ISOLATION OF A DIGITOXIN-BOUND PROTEIN FROM A BRAIN MEMBRANE PREPARATION CONTAINING Na^+, K^+-ACTIVATED ATPase

Denis R. ALEXANDER
Department of Biochemistry, Hacettepe University, Ankara, Turkey

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1. Introduction

It has been well established that the Na^+, K^+-ATPase* from a wide range of tissues contains a sub-unit, of molecular weight 84–105 000, which is phosphorylated by [^{32}P]ATP in a Na^+-dependent reaction [1–5]. There is some evidence for the involvement of a second sub-unit of lower molecular weight in Na^+, K^+-ATPase preparations from bovine brain [3], rectal gland of Squalus Acanthias [6], and canine renal medulla [7], though different results have been obtained in a Na^+, K^+-ATPase preparation from pig brain [8]. Many studies have been carried out on the binding of cardiac glycosides to various Na^+, K^+-ATPase preparations [9], but the glycoside-binding component of the system has not yet been identified.

This paper describes the isolation of the protein which binds the cardiac glycoside digitoxin. Its purification is compared, in a series of parallel experiments, with the purification of the sub-unit phosphorylated by [^{32}P]ATP in a Na^+-dependent reaction. The preliminary findings have important implications for theories of Na^+, K^+-ATPase structure and function.

2. Materials and methods

[^{32}P]ATP labelled in the γ-position was prepared as described by Rodnight and Lavin [10]. Ox brain microsomes were prepared by the method of Rodnight [11], and samples (8 mg) were labelled with [^{32}P]-ATP in the presence of 50 mM NaCl by the procedure described earlier [2], the reaction being stopped within 1 sec with 0.25 ml of 50% (w/v) SDS*. The detergent solubilises the protein and stabilises the acyl–phosphate bond formed in the Na^+-dependent labelling reaction [2].

[^{3}H]digitoxin (5.3 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, UK. The effect of SDS on the stability of digitoxin binding to protein was measured as follows: [^{3}H]digitoxin (10 μM) was added to an incubation medium containing membrane protein (2 mg), 20 mM Tris–HCl (pH 7.0), 4 mM $MgCl_2$, 3 mM ATP and 130 mM NaCl, giving a final [^{3}H]digitoxin concentration of 1 μM in a volume of 3 ml. The reaction was carried out at 37°C with rapid stirring for 10 min, after which KCl was added to a final concentration of 15 mM for 1 min, before the reaction was terminated by the addition of either 1 ml digitoxin (1 mM) or 1 ml digitoxin (1 mM) or 1 ml digitoxin (1 mM) containing 4% SDS. Samples were filtered using Centriflo membrane cones (Amicon Co., Holland), and the protein residue added to 5 ml 50 mM Tris–HCl (pH 7.4) containing 320 μM digitoxin. Dissociation of protein-bound [^{3}H]digitoxin was measured at 22°C by taking samples (0.5 ml) at intervals of 1 hr. When a higher yield of protein-bound [^{3}H]digitoxin was required for fractionation experiment, the binding reaction was carried out essentially was above, except that 8 mg of membrane protein were used, and the protein was solubilised using 0.25 ml 50% SDS.

Columns of Sepharose 4B (2.5 cm × 45 cm) and

* *Abbreviations:* Na^+, K^+-ATPase, sodium-plus-potassium ion-activated adenosine triphosphatase; SDS, Sodium Dodecyl Sulphate.

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Sephadex G-200 (2.5 cm X 45 cm) were operated by upward displacement using an eluent containing 50 mM Tris–HCl (pH 7.0), 100 mM NaCl, 0.1% SDS and 0.01% NaN_3 at room temperature. The same eluent was used for the Sephadex G-100 column (1.8 X 50 cm) only containing 1% SDS. Six proteins of known molecular weight were used to calibrate the Sephadex G-200 column, and six different proteins for the Sephadex G-100 column. The eluents used during calibration were as described above. In both cases a linear relationship was found on plotting V/V_0 against log. mol. wt. [12].

Polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [13].

3. Results and discussion

In the absence of SDS the half-life of dissociation of [^{3}H]digitoxin from microsomes was 191 min, and in the presence of detergent 3140 min. This stabilisation by SDS is in marked contrast to its action in releasing all the protein-bound [^{3}H]ouabain from brain microsomes [14]. The addition of KCl before solubilisation with detergent was essential for stabilisation of the [^{3}H]digitoxin–protein complex. In a preliminary series of experiments in which labelled protein was fractionated using Sepharose 4B, the radioactivity in the single peak of protein-bound [^{3}H]digitoxin obtained was reduced 96% compared with control when the KCl step was omitted in the initial reaction.

On fractionation of [^{3}H]digitoxin-bound protein on Sephadex G-200, a single radioactive peak of molecular weight 383 000 was obtained (peak A in fig. 1 and table 1). When protein from this peak was recycled on the same column after concentration of

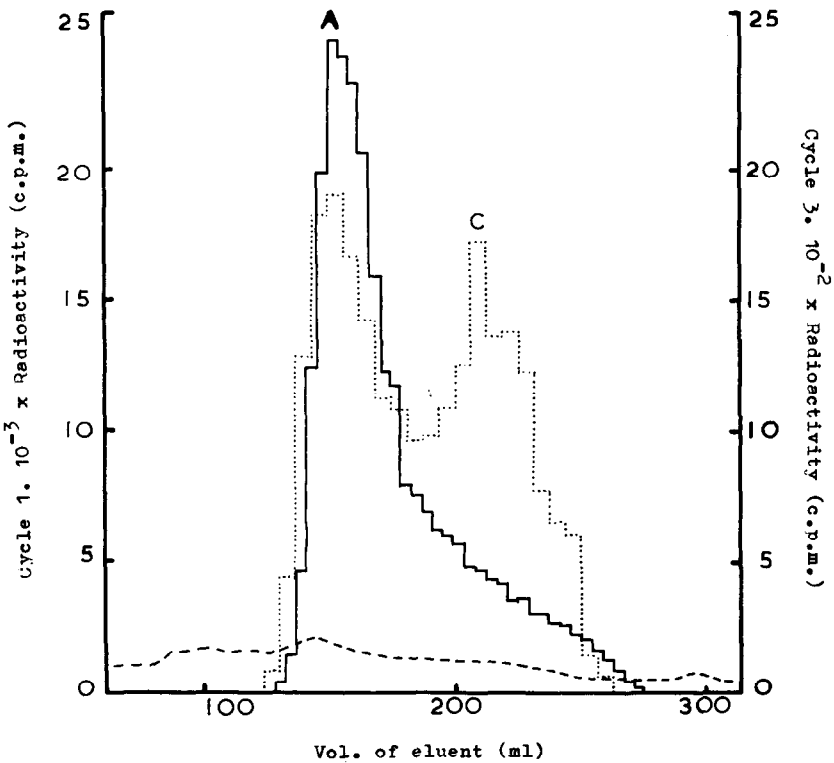

Fig. 1. The fractionation on Sephadex G-200 of membrane protein (8 mg) labelled with [^{3}H]digitoxin as described in the text. (- - - - - - - - - -), Absorbance at 280 nm of protein from the third fractionation cycle (on same scale as fig. 2); (————), radioactivity in fractions (4.0 ml) from the first fractionation cycle; (. . . . . .), radioactivity in fractions (6.0 ml) from the third fractionation cycle.

Table 1
The molecular weight of radioactive peaks isolated on Sephadex G-200

Column	Radioactive label	Peak A	Peak B	Peak C	Peak D
Sephadex G-200	[^{3}H]digitoxin	383 000 ±13 000 [17]	268 000 ±16 000 [5]	135 000 ±11 000 [3]	–
Sephadex G-200	^{32}P	387 000 ± 9 000 [6]	263 000 ±14 000 [5]	132 000 ± 9 000 [4]	93 000 ±6 000 [6]
Sephadex G-100	[^{3}H]digitoxin	–	–	–	92 000 ±1 700 [5]

Samples of membrane protein (8 mg) were labelled with [^{3}H]digitoxin and [^{32}P]ATP as described in the text. Peak C of ^{32}P labelling was obtained when ATP (final concentration 3 mM) was added to the reaction medium a fraction of 1s before solubilisation of protein with SDS. Peak D of [^{3}H]digitoxin labelling was obtained when protein from peaks A, B or C from the Sephadex G-200 column was concentrated using Centriflo filtration cones and applied to a column of Sephadex G-100. The number of estimates is in brackets.

pooled fractions, a further radioactive peak B appeared, together with peak A. On recycling of protein from both these peaks together, two peaks of labelling (A and C) were obtained as shown in fig. 1. Finally the peak C material of molecular weight 135 000 (table 1) was recycled, nearly all the labelled protein reverting to the molecular weight of the original peak A. Peaks A, B and C were obtained in varying proportions in different experiments. However, since protein from peak C consistently aggregated to give peak A, and since only three peaks were obtained (table 1), it appears that there is a specific rather than random aggregation of protein. On polyacrylamide gel electrophoresis of the labelled protein obtained following aggregation of peak C in the final cycle, two bands were visible when staining was carried out using Amido Black (though a further band of intermediate mobility appeared on staining with Coomassie brilliant blue [13]). The apparent molecular weight of the first band was 91 000 ± 2000 (average of six experiments), whilst the second band had a maximum molecular weight of 50 000. On staining for carbohydrate by the periodic acid-Schiff procedure the second band gave a posistive reaction. Since the presence of carbohydrate is likely to affect the amount of SDS bound to the protein [7], an accurate estimation of the molecular weight of the second band was not possible using this technique. Counting of radioactivity in gel slices (0.2 cm thick) showed that all the bound [^{3}H]digitoxin had been removed from the protein during electrophoresis.

When protein from peaks A, B or C from the Sephadex G-200 column was concentrated and applied to a column of Sephadex G-100 (1% SDS in the eluent), a single peak of [^{3}H]digitoxin labelling of molecular weight 92 000 (table 1) was obtained in all instances. On recycling protein from this peak on a further column of Sephadex G-100, using an eluent containing 1 M urea and 1% SDS at pH 8.7, no evidence was obtained for dissociation of the labelled protein into smaller sub-units. When the labelled protein from this column was subjected to polyacrylamide gel electrophoresis, a single band of molecular weight 91 000 was obtained, using either Amido Black or Coomassie Brilliant Blue as protein stain.

Labelling with [^{3}H]digitoxin was also carried out in the presence of 1.7 mM H_3PO_4 and absence of NaCl and ATP, and in another experiment using the same conditions as those used for labelling with [^{32}P]ATP (50 μM $MgCl_2$ and 50 μM ATP). In both cases a single peak A of labelling was obtained after fractionation on Sephadex G-200.

When protein labelled with [^{32}P]ATP was fractionated on the Sephadex G-200 column, 70–80% of the total labelling was found to have a molecular weight of 93 000 (peak D in table 1). Other peaks corresponding to peaks A and B of the [^{3}H]digitoxin labelling were also found, as shown in table 1. However, they were small in comparison with peak D. Now it is known that ATP stabilises the Na^+, K^+-ATPase

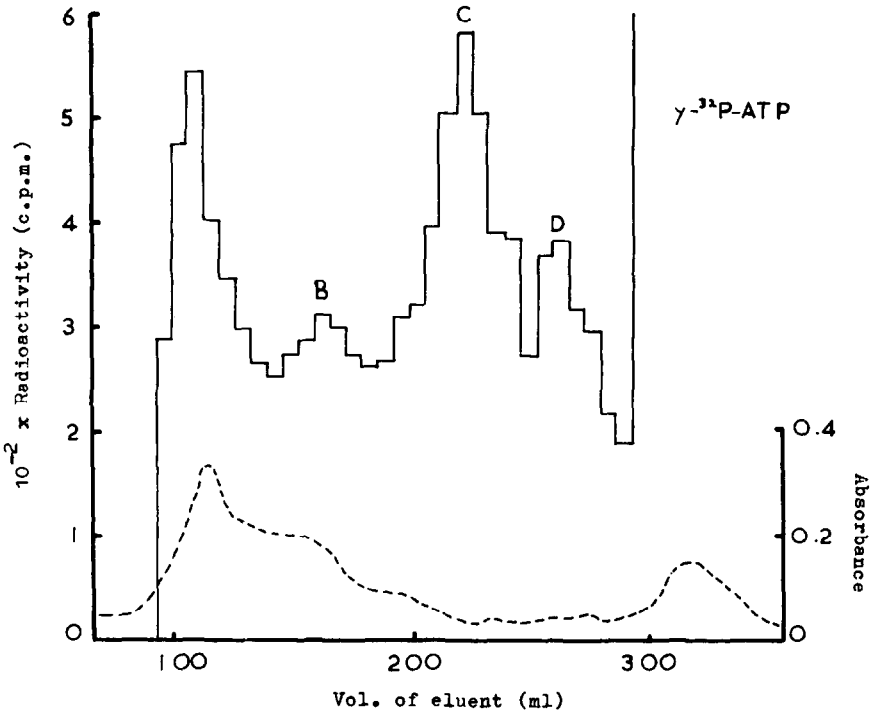

Fig. 2. The fractionation on Sephadex G-200 of membrane protein (8 mg) labelled with [^{32}P]ATP as described in the text. Before solubiliation of the protein with SDS, ATP was injected to give a final concentration of 3 mM (- - - - - - - - -) Absorbance at 280 nm; (————————), radioactivity in fractions (6.0 ml) eluted from the column.

against inhibition by various agents, such as urea [15]. Raising the ATP concentration in the phosphorylation reaction is complicated by the presence of a phosphate kinase which phosphorylates serine residues [2]. To minimise phosphorylation due to this phosphate kinase, the Na^+-dependent reaction was carried out as above, except that nonradioactive ATP was added a fraction of 1 sec before SDS, to give a final ATP concentration of 3 mM. On fractionation on Sephadex G-200 peaks A, B and D were obtained as previously, together with a new peak C of molecular weight 132 000 (fig. 2 and table 1). As with previous experiments using Sepharose 6B [2], some labelling was found in the void volume. When protein from peak C was recycled on Sephadex G-200 following concentration of pooled fractions, the main peak of labelling was found in position A with molecular weight 387 000 (table 1).

It appears from these results that the binding-site for digitoxin is found on the same sub-unit that is phosphorylated by ATP in the Na^+-dependent reaction. This conclusion is supported by the similarity in molecular weights of the various labelled peaks, and by the finding that the [^{3}H]digitoxin labelled sub-unit has an identical molecular weight to that phosphorylated by [^{32}P]ATP. Since the binding sites for cardiac glycoside and ATP are orientated at opposite sides of the membrane [9], this implies that the polypeptide of molecular weight 93 000 completely traverses the membrane, as has also recently been suggested from studies on the sulphydryl groups of the Na^+, K^+-ATPase [16]. The molecular weight estimates of the sub-unit by Sephadex chromatography and gel electrophoresis agree closely. A value of 91–93 000 for the molecular weight is probably preferable to the previous estimate of 102 000, determined using polycarylamide gels containing urea [2].

The results summarised in table 1 may be explained according to the $\alpha_n\beta_n$ theory of Na^+, K^+-ATPase

structure published by Stein et al. [17] whilst this work was in progress, except that digitoxin is bound to the α sub-unit and not the β sub-unit as their theory suggests. According to this interpretation the larger sub-unit α is closely associated with a sub-unit β of apparent molecular weight below 50 000. The $\alpha\beta$ structure is stabilised either by a high ATP concentration (3 mM), or by digitoxin-binding, prior to solubilisation with SDS, and is represented by peak C of molecular weight 132–135 000. When $\alpha\beta$ is stabilised it forms $\alpha_n\beta_n$ polymers, giving rise to peak B ($\alpha_2\beta_2$) and peak A ($\alpha_3\beta_3$). When $\alpha_n\beta_n$ is exposed to a higher concentration of SDS (1%) on Sephadex G-100 it dissociates to α and β. In absence of digitoxin and in presence of low concentrations of Mg^{2+} and ATP (50 μM) dissociation of $\alpha\beta$ is largely complete in the presence of 0.1% SDS on the Sephadex G-200 column, so yielding peak D. When the ATP concentration is raised to 3 mM the $\alpha\beta$ structure is stabilised, though a peak of α is still visible (peak D, fig 2). I have not omitted the possibility that the ^{32}P labelling in peak C may be due to phosphorylation of serine residues resulting from the short transient increase in ATP concentration prior to solubilisation with SDS. If this were the case one might speculate that it is serine residues in the β sub-unit which are being phosphorylated. Whilst there is no evidence for a connection between the cyclic AMP stimulated intrinsic protein kinase activity and the Na^+, K^+-ATPase in membrane fragments [2], the possibility has not been excluded that cyclic AMP may be a controlling parameter in the intact membrane [18].

It is not yet possible to say how many sub-units are present in the intact Na^+, K^+-ATPase. However it is of interest that estimates of molecular weight of the enzyme may be divided into approximately two groups, those estimates in the range 500–560 000 [3,19,20], and those of about 250 000 (8,21). The presence of oligomeric proteins in membrane cannot be inferred from the observation of protein aggregates in the presence of SDS. Nevertheless it remains a possibility that the Na^+, K^+-ATPase in situ may consist of a polymer of $\alpha\beta$ units [22], and that the varying estimates of molecular weight reflect the polymer state of the enzyme resulting from the particular isolation procedure employed. According to this view the phosphorylated protein of molecular weight 570 000 isolated previously on a column of Sepharose 6B [2] represents the structure $(\alpha_2\beta_2)_2$.

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