the photoactivatable derivative are not photodecomposed. Analysis of the photolabeled polypeptides on sodium dodecyl sulfate gel electrophoresis showed that over 90% of the total radioactivity incorporated was found in the large molecular weight α-chain of the kidney enzyme (M_r 93 000). The same specific labeling of the α-subunit was obtained with a crude microsomal fraction from *Electrophorus electricus*. A mild tryptic fragmentation of the subunit into two peptide fragments of M_r 58 000 and 41 000, respectively, shows that the digitalis receptor is located in the N-terminal 41 000 fragment.

The (Na^+,K^+)ATPase[1] catalyzes the cotransport of Na^+ and K^+ through the plasma membrane. The enzyme is composed of an α-chain (M_r 90 000–100 000) which is known as the catalytic subunit and of a glycosylated β-chain (M_r 45 000–58 000) which has no known function. The presence of a smaller γ-subunit which is a proteolipid component has also been described [for references see Jørgensen (1982)].

Digitalis compounds like ouabain specifically inhibit cell functions carried out by the enzyme, i.e., Na^+ and K^+ transport and ATP hydrolysis. These molecules interact with a site situated on the extracellular surface of the (Na^+,K^+)ATPase molecule. The ouabain binding site has two functionally distinct regions, one specific for the lactone ring and the steroid portion and one specific for the sugar moiety. Digitalis compounds are particularly important in heart pharmacology since they are among the most potent cardiotonic drugs. For all these reasons extensive efforts have been made to know more about the digitalis binding sites [for references see Jørgensen (1982)].

A general procedure for making compounds which provoke photosuicide inactivation of enzymes and receptors has been described recently. The procedure consists in using an aryldiazonium derivative as a photoactivatable label (Goeldner & Hirth, 1980; Goeldner et al., 1982) which produces its photodecomposition after it is complexed through the receptor site. The photodecomposition is induced by energy transfer through a tryptophan residue of the receptor.

The present paper describes the synthesis and the properties as an affinity label for the (Na^+,K^+)ATPase of ouabain *p*-aminobenzenediazonium derivative (ABD-ouabain), an ouabain derivative producing a photosuicide inactivation of the enzyme.

Experimental Procedures

Materials

Ouabain, diphenylcarbamoyl chloride (DPCC) treated trypsin, and the diTris salt of *p*-nitrophenyl phosphate were obtained from Sigma. [^{3}H]Ouabain (14 Ci/mmol) was ob-

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[1] Abbreviations: ABD-ouabain, ouabain *p*-aminobenzenediazonium derivative; APh-ouabain, ouabain *p*-aminophenol derivative; DDF, *p*-(dimethylamino)benzenediazonium fluoroborate; Boc, *tert*-butyloxycarbonyl; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TEA, triethanolamine; DPCC, diphenylcarbamoyl chloride; NMR, nuclear magnetic resonance; MS, mass spectrometry; (Na^+,K^+)ATPase, (sodium plus potassium)-activated adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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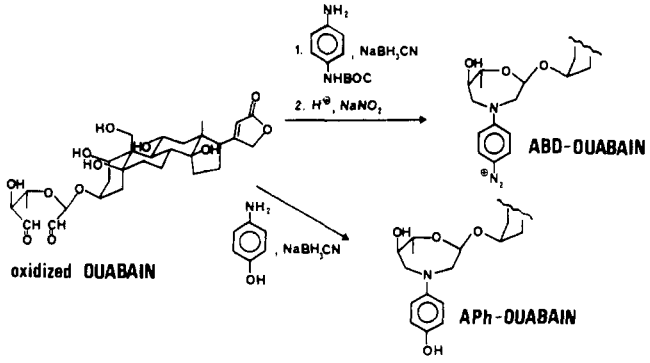

FIGURE 1: Reaction scheme for the synthesis of ABD-ouabain and APh-ouabain.

tained from New England Nuclear. Other analytical grade chemicals were from Prolabo (Paris), Merck (Darmstadt), or Fluka (Buchs). GSWP filters were obtained from Millipore (Mutzig). Bio-Gel P-2 was from Bio-Rad Laboratories (Richmond, CA). Live electric eel *Electrophorus electricus* was purchased from World Wide Scientific Animals (Ardsley, NY). Frozen dog kidneys were from Pel-Freeze (Rogers, AR). For irradiation experiments a 1000-W xenon/mercury lamp (Hanovia) with a grating monochromator (Schoeffel, GM 250) and a thermopile (Kipp & Zonen) were used.

Methods

The principles of synthesis described below are summarized in Figure 1.

Synthesis of Boc-p-phenylenediamine. A 0.3 M solution of di-*tert*-butyl dicarbonate in THF was added with stirring to a 0.3 M solution of freshly sublimated *p*-phenylenediamine (110 °C at 0.1 mmHg) in THF. The reaction mixture was stirred for an additional 4 h. After evaporation of the solvent under vacuum, the residue was hydrolyzed and extracted twice with an equivalent volume of ethyl acetate. The organic phase was dried over sodium sulfate, and after evaporation it was chromatographed by using a preparative thin-layer silica gel plate [R_f 0.5 with ethyl acetate–hexane (1:1) as developing solvent]. The compound was recrystallized from ethyl acetate and a minimum of hexane; yield 60%.

Synthesis of ABD-Ouabain. Oxidized ouabain (Rossi et al., 1980) 50 mM in absolute methanol (20 mL) was incubated with Boc-*p*-phenylenediamine. Acetic acid was added to bring the apparent pH between 5 and 6. After addition of sodium cyanoborohydride to a final concentration of 75 mM, the reaction mixture was stirred for 4 h at room temperature. Hydrolysis of the medium was followed by a 5-fold chloroform extraction and the organic phase dried over sodium sulfate. Removal of the solvent under vacuum led to a syrupy residue (about 800 mg) which was shown to be a mixture of three main compounds on analytical thin-layer silica gel chromatography: R_f 0.76 corresponded to the starting amine; R_f 0.46 corresponded to the coupled aromatic amino ouabain derivative (the spot is detected with an UV lamp 254 nm); R_f 0.06 corresponded to the starting oxidized ouabain developed in a chloroform–methanol (9:1) solvent. A minor unknown compound appeared at R_f 0.53. Chromatography on a silica gel column (50 g of silica gel) with the same eluant separated the desired pure compound (180 mg; yield 20%) in addition to a comparable amount which was contaminated with the unknown compound. The Boc-protecting group was easily detected by NMR spectroscopy (sharp singlet at 1.54 ppm).

Diazotization of the Aromatic Amine. The amino-protecting Boc group was removed by stirring in a 0.2 M TFA solution (0.5 mL) at −5 °C for 45 min under nitrogen. The free aromatic amine was then directly diazotisized by treatment with sodium nitrite (10% excess) at −10 °C. Solid nitrite was added during 20 min in the absence of light. After stirring for another 20 min under the same conditions, TFA was lyophilized. The yellow residue was taken up in 0.3 M tetrafluoroboric methanolic solution (0.6 mL) and immediately chromatographed on a Bio-Gel P-2 column (100 g) and eluted with water in the absence of light. After a 80-mL head fraction, the diazonium salt was eluted in four fractions of 15 mL; estimated yield (by UV analysis) 60%.

Synthesis of APh-Ouabain. This compound was synthesized by coupling *p*-aminophenol to oxidized ouabain in the presence of sodium cyanoborohydride as for the precursor of ABD-ouabain. APh-ouabain was purified on preparative thin-layer chromatography with development in acetone–methanol (9:1): R_f 0.40; yield 32%. The spectroscopic properties of this compound are the following: UV gave λ_{max} 304 nm (ϵ 1450). MS analysis was performed on a Thomson TH-208B double focusing instrument with direct introduction in the ionization chamber on a gold support (Constantin & Hueber, 1982). The temperature was 280 °C, the electron energy was 70 eV, and the ion acceleration was 8 kV. MS: $M^+ = 659$ (molecular ion), m/e 223 (100: substituted sugar moiety). NMR was taken in acetone-d_6 on a Cameca 250 MHz with Fourier transform. NMR δ 6.75 and 6.71 (aromatic protons, 4 H), 5.88 (s, C-22 proton), and δ 4.98 and 4.85 (AB quartet, J = 20 Hz, C-21 methylene, 2 H).

Photodecomposition of ABD-Ouabain. A 0.35 mM solution of ABD-ouabain was irradiated in a Pyrex reactor with a Phillips lamp HPK 125-W type 572033/00. After lyophilization the properties of the phototransformed derivative were compared to those of APh-ouabain. Analytical thin-layer chromatography showed a mixture of three compounds. The main compound formed comigrated with synthetic APh-ouabain. The UV spectra of the photolyzed sample as well as of the synthetic APh-ouabain are shown in Figure 2. We did not try to identify the unknown compounds.

Synthesis of Radioactive ABD-Ouabain. A 33 mM methanolic solution of oxidized [^{3}H]ouabain (1 mCi, specific radioactivity 1 Ci/mmol) was incubated in an ice bath with an equivalent amount of Boc-*p*-phenylenediamine and sodium cyanoborohydride at a final concentration of 66 mM. The pH was adjusted to 6.2 with a 2% acetic acid solution in methanol, and the reaction mixture was kept in the cold overnight. The crude reaction mixture was chromatographed directly on an analytical thin-layer silica gel plate (about 6 cm wide and 10 cm high) with chloroform–methanol (8.5:1.5) as the developing solvent. A 0.5 cm wide lateral strip was removed and analyzed for radioactivity. The band containing the desired compound (R_f 0.54 detectable with UV lamp at 254 nm) represented about 40% of the total radioactivity. Extraction of the compound from silica gel with methanol resulted in a 17% yield. After removal of the silica gel over glass wool and centrifugation, the methanolic solution was dried under a nitrogen stream and used directly for the diazotization. The procedure of diazotization was the one previously described for the unlabeled compound [sodium nitrite was added in a water solution (13 mM)]. The photochemical decomposition of an aliquot of the radiolabeled ABD-ouabain led to the same mixture of compounds as the photodecomposition of the unlabeled sample.

The overall yield was 12% for a compound having a specific radioactivity of 1 Ci/mmol.

(Na^+,K^+)ATPase Preparations. (Na^+,K^+)ATPase from the electric organ of *Electrophorus electricus* was purified ac-

cording to Dixon & Hokin (1978). (Na^+,K^+)ATPase from dog kidney was prepared as described by Jørgensen (1974). The microsomal fraction of the electric organ was prepared following the method of Agnew et al. (1978).

Enzyme Assays. (Na^+,K^+)ATPase activity was measured spectrophotometrically at 340 nm with a pyruvate kinase–lactate dehydrogenase linked system as previously described by Gache et al. (1976).

Reversible Binding of [^3H]Ouabain and [^3H]ABD-Ouabain to (Na^+,K^+)ATPase. Specific radioactivities of [^{3}H]ouabain and [^{3}H]ABD-ouabain were respectively 14 and 1 Ci/mmol. Purified (Na^+,K^+)ATPase from *Electrophorus electricus* (50 μg) was incubated for 45 min at room temperature in the absence of light in 1 mL of 50 mM TEA-HCl buffer (pH 6.8) containing 2 mM ATP, 2 mM $MgCl_2$, 100 mM NaCl, and various concentrations of radiolabeled ligand. Following incubation, an aliquot was taken to determine the (Na^+,K^+)-ATPase activity remaining, and the rest of the reaction mixture was filtered through GSWP Millipore filters (0.22 μm). The filters were rinsed 3 times with 5 mL of 50 mM TEA, pH 6.8, to remove the uncomplexed ligand. Nonspecific binding was estimated from parallel experiments in which the enzyme preparation was preincubated with 1 mM unlabeled ouabain.

Irradiation Experiments. The samples were irradiated in a quartz cell with a 1-cm light pathway by using monochromatic light from a 1000-W Xe/Hg lamp connected to a monochromator. The resulting light beam was focused with a lens to a spot of about 10 mm high and 2 mm wide, and its intensity was measured with a thermopile coupled to a microvoltmeter. For example, at 350 nm a light intensity measurement of 1 mV corresponds to an incident energy of 2.17×10^{-6} einstein s^{-1} cm^{-2}.

The irradiated solutions (typically a 0.5-mL buffer solution containing the purified enzyme at 0.25 mg/mL and ABD-ouabain at 10^{-6} M) were maintained at 4 °C and stirred magnetically. We checked that irradiations at 290 nm (incident energy 20 μV) in the absence of ABD-ouabain did not affect the enzyme.

Irreversible Binding Experiments. Association of [^{3}H]-ABD-ouabain (1 Ci/mmol) with its receptor was obtained by using the incubation conditions described above. After a 5-min incubation of 1 μM [^{3}H]ABD-ouabain at room temperature in the dark, the sample was irradiated as described previously. Aliquots containing 5 μg of protein were withdrawn at different times and added to 1 mL of a 5% trichloroacetic acid solution. The precipitated protein was filtered through glass fiber filters (Whatman GF/C) and rinsed twice with 5 mL of 50 mM TEA solutions (pH 7.5). The extent of covalent incorporation was estimated by counting the radioactivity trapped on filters. Nonspecific covalent labeling was determined in experiments in which enzyme preparations were first incubated with 1 mM unlabeled ouabain for 10 min.

Mild Tryptic Digestion of [^3H]ABD-Ouabain-Labeled Enzyme. Purified (Na^+,K^+)ATPase (0.75 mg/mL) from dog kidney was irradiated at 290 nm for 1 h at 4 °C in the presence of 2 μM [^{3}H]ABD-ouabain. The labeled enzyme was centrifuged at 45 000 rpm for 45 min, and the pellet obtained was resuspended in 2 mL of a solution containing 25 mM imidazole and 1 mM EDTA (pH 7.5) and centrifuged again. The final pellet was resuspended in a medium containing 25 mM imidazole, 1 mM EDTA, and 100 mM KCl to a final protein concentration of 1 mg/mL. Trypsinolysis was started by addition of 4 μg of DPCC-treated trypsin at 37 °C. After 7 min the reaction was stopped by addition of a 2-fold molar excess of soybean trypsin inhibitor.

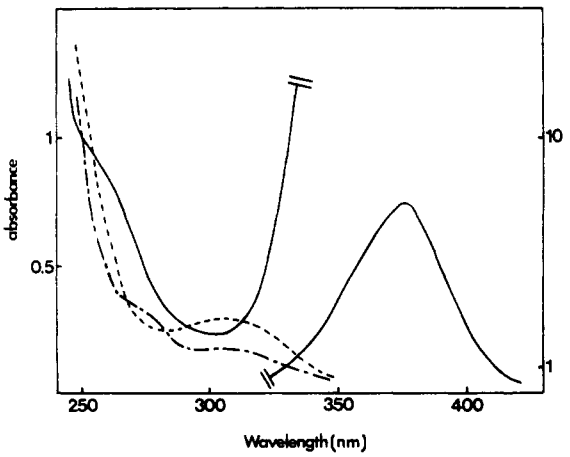

FIGURE 2: Absorbance spectra. ABD-ouabain 0.2 mM in water (—); note the change in scale on the right side of the spectrum. ABD-ouabain 0.2 mM in water after photolysis (- - -). APh-ouabain 0.2 mM in water (- - -).

Gel Electrophoresis. Intact subunits or tritiated tryptic peptides were separated on analytical 10% SDS–polyacrylamide gels according to Laemmli (1970). Calibration of molecular weights was obtained with bovine serum albumin (M_r 68 000), catalase (M_r 58 000), ovalbumin (M_r 45 000), lactate dehydrogenase (M_r 33 000), chymotrypsinogen (M_r 25 000), and myoglobin (M_r 17 800). After staining, the gels were incubated at 80 °C in 0.3 mL of 30% H_2O_2 for 16 h before counting.

Results

Characterization of ABD-Ouabain. The synthesis of this derivative (Figure 1) is described under Methods. In fact the filtration of the diazotization reaction mixture on a Bio-Gel P-2 column constitutes a very practical method of synthesizing diazonium salts in an aqueous solution on a small scale basis. The solutions can be very easily analyzed by UV spectroscopy. The UV absorption spectrum of ABD-ouabain (Figure 2) shows the presence of a *p*-(dialkylamino)benzenediazonium chromophore and resembles closely the sum of the UV spectra of DDF (Sukigara & Kikuchi, 1967) and ouabain. The wavelength of the absorption maximum of ABD-ouabain (375 nm) is slightly shifted compared to that of DDF (378 nm). The extinction coefficient of DDF (ϵ_M 38 000 at λ_{max} 378 nm) was used to calculate the concentration of ABD-ouabain in the different fractions. The purity of the samples was estimated by the ratio of optical densities at 375 and 250 nm, a ratio of about 7.5 corresponding to a theoretically pure sample. In the synthesis of the radioactive derivative, the analysis of the fractions containing ABD-ouabain showed a constant ratio of about 7 for about 90% of diazonium formed. The radioactivity was directly proportional to the concentration of ABD-ouabain which indicates that the compound is not contaminated with an unidentified radiolabeled ouabain derivative.

Attempts to isolate ABD-ouabain in a crystalline state failed, and therefore, we could not characterize this chemical by NMR or MS. It has been further characterized by the demonstration of its photochemical transformation to the corresponding ouabain *p*-aminophenol derivative (APh-ouabain). This phototransformation was not stoichiometric as was the case for the photodecomposition of DDF (Kieffer et al., 1981). Nevertheless the major compounds formed during this reaction comigrate on TLC with the synthetic APh-ouabain (see Methods). the UV spectra of the synthetic APh-ouabain and the photolyzed ABD-ouabain (Figure 2) show a maximum absorption around 300 nm which is characteristic of *p*-

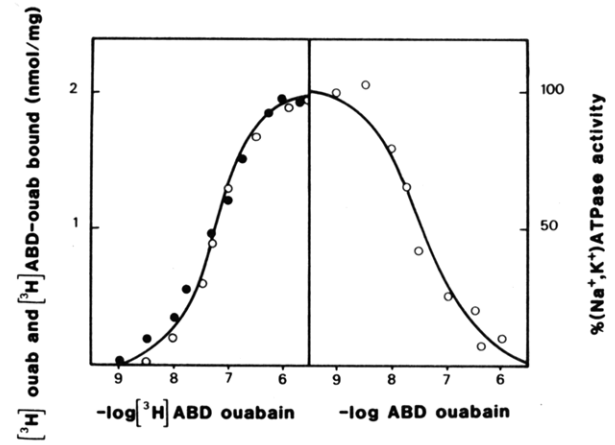

FIGURE 3: Reversible binding of [^{3}H]ABD-ouabain (○) and [^{3}H]-ouabain (●) to (Na^+,K^+)ATPase. Purified (Na^+,K^+)ATPase from *Electrophorus electricus* (50 μg) was incubated for 45 min at room temperature in the dark in 1 mL of TEA-HCl (pH 6.8) containing 2 mM of ATP, 2 mM $MgCl_2$, 100 mM NaCl, and various concentrations of the radiolabeled ligand (1 Ci/mmol). (Left) The reaction medium was filtered through GSWP Millipore filters (0.22 μm). The filters were rinsed 3 times with 5 mL of 50 mM TEA, pH 6.8, to remove excess ligand. The specific binding was calculated by subtracting the contribution of a sample previously incubated with 1 mM unlabeled ouabain. (Right) One aliquot of the above medium was taken to determine the (Na^+,K^+)ATPase activity.

aminophenol derivatives, and it agrees well with the spectrum of *p*-(*N*,*N*-dimethylamino)phenol (λ_{max} 296 nm, ϵ_M = 1415).

The general physical characteristics of the synthetic APh-ouabain derivative are summarized under Methods. The mass spectrum of APh-ouabain provides convincing results concerning the structural characterization. Besides the finding of the expected mass value, all the major fragmentation peaks could be explained. The fact that the largest peak (*m*/*e* 223) corresponds to the sugar moiety substituted by the *p*-aminophenol group is good evidence that the reductive amination of oxidized ouabain has taken place as expected.

Reversible Binding of ABD-Ouabain Derivative to the Digitalis Receptor in the Dark. The dissociation constant of the ABD-ouabain–receptor complex was determined both by direct binding studies and by following the inhibition of the (Na^+,K^+)ATPase activity. The data presented in Figure 3 (left) show that ABD-ouabain interacts with the digitalis binding site with a dissociation constant of 50 nM with a maximum binding capacity of about 2 nmol/mg of protein. Over 90% of this binding is protected by a large excess (1 mM) of unlabeled ouabain. These binding characteristics are very near to those found for the binding of [^{3}H]ouabain to the (Na^+,K^+)ATPase under the same conditions. The inhibition of the enzymatic activity (Figure 3, right) parallels the binding of [^{3}H]ABD-ouabain to the purified enzyme. The half-maximum inhibition of the (Na^+,K^+)ATPase is observed at 40 nM of ABD-ouabain.

Irreversible Binding of ABD-Ouabain to the (Na^+,K^+)ATPase. After irradiation of the [^{3}H]ABD-ouabain–receptor complex under energy transfer conditions (λ_{exc} 290 nm), a covalent incorporation of the radiolabeled probe was observed. The kinetic properties of the covalent incorporation are shown in Figure 4 which indicates that a plateau value corresponding to about 8% of the total ouabain binding sites available in the incubation medium was obtained after 15 min of irradiation.

The identification of the polypeptide chains which are covalently labeled by [^{3}H]ABD-ouabain (1.2 μM) is shown in Figure 5 by using either a purified preparation of (Na^+,K^+)ATPase or a crude membrane preparation of the electric organ of electric eel. Quite clearly in both cases most

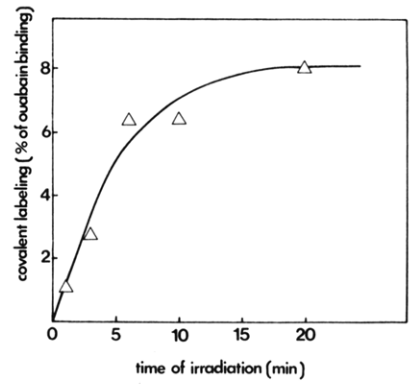

FIGURE 4: Kinetics of the covalent incorporation of [^{3}H]ABD-ouabain. Purified (Na^+,K^+)ATPase from *Electrophorus electricus*, 0.25 mg/mL, was incubated in the dark with 1.2 μM [^{3}H]ABD-ouabain (1 Ci/mmol) for 5 min at 20 °C. The sample was then irradiated at λ 290 nm. At the indicated times an aliquot containing 25 μg of protein was withdrawn and precipitated in 1 mL of 5% trichloroacetic acid and then filtered through Whatman (GF/C) filters. The non-specific incorporation of [^{3}H]ABD-ouabain was obtained by repeating the same experiment in the presence of an excess of unlabeled ouabain (50 μM). The specific covalent incorporation represents 65% of the total incorporation.

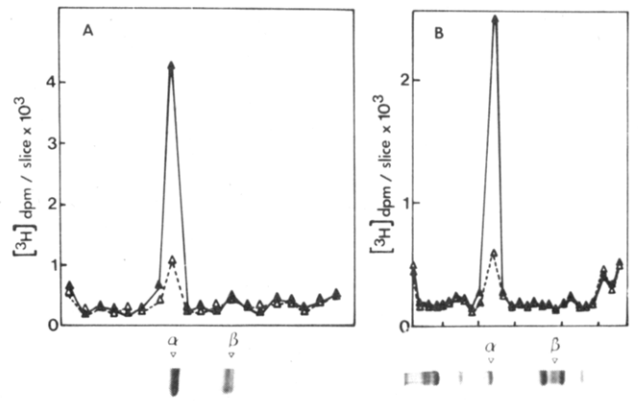

FIGURE 5: SDS gel electrophoresis of two preparations of (Na^+,K^+)ATPase from *Electrophorus electricus*. The labeling of (Na^+,K^+)ATPase was obtained as described in Figure 4. Samples containing 20 μg of protein were then analyzed on a 10% polyacrylamide slab gel according to Laemmli (1970). The gel was then sliced in 3 mm thick sections and dissolved in 30% H_2O_2 for 16 h at 80 °C. (A) Purified (Na^+,K^+)ATPase from *Electrophorus electricus*. (B) Crude microsomal fraction of electric organ from *Electrophorus electricus* (▲) enzyme preparation and (△) after its incubation with unlabeled ouabain (50 μM).

of the incorporated radioactivity is localized in the α-subunit (M_r ≃93 000) of the enzyme, and this labeling is reduced by 80% in the presence of an excess (50 μM) of unlabeled ouabain. No specific incorporation of radioactivity is observed in the β-subunit (M_r 47 000) or in the low molecular weight region which contains the proteolipid component.

An identical pattern of radioactivity distribution is observed for a mammalian source of (Na^+,K^+)ATPase (Figure 6, top).

Trypsin Cleavage of the [^{3}H]ABD-Ouabain-Labeled α-Subunit from Dog Kidney (Na^+,K^+)ATPase. It has been previously reported that in the presence of KCl the catalytic subunit is cleaved by trypsin in two main fragments: an N-terminal peptide (M_r 41 000) and a C-terminal fragment (M_r 58 000) (Jørgensen, 1975; Castro & Farley, 1979). Figure 6 (bottom) shows that most of the radioactivity incorporated in the α-subunit (Figure 6, top) is recovered after trypsinolysis in the presence of KCl in a polypeptide of apparent M_r 41 000. No significant incorporation of radioactivity was visible in the C-terminal polypeptide of M_r 58 000.

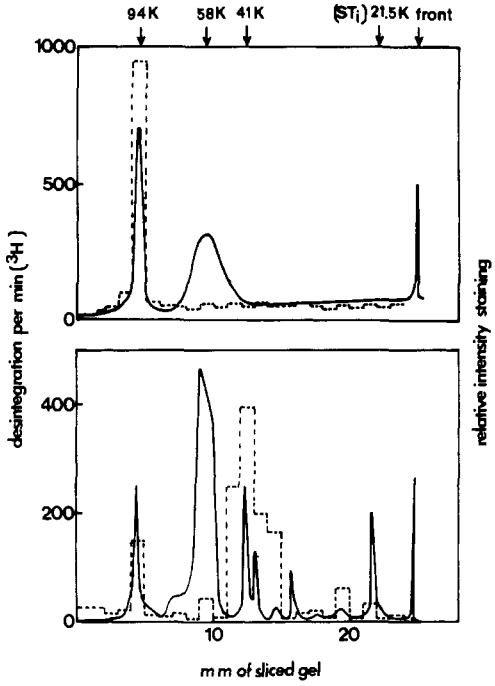

FIGURE 6: Coomassie blue staining (—) and radioactivity profile (---) of intact and trypsin-digested (Na^+,K^+)ATPase after labeling with [^{3}H]ABD-ouabain. The purified (Na^+,K^+)ATPase from dog kidney was labeled as described under Methods. (Top) Intact enzyme; (bottom) digested enzyme. The labeled enzyme (60 μg) was trypsinolyzed in the presence of 100 mM KCl with a trypsin/ATPase weight ratio of 1/15 at 37 °C for 7 min. The reaction was stopped by addition of a 2-fold molar excess of soybean trypsin inhibitor.

Discussion

The most recent attempts to covalently label the digitalis site have been made with (nitroazidobenzoyl)ouabain (Forbush et al., 1978; Forbush & Hoffman, 1979), (nitroazidophenyl)ouabain and (nitroazidophenyl)strophanthidin (Rogers & Lazdunski, 1979a,b), [(nitroazidophenyl)glycyl]digitoxigenin and (diazomalonyl)digitoxin (Hall & Ruoho, 1980), and [(nitrophenyl)triazene]ouabain (Rossi et al., 1980). All but the last derivative are photoactivatable reagents; [(nitrophenyl)triazene]ouabain is an alkylating digitalis compound. When photoactivatable compounds have their light-sensitive group in the steroid portion, only the α-subunit is labeled (Rogers & Lazdunski, 1979a,b). When the light-sensitive group of the digitalis derivative is on the first or second sugar moiety of the structure, the α-subunit and the γ-subunit are covalently labeled (Forbush & Hoffman, 1979; Rogers & Lazdunski, 1979a,b; Hall & Ruoho, 1980). Labeling of the β-subunit requires insertion of a carbene precursor in the 4′ portion of the third sugar residue of digitoxin. The alkylating group in [(nitrophenyl)triazene]ouabain is situated on the sugar moiety of ouabain, and it incorporates covalently exclusively in the α-chain.

The idea of the work presented here started with the observation by Fortes (1977) that energy transfer occurs between a tryptophan residue in the dog kidney (Na^+,K^+)ATPase and anthranoylouabain bound to the digitalis site. There are structural analogies between ABD-ouabain and anthranoylouabain, and the UV spectrum of ABD-ouabain indicates that the derivative has the properties of a photoreagent which can be decomposed by an energy transfer reaction from an excited tryptophan residue (irradiation wavelength 290 nm). For these two reasons it was expected that covalent labeling of the digitalis binding site with ABD-ouabain could be obtained by the following mechanism:

$$\text{receptor } (R_{Trp}) + \text{ABD-ouabain} \longrightarrow R_{Trp}\text{-ABD-ouabain}$$
$$\downarrow h\nu = 290\ \text{nm}$$
$$R_{Trp}^{*}\text{-ABD-ouabain}$$
$$\downarrow \text{energy transfer}$$
$$R_{Trp} = \text{ABD}^{*}\text{-ouabain}$$

In the dark ABD-ouabain binds reversibly to the digitalis receptor, and this binding produces (Na^+,K^+)ATPase inactivation. The dissociation constant of the ABD-ouabain–receptor complex is 50 nM. ABD-ouabain is covalently incorporated into the digitalis receptor after irradiation at 290 nm, i.e., under conditions of energy transfer induced photoactivation. Of course, under these conditions only ABD-ouabain molecules bound to digitalis sites are photoactivated, free ABD-ouabain molecules are not, and this is the great interest of the technique. Covalent incorporation reaches 8% of the total ouabain binding capacity of the enzyme (Figure 4).

Unlike other ouabain derivatives having the photolabel on the sugar moiety, ABD-ouabain labels exclusively the α-subunit of the purified (Na^+,K^+)ATPase from both electric eel and dog kidney. Very specific labeling can also be obtained with a crude microsomal fraction of the *Electrophorus electricus* electric organ (Figure 5B). Therefore, ABD-ouabain can be considered as a better photoactivatable derivative of ouabain (or of other digitalis compounds) than those which have already been described (better yield of covalent incorporation and better selectivity for the α-chain).

The localization of the ABD-ouabain site in the α-chain sequence was obtained after mild trypsinolysis (Figure 6). Trypsinolysis cleaves the α-chain into two fragments: an N-terminal peptide of M_r 41 000 which contains the ATP phosphorylation site and a C-terminal peptide of M_r 58 000 which is labeled with one of the hydrophobic probes, [^{3}H]-adamantanyldiazirine (Farley et al., 1980). ABD-ouabain is covalently and selectively incorporated in the M_r 41 000 fragment. This is a further indication that ABD-ouabain used as a photosuicide reagent is much more specific than other photoactivatable derivatives. (Nitroazidophenyl)ouabain (NAP) labels both the M_r 58 000 and M_r 41 000 fragments (Jørgensen et al., 1982).

The results obtained with ABD-ouabain after photoactivation through energy transfer are very similar to those obtained with the alkylating ouabain derivative [(nitrophenyl)-triazene]ouabain (NPT). NPT-ouabain also selectively labels the α-chain (Rossi et al., 1980, 1982) and also incorporates uniquely in the M_r 41 000 fragment.

ABD-ouabain is the second example of a successful photosuicide reagent (Goeldner & Hirth, 1980). The reagent is stable in the absence of light. When it is irradiated, it generates a very reactive aryl cation [aryl cations are able to react with molecular nitrogen (Ambroz & Kemp, 1979), amide bonds (B. Kieffer, personal communication) and C–H bonds (Stumpe, 1980)]. The procedure also largely eliminates the nonspecific covalent labeling often observed with classical photoaffinity reagents which is due in part to the indiscriminate photoactivation of the specifically bound ligand as well as the free ligand molecules. Moreover the ionic character of diazonium salts renders them less hydrophobic than the corresponding azido derivatives, thereby decreasing their tendency for nonspecific binding to the membrane outside of the specific receptor site.

It has been shown recently that the ouabain receptor is internalized in HeLa cells (Cook et al., 1982), and we have

made the same observations with the epithelial dog kidney cell line MDCK (unpublished observations). One usually studies internalization of hormones, growth factors, or lipoproteins by following a reporter signal (often radioactive or fluorescent) placed on the ligand molecule. A particularly interesting application of ABD-ouabain would be to study the mechanism of internalization and recycling of the digitalis receptor by directly labeling it radioactively.

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Registry No. ATPase, 9000-83-3; ABD-ouabain, 83859-88-5; Boc-*p*-phenylenediamine, 71026-66-9; APh-ouabain, 86569-16-6; di-*tert*-butyl dicarbonate, 24424-99-5; *p*-phenylenediamine, 106-50-3; oxidized ouabain, 73165-88-5; *p*-aminophenol, 123-30-8.

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Mechanism of Folding of Ribonuclease A. Slow Refolding Is a Sequential Reaction via Structural Intermediates†

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ABSTRACT: Two models have been proposed to explain the observed folding kinetics of small proteins. The sequential model assumes that folding proceeds on an ordered pathway via structural folding intermediates, whereas the simple model of folding involves only multiple unfolded forms of the protein and a single native state. In the latter model, refolding is limited by interconversion reactions in the unfolded protein; accumulation of structural intermediates during folding is excluded. Here, two experimental tests are presented to discriminate between these models for the major slow folding species of ribonuclease A. The first test shows that a nativelike intermediate accumulates during folding, which unfolds rapidly compared to native ribonuclease A, and the second test demonstrates that refolding is a sequential reaction, resulting in the transient accumulation of an intermediate and in a lag in the formation of fully native protein. Both results rule out the simple model of folding and agree with the sequential model via structural intermediates. The nativelike intermediate is stable toward unfolding and is on the pathway of refolding for denaturant concentrations up to 2 M guanidine hydrochloride at pH 6 and 10 °C.

Structural intermediates have been detected in the reversible folding reactions of ribonuclease A (RNase A)[1] [for a review, see Kim & Baldwin (1982)] and of other small protein molecules (Ko et al., 1977; Crisanti & Matthews, 1981; Desmadril & Yon, 1981; McPhie, 1982; Goto & Hamaguchi, 1982; Nall, 1983). The model of sequential folding (Kim & Baldwin, 1982) states that protein folding proceeds on an ordered pathway via a definite sequence of structural folding

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5) with disulfide bonds intact; U_S and U_F, slow- and fast-folding species of unfolded RNase A, respectively; Gdn·HCl, guanidine hydrochloride; τ, time constant of chemical reaction (reciprocal of the apparent rate constant, k^{-1}); I_1 and I_N, folding intermediates; 2′-CMP, cytidine 2′-phosphate; 2′,3′-CMP, cytidine 2′,3′-phosphate.

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