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Photoaffinity labeling of erythrocyte membrane ($Na^+ + K^+$)-ATPase with high specific activity [^{125}I]iodoazidogalactosyl digitoxigenin

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Photoaffinity labeling of ($Na^+ + K^+$)-ATPase in erythrocyte membranes with cardiotonic steroid derivatives, followed by gel electrophoresis, requires a radiolabel of very high specific activity, since the enzyme represents less than 0.05% of the total membrane protein. We report the synthesis of a radioiodinated, photosensitive derivative of the cardiac glycoside, 3-β-*O*-(4-amino-4,6-dideoxy-β-D-galactosyl)digitoxigenin, with very high specific activity. The product, [^{125}I]iodoazidogalactosyl digitoxigenin ([^{125}I]IAGD), is carrier-free with a specific activity of 2200 Ci/mmol. Incubation of [^{125}I]IAGD (1.8 nM) with human erythrocyte membranes (300 μg protein), followed by photolysis and analysis by SDS-PAGE, showed specific radiolabeling of a polypeptide that had the same molecular weight as catalytic α subunit (100000 M_r) of ($Na^+ + K^+$)-ATPase in eel electroplax microsomes. Photoaffinity labeling of erythocyte and electroplax membranes by [^{125}I]IAGD was specific for the cardiac glycoside binding site of ($Na^+ + K^+$)-ATPase since radiolabeling of the α subunit was inhibited when ouabain was included in the pre-photolysis incubation. [^{125}I]IAGD can, therefore, be used as a probe in structural studies of human erythrocyte membrane ($Na^+ + K^+$)-ATPase.

Erythrocytes have been used extensively for the study of ($Na^+ + K^+$)-ATPase properties. Since blood is a readily available human biopsy tissue, the properties of the erythrocyte enzyme have been studied in a number of clinical conditions. Several disorders appear to be associated with abnormal erythrocyte ($Na^+ + K^+$)-ATPase activity [1]; these include bipolar manic-depressive disorder [2] and essential hypertension [3]. Our aim was to develop a radiolabeled marker for the ($Na^+ + K^+$)-ATPase in erythrocyte membranes which would facilitate structural studies of the enzyme in these and other clinical disorders. In order to obtain sufficient labeling of the erythrocyte membrane protein for analysis by SDS-PAGE, a marker of very high specific radioactivity was needed. This is because, in erythrocyte membranes, the enzyme represents less than 0.05% of the total membrane protein – a much lower proportion than is found in other tissues such as kidney and brain.

Photoactive cardiotonic steroid derivatives labeled with ^{14}C, ^{3}H, or ^{125}I have been used with a variety of tissues to obtain stable covalent photo-

Abbreviations: [^{125}I]IAGD, [^{125}I]iodoazidogalactosyl digitoxigenin; azidogalactosyl digitoxigenin, *N*-(2-hydroxy-4-azidobenzoylamide)galactosyl digitoxigenin; galactosyl digitoxigenin, 3-β-*O*-(4-amino-4,6-dideoxy-β-D-galactosyl)-digitoxigenin; NHS-ASA, *N*-(2-hydroxy-4-azidobenzoyloxy)succinimide (the succinimide ester of 4-azidosalicylic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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labeling of $(Na^+ + K^+)$-ATPase [4–10]. However, the specific radioactivities that were reported for these photolabels are too low for the compounds to be useful in photolabeling the enzyme in erythrocyte membranes. Formation of the acyl phosphate-enzyme complex with high specific activity ^{32}P has been used to identify erythrocyte $(Na^+ + K^+)$-ATPase after electrophoresis on paper [11] and on polyacrylamide gels [12,13]; this method is of limited use since the instability of the acyl phosphate restricts procedures to those which can be carried out at low pH. A photo-activated ATP analog has been used to label cation-stimulated ATPases in human erythrocyte membranes but the labeling is less specific than with cardiotonic steroid derivatives [14].

We describe here the synthesis and use of [^{125}I]IAGD, a carrier-free radioiodinated photoaffinity label for the ouabain binding site of $(Na^+ + K^+)$-ATPase. The specific radioactivity of this ligand is much higher (2200 Ci/mmol) than that of any previously described photolabels for the enzyme (e.g. 10 Ci/mmol for [^{125}I]iodoazidocymarin [10]). With this compound, the erythrocyte membrane $(Na^+ + K^+)$-ATPase can be specifically radiolabeled so that the enzyme can be identified by autoradiography after separation by gel electrophoresis techniques.

The synthesis of azidogalactosyl digitoxigenin (Fig. 1) involved reaction of the cardiac glycoside with *N*-(2-hydroxy-4-azidobenzoyloxy)succinimide (NHS-ASA) (Pierce Chemical Co., Rockford IL). Ji and Ji [15] first reported the use of NHS-ASA as a photoactivatable heterobifunctional reagent which could be radioiodinated. These authors radioiodinated NHS-ASA directly and used the product, without purification, to derivatize the lectin, concanavalin A. We have used NHS-ASA in an alternative procedure to form a carrier-free, radioactive photoactivatable derivative of a cardiac glycoside. Galactosyl digitoxigenin (Ash Stevens, Inc., Detroit, MI) was reacted with unlabeled NHS-ASA to form azidogalactosyl digitoxigenin (structure as shown in Fig. 1), which was characterized chemically. This product was then iodinated with carrier-free Na^{125}I (New England Nuclear, Boston, MA).

For the iodination step, the following were added to a combi-V-vial (New England Nuclear)

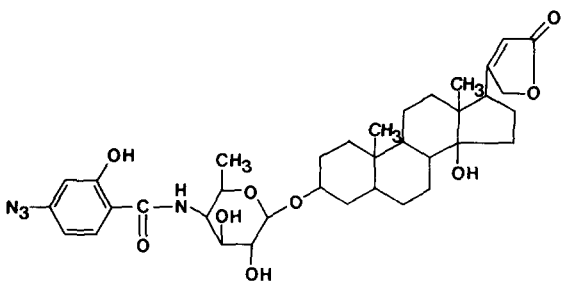

Fig. 1. The structure of 3-*β*-*O*-[*N*-(2-hydroxy-4-azidobenzoylamide)-4-amino-4,6-dideoxy-*β*-D-galactosyl]digitoxigenin (azidogalactosyl digitoxigenin). Azidogalactosyl digitoxigenin was synthesized in the following manner: Galactosyl digitoxigenin (10 mg, 16.8 μmol) in a 6×50 mm test tube was dried for 24 h under vacuum in a drying pistol which was heated with refluxing ethanol and the dried compound was then dissolved in 20 μl of dry *N*,*N*-dimethylformamide. Triethylamine (2.4 μl, 16.8 μmol) was added to the solution to neutralize the amino group of galactosyl digitoxigenin. To start the reaction, 20 μl of NHS-ASA (7 mg, 25.3 μmol) was added to the solution in the dark. The reaction tube was corked, sealed with parafilm and left at 25°C for 18 h. Absolute ethanol (0.5 ml) was added to stop the reaction and the mixture was dried by vacuum pump for 5 h. To ensure that all the *N*,*N*-dimethylformamide was removed, the residue was dissolved in ethanol (0.5 ml) and dried by vacuum for three times. The residue in the reaction tube was dissolved in 100 μl ethanol, applied to a thick layer (1 mm) silica gel plate (silica gel 60 F-254, Sigma Chemical Co., St. Louis, MO), and developed with benzene/acetonitrile (1:1, v/v). The band of silica gel that contained the reaction product (UV positive, $R_F = 0.5$) was removed and extracted five times with 25 ml ethanol. The extract was reduced in volume by rotovaporation in a 250 ml round bottom flask and was then transferred to a 5 ml round bottom flask and reduced to an oily residue. This residue was taken up in 0.5 ml ethanol, then 1.5 ml water were added; this resulted in the formation of a white flocculent precipitate. The suspension was lyophilized. The product (60% yield) appeared to be homogeneous when analyzed on precoated silica gel TLC plates, 60 F-254 (MCB Manufacturing Chemists, Inc., Cincinnati, OH), developed with either benzene/acetonitrile (1:1, v/v) or toluene/methanol/acetic acid (45:2:1, v/v). The product on the thin-layer plate was positive for cardiac glycoside, as determined by staining with ceric ammonium sulfate, and was negative for a free amino group, as determined by staining with ninhydrin. The infrared spectrum of the purified product showed peaks at 2100 cm^{-1}, which is characteristic of an azide group, and at 1730 cm^{-1}, which is characteristic of an α,β unsaturated lactone of a cardiac glycoside.

which contained 10 μl 0.1 M NaOH and 2 mCi Na^{125}I: 10 μl 0.1 M HCl, 20 μl sodium phosphate buffer (0.5 M, pH 7.4), and 2 μl azidogalactosyl digitoxigenin (1 mg/5 μl *N*,*N*-dimethylform-

amide). The reaction was started with the addition of 20 μl chloramine-T (5 mg/10 ml phosphate buffer). The reaction was stopped after 15 seconds with the addition of 100 μl sodium metabisulfite (5 mg/ml water). The mixture was transferred to a 12 ml conical test tube and extracted four times with 200-μl aliquots of ethyl acetate to recover the radioactive product. The volume of the ethyl acetate was reduced under a gentle stream of N_2 and the concentrated material was applied to a pre-coated TLC plate. A paper wick made with Whatman 3MM chromatography paper was clamped to the top of the TLC plate with a glass rod and two binder clips. The plate was developed for 4.5 hours with toluene/methanol/acetic acid (45 : 2 : 1, v/v). The solvent front reached the top of the plate in approximately one hour, and thereafter evaporated from the paper wick, which extended to the outside of the chromatographic tank.

The starting material, azidogalactosyl digitoxigenin, migrated as a single ultraviolet light-absorbing band on the TLC plate. Autoradiography showed two radioactive products; these migrated ahead of the azidogalactosyl digitoxigenin. The major radioactivity product was extracted from the silica gel (2 ml ethanol, three times) and used as the photoaffinity label, [^{125}I]IAGD. It was carrier-free, with a specific activity of 2250 Ci/mmol. The minor radioactive product migrated just ahead of [^{125}I]IAGD. This may be a diiodinated product since two iodine atoms can be added to the 2-hydroxy-4-azidobenzoyl moiety [16], which is the functional group of azidogalactosyl digitoxigenin.

For photoaffinity labeling, membranes were incubated for 60 minutes at 30 °C in the dark with 200 μl of Solution A (3 mM Tris-phosphate, 4 mM $MgCl_2$, and 50 mM imidazole-HCl, pH 7.2) which contained 1% *N,N*-dimethylformamide, and 1.8 nM [^{125}I]IAGD. Ouabain (1 mM) was present in a second incubation tube for each membrane sample. At the end of the incubation period, the samples were diluted to 10 ml with ice cold Solution A and centrifuged at 150000 × *g* for 45 minutes to remove unbound [^{125}I]IAGD. The pellets were resuspended and diluted to 5 ml with ice cold Solution A. The samples were then photolyzed for 10 s at a distance of 10 cm from a 1 kW high pressure mercury lamp (AH-6, Advanced Radiation, Santa Clara, CA). The photolysis was carried out in thick walled pyrex glass test tubes in order to minimize damage to the protein by ultraviolet irradiation during photolysis. All photolyzed samples were diluted to 10 ml with ice cold Solution A and left on ice for 3 h. The membranes were pelleted by centrifugation at 150000 × *g* for 45 min and were then frozen at −20 °C. The pellets were thawed the following day and analyzed for covalent incorporation of radioactive ligand by

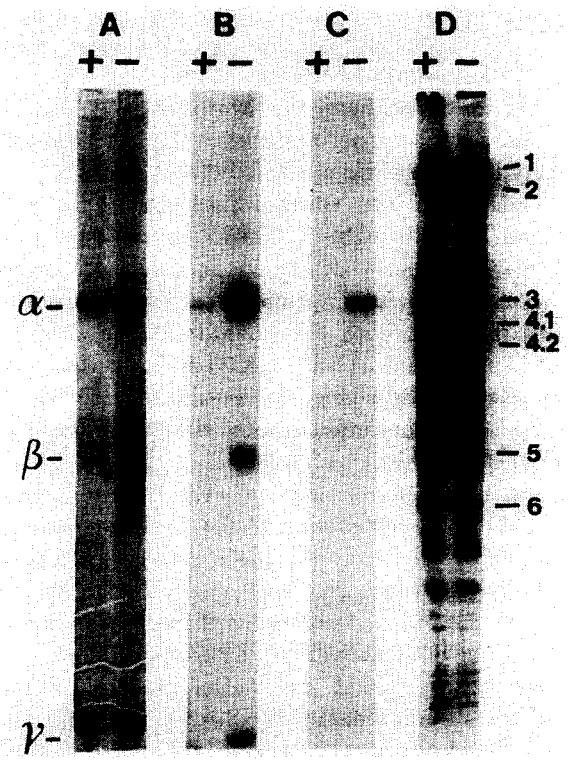

Fig. 2. Identification of ouabain-protectable [^{125}I]IAGD-photoaffinity labeled ($Na^+ + K^+$)-ATPase subunits in eel electroplax microsomes and human erythrocyte membranes. Incubation and photolysis conditions, analysis by SDS-PAGE (7–14%), and autoradiography were as described. Membrane preparations were incubated with [^{125}I]IAGD in the presence (+) or absence (−) of 1.0 mM ouabain. A, B: eel electroplax microsomes, 50 μg of protein; C, D: human erythrocyte membranes, 300 μg of protein. A, D: Coomassie blue protein staining of the gel tracks; B, C: Autoradiograms of radiolabeled protein in the gel tracks. The locations of the α, β, and γ subunits are indicated for the electroplax microsomes; erythrocyte membrane protein bands are numbered according to Fairbanks et al. [21].

SDS-PAGE (7–14% linear gradient) as previously described [10].

$(Na^+ + K^+)$-ATPase, purified from several sources (for reviews see Refs. 17 and 18), consists of at least two polypeptides: an α or an $\alpha(+)$ subunit (100000–102000 M_r) and a carbohydrate-containing β subunit (44000–56000 M_r). In addition, small peptides (12000–15000 M_r) referred to as proteolipids or γ subunits, have been identified. The α subunit is the catalytic subunit, and it contains the cardiac glycoside binding site. [^{125}I]IAGD was established as a photoaffinity label for $(Na^+ + K^+)$-ATPase by demonstration of radiolabeling of the α subunit of the enzyme in microsomal membranes from electroplax tissue of the electric eel (*Electrophorus electricus*). Electroplax microsomes, which were prepared as described [19], are a highly enriched source of $(Na^+ + K^+)$-ATPase. Samples (50 μg of protein) were photolabeled as described above. The labeling pattern with [^{125}I]IAGD, which is shown in the autoradiogram in Fig. 2B, indicated that most of the incorporated radioactivity was associated with the α subunit; this subunit can be seen as a major membrane constituent in the protein stained gel shown in Fig. 2A. There was also radioactivity associated with the β subunit and a proteolipid component (γ). The radiolabeling of all the $(Na^+ + K^+)$-ATPase polypeptides was inhibited with ouabain in the pre-photolysis incubation (Fig. 2B). These results are similar to those described for photoaffinity labeling of eel electroplax microsomes with low specific activity [^{125}I]iodoazidocymarin [10]. Radiolabeled $\alpha(+)$ catalytic subunit in eel electroplax microsomes was observed with [^{125}I]iodoazidocymarin [10]; however, the [^{125}I]IAGD-labeled sample did not show two distinct α and $\alpha(+)$ subunits on the autoradiogram (Fig. 2).

Human erythrocyte membranes were prepared as described [20], and samples (300 μg of protein) were photolabeled with [^{125}I]IAGD and submitted to SDS-PAGE. Autoradiograms of the gels revealed a highly specifically radiolabeled polypeptide (Fig. 2C,D). This polypeptide can be identified as the catalytic α subunit of $(Na^+ + K^+)$-ATPase since the radiolabeling was blocked when ouabain was included in the pre-photolysis incubation to occupy the cardiac glycoside binding site of the enzyme; the molecular weight of the polypeptide was the same as that for the α subunit of eel electroplax $(Na^+ + K^+)$-ATPase. In an experiment similar to the one described above, the efficiency of [^{125}I]IAGD labeling was calculated to be roughly of the order of 1%. This is similar to the efficiency of [^{125}I]iodoazidocymarin labeling of eel electroplax enzyme [10]. Photolabeling of the β subunit and the proteolipid components of the erythrocyte enzyme were not detected.

The α subunit of erythrocyte $(Na^+ + K^+)$-ATPase is a very minor constituent of the polypeptides that migrate as Band 3 (nomenclature of Fairbanks et al. [21]) when the total membrane proteins are separated by SDS-PAGE (see Fig. 2D); the major constituent of Band 3 is the anion channel protein. Knauf et al. [12] showed that this could be proteolyzed by incubation of intact erythrocytes with pronase prior to the preparation of membranes, and that this procedure did not affect either $(Na^+ + K^+)$-ATPase activity or the molecular weight of the ^{32}P-labeled α subunit after separation by SDS-PAGE. We found that the 100000 M_r α subunit of the enzyme was effectively photoaffinity labeled with [^{125}I]IAGD in membranes made from erythrocytes in which the anion channel had been proteolyzed by treatment of the intact cells with pronase (data not shown).

Specific [^{125}I]IAGD photoaffinity labeling, using the erythrocyte as a biopsy tissue, will allow structural analysis of human $(Na^+ + K^+)$-ATPase by techniques such as isoelectric focusing and peptide mapping. This may help elucidate the basis of the abnormality in disorders which appear to be associated with abnormal activity of the enzyme [1–3].

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