GEL CHROMATOGRAPHY AND GEL ELECTROPHORESIS OF CARDIAC GLYCOSIDE-Na,K-ATPase COMPLEXES IN SODIUM DODECYL SULFATE: A CAUTION

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1. Introduction

It is now well accepted that the Na,K-ATPase is the in vitro manifestation of the Na-pump and that this enzyme system specifically binds cardiac glycosides [1–4]. With the development of numerous procedures for the purification of Na,K-ATPase, several investigators are attempting to further resolve the nature of this binding of cardiac glycosides to the Na,K-ATPase. Recently four separate reports appeared in the literature in which the authors concluded that the cardiac glycoside receptor is localized on the 95 000 dalton catalytic subunit of the Na,K-ATPase [5–8]. In two of these studies [5,6] a covalent attachment of the cardiac glycoside to the Na,K-ATPase was formed and it was then determined that the 95 000 dalton subunit was the protein moiety involved. In contrast, two other investigators [7,8] have also reported the identification of this protein as the receptor, but *without* the prior formation of a covalent bond between the Na,K-ATPase and the glycoside.

Alexander [7] and Alexander and Rodnight [9] reported the partial characterization of a component of the Na,K-ATPase which is phosphorylated by [^{32}P]ATP and labeled with [^{3}H]digitoxin. In the latter case, ox brain microsomes were incubated with [^{3}H] digitoxin in the presence of $MgCl_2$, ATP and NaCl. Potassium chloride was added and the reaction was terminated by the addition of SDS and unlabeled digitoxin. The labeled protein fraction was then subjected to gel chromatography in the presence of SDS on columns of Sephadex G-200 and G-100, followed by gel electrophoresis in the same detergent. They concluded that [^{3}H]digitoxin binds to the catalytic subunit and that KCl stabilizes this complex in the presence of SDS. It was suggested that the variation in distribution of the radioactive peak on successive column elutions represented aggregate forms of the catalytic and glycoprotein subunits.

More recently, Kott et al. [8] also concluded that the cardiac glycoside receptor resided on the 95 000 dalton subunit, utilizing SDS-polyacrylamide gel electrophoresis. They further concluded that SDS, in the absence of KCl, stabilizes the [^{3}H]ouabain–Na,K-ATPase complex, and that this complex (catalytic subunit plus ouabain) can be isolated by electrophoresis.

The techniques employed in these two studies [7,8] are much simpler than those of Ruoho and Kyte [5] and Hegyvary [6], and the authors' conclusions are essentially the same; i.e. that the catalytic subunit is, at least, part of the cardiac glycoside receptor.

We attempted to carry out similar experiments and to repeat those of Alexander [7] and of Kott et al. [8]. We found that digitoxin and ouabain exhibit some unexpected properties with respect to gel chromatography, gel electrophoresis and dialysis

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Abbreviations: Na,K-ATPase sodium-plus-potassium ion-activated adenosine triphosphatase; SDS, sodium dodecyl sulfate; CHDTA, 1,2-cyclohexanediaminotetraacetic acid.

in the presence of SDS. In the absence of prudent controls, this anomalous behavior can result in misinterpretations of experiments in which these techniques are used to resolve non-covalent digitalis–Na,K-ATPase complexes.

2. Materials and methods

2.1. *SDS-gel chromatography of [^{3}H]digitoxin–Na,K-ATPase*

Six mg of Na,K-ATPase purified from sheep kidney by a modification of our previous procedure [10] was incubated for 7 min at 30°C with 2.5 mM $MgCl_2$, 2.5 mM Tris-ATP, 100 mM NaCl, 50 mM Tris-Cl, pH 7.0, and 2.5 μM [^{3}H]digitoxin (266 cpm/pmole) in a final volume of 5 ml. Potassium chloride was then added to a concentration of 16 mM and the mixture was incubated for an additional 2 min. The reaction mixture was cooled and centrifuged at 165 000 *g* for 20 min. To remove unbound [^{3}H] digitoxin the pellet was homogenized in 6 ml of ice-cold 25 mM imidazole, pH 7.2, containing 16 mM KCl, plus and minus 100 mM NaCl, and 10^{-5} M unlabeled digitoxin and recentrifuged. The washed pellet was solubilized at 25°C in 0.9 ml of 25 mM imidazole, pH 7.2, 100 mM NaCl and 9% SDS. An aliquot was removed for the determination of bound [^{3}H]digitoxin, and a small amount of *p*-nitrophenol and pre-chromatographed blue dextran was added to the remainder of the fraction.

The sample, which contained approximately 6 mg protein and 4×10^6 cpm (2500 pmol digitoxin per mg protein), was immediately applied to a 6% agarose column (Bio-Gel A-5m, 100–200 mesh; 1.6 × 87 cm) which had been equilibrated with 25 mM imidazole, pH 7.0, 100 mM NaCl, 0.1% SDS and 0.02% NaN_3 at a flow rate of about 8 ml/h. Na,K-ATPase alone and [^{3}H]digitoxin alone were similarly chromatographed. The agarose column was calibrated with proteins of known mol. wt. which had been solubilized in SDS and 2-mercaptoethanol. The distribution coefficient (K_D) of each protein and radioactive peak was calculated from $K_D = (V_e - V_o)/(V_t - V_o)$, where V_e = elution volume for the peak, V_o = void volume of the column (blue dextran), and V_t = total solvent volume of the column (*p*-nitrophenol).

2.2. *SDS-polyacrylamide gel electrophoresis of [^{3}H] ouabain–Na,K-ATPase*

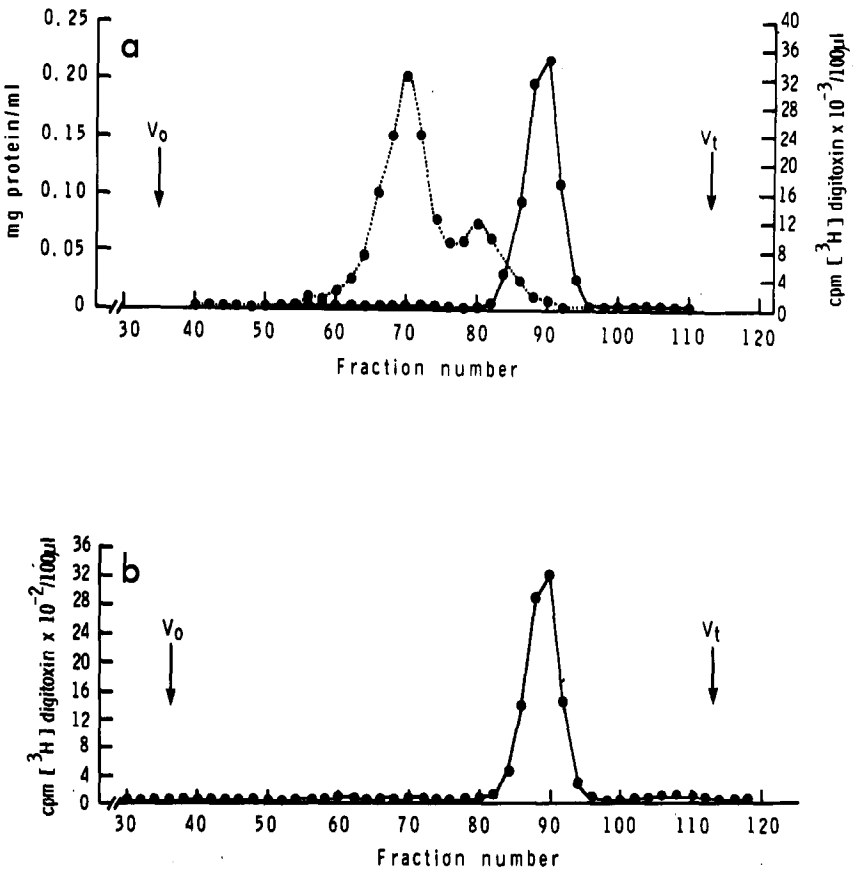
600 μg of purified sheep kidney Na,K-ATPase was incubated with 2 mM $MgCl_2$, 135 mM NaCl or 5 mM KCl, 2 mM CHDTA, 25 mM imidazole, pH 7.2, 2 mM Tris-ATP and 3×10^{-5} M [^{3}H]ouabain (75 cpm per pmole) in a final volume of 5 ml for 15 min at 37°C. The mixture was cooled and centrifuged at 100 000 *g* for 60 min. The supernatant was decanted and the tube walls wiped dry. The pellets were suspended in 0.5 ml of either ice-cold 3 mM sodium borate buffer, pH 8.5, or 10 mM Tris-phosphate, pH 7.1, both containing 1% SDS, 1% 2-mercaptoethanol, 5 mM NaCl, 5% glycerol and bromphenol blue. After 30 min at 3°C the tubes were centrifuged and the supernatants collected.

Tris-glycine (pH 8.9) 7.5% polyacrylamide gels containing 0.1% SDS and buffers were prepared as described by Laemmli [11], and pH 7.1 5% gels were prepared according to Weber and Osborn [10,12], except that 50 mM Tris-phosphate was substituted for their 100 mM Na-phosphate. 20 to 50 μl aliquots of the solubilized Na,K-ATPase were assayed for bound [^{3}H]ouabain and applied to the tops of the gels. The Tris-glycine gels were electrophoresed toward the anode at 0.5 mA/gel for 30 min and at 1 mA/gel for 4 h and the Tris-phosphate gels were run at 1 mA/gel for 30 min and 3 mA/gel for 4 h. After electrophoresis, the gels were either stained with Coomassie brilliant blue and then scanned at 560 nm or frozen, sliced into 2 mm sections and assayed for radioactivity without solubilization. [^{3}H]ouabain alone was electrophoresed using both gel systems, plus and minus SDS, and on the Tris-glycine-SDS gels with the 50 mM Tris-Cl, pH 6,9, sample buffer described by Laemmli [11].

3. Results and discussion

3.1. *SDS-Gel chromatography*

As shown in fig.1a, the two polypeptides of the Na,K-ATPase are only partially separated on this 6% agarose column. The calculated mol. weights of the two polypeptides (87 000 and 39 000) are similar to those determined previously by both gel chromatography and electrophoresis in SDS [10,13–16]. There are no detectable aggregate forms of the poly-



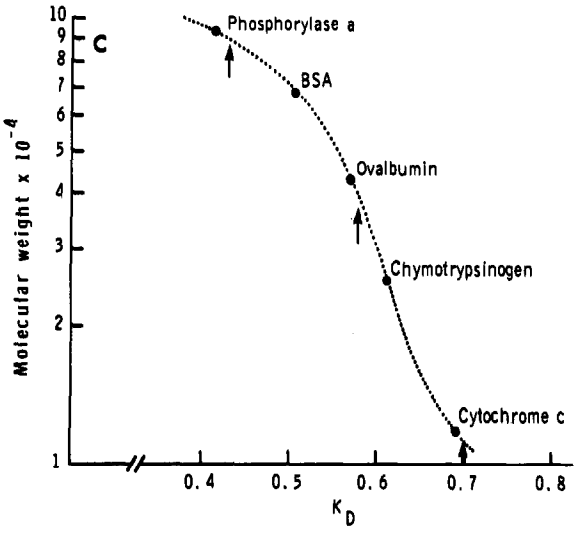


Fig.1. Chromatography of SDS-solubilized [^{3}H]digitoxin–Na,K-ATPase on 6% agarose. (a) The [^{3}H]digitoxin–Na,K-ATPase complex was solubilized in SDS and chromatographed in 0.1% SDS as described in Materials and methods. The fractions (1.5 ml) were assayed for radioactivity (———) and for protein (- - - -) by the method of Lowry et al. [22]. Blue dextran (V_o) and *p*-nitrophenol (V_t) were measured at 254 nm and 410 nm, respectively. Na,K-ATPase alone and [^{3}H]digitoxin added to SDS-solubilized Na,K-ATPase were similarly chromatographed. (b) [^{3}H]digitoxin (3.3×10^5 cpm) was diluted into SDS and chromatographed exactly as described above. (c) Calibration of the 6% agarose column. Phosphorylase *a*, bovine serum albumin (BSA), ovalbumin, chymotrypsinogen and cytochrome *c* were solubilized in SDS and chromatographed as described above for the Na,K-ATPase and [^{3}H]digitoxin. The logarithm of the molecular weight of each standard protein is plotted as a function of its K_D. Arrows, mark the distribution coefficients of the catalytic subunit, glycoprotein and [^{3}H]digitoxin.

peptides. Approx. 86% of the [^{3}H]digitoxin that was bound to the Na,K-ATPase prior to the addition of SDS was eluted in the single peak of radioactivity which overlaps the trailing edge of the glycoprotein peak.

The location and shape of this [^{3}H]digitoxin peak is the same when *free* [^{3}H]digitoxin is chromatographed in the absence of any protein (fig.1b), and when the [^{3}H]digitoxin is mixed with SDS-solubilized Na,K-ATPase just before application to the column.

Free [^{3}H]digitoxin migrates with an unusually high apparent mol. wt.; i.e. 11 000, in contrast to its formula weight of 765. Its position on the column, relative to that of the two polypeptides, is similar to that reported by Kyte [13] for phospholipids, and this may be due to the formation of SDS-digitoxin micelles. This irregular distribution of digitoxin may, in part, account for Alexander's assumption that it was co-migrating with, and therefore bound to, the Na,K-ATPase. Also, with the much shorter gel filtration columns employed by Alexander it would have been difficult to actually separate the protein and [^{3}H]digitoxin peaks.

One other major difference between the present study and that of Alexander is the assignment of mol. wts. to the eluted peaks of protein and radioactivity. This appears to be due to some confusion as to whether or not SDS denatures the standard proteins used for calibrating the gel filtration columns [7,9]. There is, however, considerable evidence [17–20] which strongly suggests that most proteins (including those used here, fig.1c) do not retain their native oligomeric structures or molecular weights when treated with concentrations of SDS greater than 8×10^{-4} M.

We also attempted to measure directly the stability of the [^{3}H]digitoxin–Na,K-ATPase complex in the presence of SDS-column buffer using cellulose dialysis tubing (pore diameter = 4.8 nm). We found, however, that [^{3}H]digitoxin, in the absence of protein, did not readily pass through the dialysis membrane, and after 24 h of vigorously stirred dialysis at 25°C only 9% of the [^{3}H]digitoxin initially present had diffused into the dialysate.

It is obvious that with the conditions described here there is no evidence that [^{3}H]digitoxin remains bound to either of the Na,K-ATPase subunits after treatment with SDS. Digitoxin in SDS does exhibit some anomalous behavior with both gel filtration and dialysis which, under some experimental conditions, could be misinterpreted as evidence for such an association.

3.2. *SDS-gel electrophoresis*

Fig.2a is an illustration of the typical profile

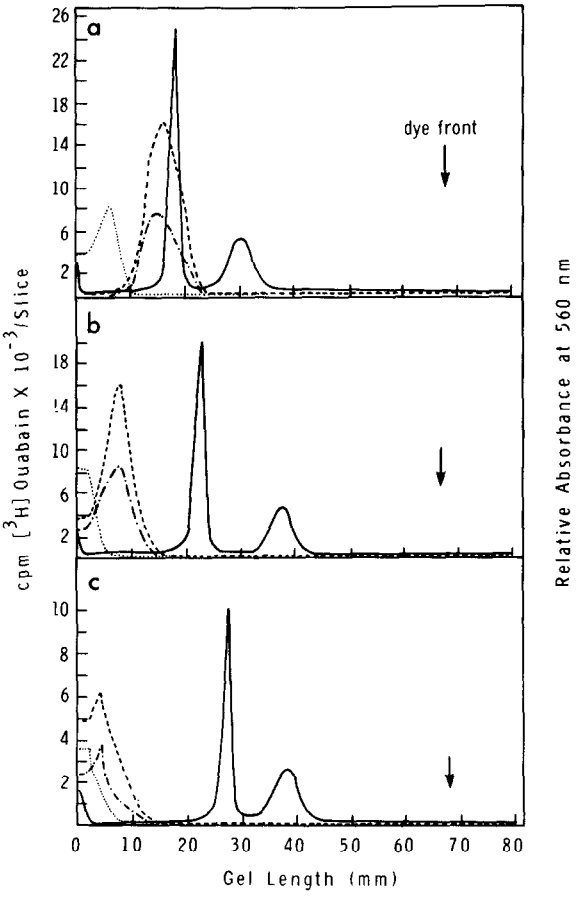


Fig.2. Electrophoresis of SDS-solubilized [^{3}H]ouabain–Na,K-ATPase. (a) Na,K-ATPase labeled with [^{3}H]ouabain (- - - -) and [^{3}H]ouabain alone (– . – . –) were solubilized with SDS in sodium borate buffer, pH 8.5, and applied to 7.5% Tris-glycine gels as described in Materials and methods. Coomassie brilliant blue stained gels were scanned at 560 nm (———) and 2 mm slices of frozen gels were assayed for radioactivity. [^{3}H]ouabain (. . . .) in sodium borate buffer minus SDS was electrophoresed under the same conditions except that SDS was omitted from both the gels and the running buffer. (b) Same was (a), with 50 mM Tris-Cl, pH 6.9, sample buffer. (c) Same as above, with 5% gels containing 50 mM Tris-phosphate, pH 7.1, and 10 mM Tris-phosphate, pH 7.1, sample buffer.

obtained when the [^{3}H]ouabain–Na,K-ATPase complex is solubilized with SDS in sodium borate buffer (pH 8.5) and electrophoresed on Tris-glycine gels (pH 8.9) as described by Kott et al. [8]. This is very similar to their profile in that the [^{3}H]ouabain peak overlaps that of the catalytic subunit. However, we also observed that *free* [^{3}H]ouabain, in the absence of protein, exhibits exactly the same relative mobility when electrophoresed under the same conditions.

Furthermore, when either free [^{3}H]ouabain or the [^{3}H]ouabain–Na,K-ATPase complex is solubilized in 50 mM Tris-Cl, pH 6.9, (containing SDS, 2-mercaptoethanol, NaCl and glycerol) and electrophoresed on the Tris-glycine gels, the relative mobility of the [^{3}H] ouabain peak is reduced by 40–50%, and it no longer overlaps the catalytic subunit (fig.2b). If the same samples are solubilized in the Tris-phosphate buffer, pH 7.1, and electrophoresed on Tris-phosphate gels, the [^{3}H]ouabain peak lags even farther behind the catalytic subunit (fig.2c). When [^{3}H]ouabain alone is electrophoresed in any of the above buffer and gel conditions, minus SDS, the radioactive peak is found either near or at the top of the gels.

In our experiments, therefore, the fortuitous co-electrophoresis of [^{3}H]ouabain and the catalytic subunit of the Na,K-ATPase appears to be due solely to the particular gel and sample buffer conditions, and does not reflect an association of ouabain with either of the Na,K-ATPase subunits.

This is not to suggest that the catalytic subunit is not at least part of the cardiac glycoside receptor. The work of Ruoho and Kyte [5] and particularly that of Hegyvary [6], in which approximately 40% of the Na,K-ATPase molecules were covalently labeled with oxidized [^{3}H]ouabain prior to SDS-gel electrophoresis, is good evidence that the receptor is located on the catalytic subunit.

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